ECDYSIS TRIGGERING HORMONE IN AEDES AEGYPTI: FOCUS ON INKA CELL MAPPING

By

Lena Lebada

A capstone project submitted for

Graduation with University Honors

June 15, 2020

University Honors

University of California, Riverside

APPROVED

_____

Dr. Michael Adams

Department of Entomology and Cell, Biology & Neuroscience

_____

Dr. Richard Cardullo, Howard H Hays Jr. Chair, University Honors

ABSTRACT

The peptide hormone ETH is produced by endocrine Inka cells in all insect species examined thus far. Immunohistochemistry has been an effective strategy for mapping Inka cells previously. The first objective of this study is to map Inka cell distribution in male and female yellow fever mosquitoes. During the adult stage of the yellow fever mosquito, we hypothesize ETH functions as an allatotropin that mediates reproductive potential in males. This hypothesis will be tested by knockdown of the ETH receptor using RNA interference and assessment of its impact on fecundity following mating with control females.

ACKNOWLEDGMENTS

I would like to express my special thanks of gratitude to my Professor Michael Adams as well as postgraduate student Yike Ding, who provided me the support and opportunities to do this wonderful project on the topic, mapping Inka cells within *Aedes aegypti*. I would like to thank Professor Michael Adams for this wonderful opportunity to join his lab and partake in this research. Due to the kindness of my professor and colleagues, I came to know about so many new things due to them and this wonderous endeavor. I am really thankful to them. Secondly, I would also like to thank my colleagues and labmates who helped me a lot in finalizing this project within the limited time frame.

Table of Contents:

ACKNOWLEDGMENTS	1
INTRODUCTION AND PURPOSE	3
PROCEDURE AND METHODS	5
RESULTS	10
DISCUSSION	12

INTRODUCTION AND PURPOSE

The Inka cells, receptors, and hormones expressed are very significant to mosquitoes, specifically *Aedes aegypti* and their survival from larva to adult. Inka cells produce the ecdysis triggering hormone (ETH), which facilitates completion of molting through ecdysis, or shedding of the old cuticle. Locating Inka cells and their receptors in mosquitoes has required the use and the learning of such procedures as immunohistochemistry, mounting slides, and raising mosquitoes. This is crucial to the understanding of these cells, receptors, and hormones. Firstly, Inka cells are very involved in expressing a hormone called ETH. This ETH hormone, also known as Ecdysis Triggering Hormone, is involved in molting, which allows the mosquitos to go through different stages of becoming an adult. This specific yellow fever mosquito, also called *Aedes aegypti*, was involved in carrying a gene for a disease. This mosquito can be further analyzed and studied in order to find ways for them to stop carrying this gene in order to indirectly stop disease from spreading. It is crucial to learn more about these hormones and Inka cells to help us understand and expand our knowledge about the mosquito and continue into further research on disease prevention and diseases vectors.

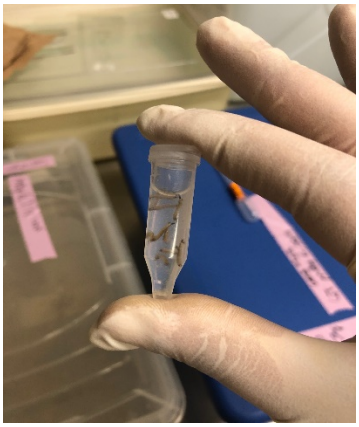

Figure 1: These are adult dissected mosquitos that are undergoing molting and ecdysis. immunohistochemistry.

Inka cells have shown importance in assisting with the molting process as the mosquito goes through its process of maturation. These Inka cells are not only important for their ability to carry the ETH hormone, they are also important for the molting and maturation. The mosquitoes go through several phases that cause them to shed their cuticle and this hormone is involved in allowing the

mosquito to shed that cuticle. Without this hormone the mosquito would die from being unable to emerge from its cuticle to move onto its next stage of maturation.

There are a few major hormones in the *Aedes aegypti* such as Juvenile Hormone, Ecdysone, and ETH. Studied show that the juvenile hormone is important for controlling the development and reproduction in mosquitoes. Then the ecdysone hormone is important for the molting process, and if it is removed then the animal will not be able to molt and will die at that stage. The last hormone ETH has to do with the ecdysone hormone in order to help with initiating molting and is a triad hormone that helps the animal switch from being in a reproductive state to being in a survival state.

In this paper the focus will be on constructing a consistent map of Inka cells of male and female mosquitoes. The idea is that there will be Inka cells situated on the respiratory tracheal system of the mosquito. The corpora allata is another suspected place to have the ETH, therefore it is important to dissect this area as well. The main area of the Inka cells along the tracheal tubes is done by an easy dissection by opening the mosquito from the dorsal side in order to maintain the normal configuration of the tracheal system and CNS. In my research, the focus is to locate Inka cells and retain their normal position following dissection and immunohistochemistry. A control of finding pyrokinin neurons, which also will be stained with an antiserum that cross-reacts with both ETH and pyrokinin peptides. The ETH producing blood fed females should be the most effective type of mosquito to observe. This is due to the steroid increasing and allowing the Inka cells to contain more ETH levels than normal. After blood feeding the amount of protein in the body will increase, causing the Inka cell to become larger and allow the immunohistochemistry to become more obvious after staining.

PROCEDURE AND METHODS

When raising the mosquitoes, it is important to stick with a tentative schedule for raising the mosquitos and a consistency among all of the procedures for immunohistochemistry. The mosquitos were very simple to raise and it was a simple set of steps to follow immunochemistry. After conducting a few trials of immunohistochemistry, we have started to collect some data that will be put together and presented at a later date. As for now we have these few mosquitos that we have performed immunohistochemistry in order to map the location and presence of the Inka cells. Inka cells have been found previously in *Drosophila* and the goal in this project is to document their presence in mosquitoes. Inka cells are important for insects like *Drosophila* and mosquitoes because they produce certain hormones such as ETH. These Inka cells are found along the insect's trachea, they have one on the left side and one on the right side. The insects have segments throughout their body and there is one pair of Inka cells per body segment.

It is also important to learn how to mount the slides after performing immunohistochemistry, we used a green fluorescent dye in order to stain the Inka cells. After immunohistochemistry was performed, we took the mosquito larvae or adult and put it onto the slide with PBS. Then we added small pieces of clay to the edges to seal the top plate of glass that covers it. Then we would observe the cells under a microscope in order to see the presence of these Inka cells and location. Later we would like to take the approach of using a confocal microscope in order to make three-dimensional images of these mosquitoes with the possibility of not having to peel off the cuticle. It takes a lot of time to be able to peel the cuticle off of the mosquito correctly and it is possible to move or take off a section with an Inka cell.

As for the ETH receptor cells, we also perform immunohistochemistry in order to locate them on a fluorescent or confocal microscope. Before we perform immunohistochemistry, we

have to make sure that the mosquitoes are blood fed at exactly 24 hours before dissection and fixation. We do the dissection 24 after blood feeding in order to see the expression of the ETH in the receptor cells very strongly, they produce the most hormone right after blood feeding. It is very difficult to dissect the mosquitoes since they are so soft and we have to make sure not to damage any tissue in order to see the cells clearly later. It is important that we use the correct antibody to stain these cells. Then since we have now seen these cells along the trachea we can explore these cells in the brain, corpora allata, as well as reproductive tracts such as the ovaries.

I hope to try other techniques such as different ways of dissecting the mosquito to find an easier way to see the cells. Looking at the whole body has not been too successful but possibly looking at the whole body with a confocal microscope or making more ways for the antibody to go into the animal could lead to a better result. Personally, I think that the only way to see the cells clearly, whether is the Inka cells or the ETH receptor cells, is to have the mosquito after blood feeding, with the mosquito dissected to be open. It is important to have the mosquito open to see the trachea visibly where the cells are thought to be expressed the most. The cells are not very visible from the outside of the body, even with the cuticle peeled because the mosquitos are not that transparent. So it just makes sense to have the mosquito dissected open, and have anything possibly blocking the view of the trachea removed, such as the gut, in order to get the clearest view.

In the last five years a lot has been discovered, but a comprehensive map of the Inka cells and ETH receptor cells have not been demonstrated in the mosquito. During the course of starting this research we have already begun to see the Inka cells and the receptors for the hormones along the trachea, so we know we have to look more at the mosquito. Although the

dissections seem to take time, I hope to maybe find an easier way to see the cells in the trachea in order to have more time to look at different parts such as the corpora allata.

Raising mosquitoes:

In order to raise the mosquitos, wild-type *Aedes aegypti* eggs were taken from adults. The eggs were acquired by putting a napkin soaked in some water into a cage of female and male adults. They had a 10% sucrose solution as a source of food. The mosquitos would take about two days to mate and lay the eggs in order for us to use them. The mosquito eggs were stored in plastic containers with a moist paper towel to prevent them from drying out. Once adult or larva were needed, the mosquitos were placed in a bin of water and fed every day. The diet for the mosquitoes hatching from eggs consisted of a mix of dog food, yeast and lactalbumin. Once the mosquitoes became pupa, they were transferred into a small cup with water, then placed into a cage to allow them to hatch and mature into adults. Before the mosquitos were taken for dissection, they are blood fed in order to increase the levels of steroid, causing the Inka cells to become larger and full of ETH. This allows the observation of the Inka cell after immunohistochemistry to be more visible. The female mosquitoes were placed into a small mesh cage, allowing them to insert their proboscis through the material to suck blood. The mosquitoes were then fed blood that had been warmed using a device that continuously runs water over the blood feeding in order to create a simulation of a live animal. After about an hour the mosquitoes are then placed back into their room for about twenty-four hours.

Dissection:

When the mosquitoes are ready to be taken for dissection, a carefully designed suction pipet that allows the mosquitos to be unharmed is used to transfer the mosquitos. For the larva dissection it is important to make sure that the scissors and forceps being used are all sharpened

Figure 2: This is an adult mosquito that has been dissected and pinned down. The dissected mosquito is also covered in PBS in order to keep the tissue from drying out during the dissection.

and sterilized. Wild type adult female mosquitos twenty-four hours post blood meal or larval mosquitos in their fourth instar are then placed the mosquitos in ice for about ten minutes in order to anesthetize them. Transfer mosquito to Sylgard dissecting chamber in order to pin the specimen out with minute pins. Then since there is no real hard cuticle to peel, it is important to puncture some holes in the left and right side in order for the staining to go to the Inka cells. The dissection of the larva differs slightly from the adult since the adult has legs and wings. The first step of the adult dissection is to peel off the wings and pin down the mosquito with the ventral side down. Then pin the head and the tail down in order to ensure the mosquito will not rip when cutting the mosquito open. The next step is to use scissors to cut the mosquito on the dorsal side from the head down to the tail. Then make sure to pin the mosquito completely flat and then add PBS in order for it to not curl when removing the pins and moving it to a tube for fixation overnight.

Immunohistochemistry:

For the immunohistochemistry procedure for both larva and male adult mosquitoes were chosen for dissection. To ensure the right gender was selected for the process the mosquitos were chosen one at a time from a container of mosquitos. Then the solution was prepared for the mosquitos to be fixed in PBS: .05% triton-X in PBS. NGS: normal goat serum (SIGMA), Primary & secondary antibodies, mounting solution (aqua and poly mounting media) were stored

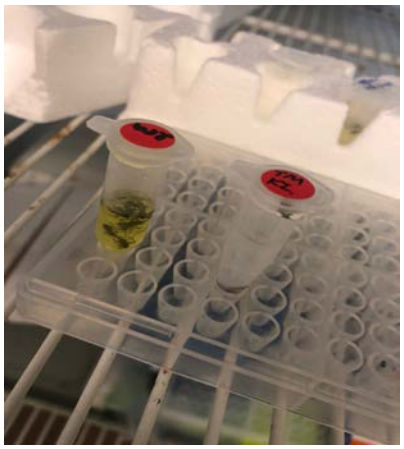

Figure 3: These are tubes containing WT mosquitos going through fixation.

in the fridge in advance for after dissection. Then the mosquitoes were dissected following the procedure of exposing the entire tracheal tube system.

Then the mosquito was fixed after dissection using a small tube containing 4% paraformaldehyde (PFA), the mosquito is transferred using forceps. It is important to be cautious not to break the cuticle or disrupt the location of the tissues when transferring the insect. The mosquito is placed in this tube at 4 degrees Celsius overnight.

Next, is the blocking step which involves washing the insect with PBS 3 times and then with PFA 3 times. The process of washing consists of removing the fixing solution that was put in for overnight then replacing it with PBS. Each wash is about 5 minutes, and the tube is inverted constantly. This process is repeated three times then is washed with PBS with Triton X for about 5 minutes which is used specifically with insects and invertebrates. The function of PBST is to open the space cell to cell and open small holes on the cell membrane. Then 3-5% NGS was added, specifically 5% in order to make the tissue clean because the tissue was thought to be dirty. The entire tube always consists of about 500 milliliters each time, the rest of the tube is filled and is left for 1-2 hours at room temperature.

The next step is to add Primary Antibody Treatment by using GFP after removing the NGS blocking solution. The primary solution consists of a (1:500) dilution for a total 500 ul = 2ul rabbit anti-GFP (50% with glycerol) +15uL NGS + 483 uL PBST. Incubate at 4 degrees celsius overnight with rocking. Next is the Secondary Antibody treatment but first its important to wash again with PBS 3-6 times for five minutes each. Add secondary antibody solution

(1:500) For total 500 uL = 2 uL Alexa Fluor 488 anti-rabbit (50% glycerol) + 15 uL NGS+ 483 uL PBST. Then incubate at 4^{o}C for overnight or less with rocking

Mounting:

To start mounting, specimens were washed with PBST 6 times with rocking, then washed with PBS 2 times. Prepare mounting solution in some ice, clean slide glass, cover glass for on top of slide, clean sharp forceps, mounting clay, clear nail polish to seal slide. Start transferring the tissue using pipette and use forceps to reposition the tissue. Using Kimwipes, remove as much PBS as possible. Using another pipette put 2-3 drops of mounting media without bubbles. Continue to reposition tissue on each corner of cover glass, put a small amount of clay (this side down and attached to slide glass). After removing extra mounting media outside of the cover glass, seal with nail polish. Then label the slide and keep in 4 degrees Celsius. Take a picture with fluorescent microscope or confocal microscope, it is important to remember the fluorescent will only last about 2 weeks. GFP (1:500) goat anti rabbit antibody is detected under wavelength 488.

RESULTS

Mapping the Inka cells using immunohistochemistry was performed on both fourth instar larvae and on blood fed adult female mosquitoes. The Inka cells are positioned on the tracheal tubes in the mosquito, which is best seen when looking into the mosquito from the dorsal side, since the central nervous system of the mosquito is towards the ventral. The tracheal tubes can be followed throughout the mosquito and there is one pair of Inka cells per segment. There is one Inka cell on the left and one on the right per segment; it is estimated that there are about eight Inka cells along the tubes and in total about sixteen Inka cells in total, which are brightly illuminated. After immunohistochemistry was performed and the GFP antibody was able to

penetrate the cells in order to stain the ETH in the Inka cells, the fluorescence was now able to be observed under a microscope.

It was also important to make sure there was a control for the staining of the mosquito to ensure that the staining by immunohistochemistry process was performed correctly. The control for this was looking for a pair of pyrokinin neurons that would brightly fluoresce in every mosquito that had the Inka cell staining done correctly. These neurons produce peptides, which have conserved amino acid sequence motifs similar to that of ETH peptides [1]. After this pair of cells were found, then the mosquito was examined for the Inka cells mapping.

After observing the visible pyrokinin control cells the Inka cells were also visible. The Inka cells are shown very brightly following along the tracheal tubes. The segmentation is clearly shown along the tubes and also the cells have been penetrated well by the antibody in order to show this green fluorescence. The microscope that was used was a semi confocal microscope that had a specific lens for being able to observe the GFP fluorescence. The wavelength used for observing the mosquito slides was 395 nanometers in order for the fluorescence in the Inka cells to show. The Inka cells are visibly seen along the right side of the

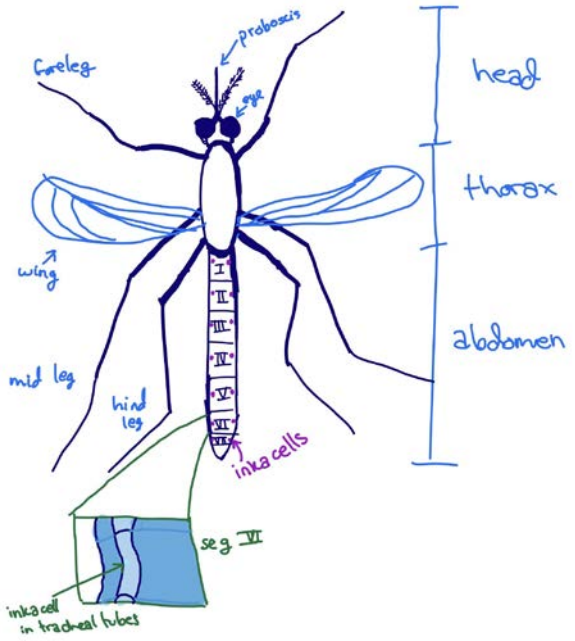

Figure 4: This drawing depicts the segments of the mosquito and where Inka cells would be located within these segments.

mosquito, there are 3 Inka cells shown here. It is important to notice that there is just one cell per segment.

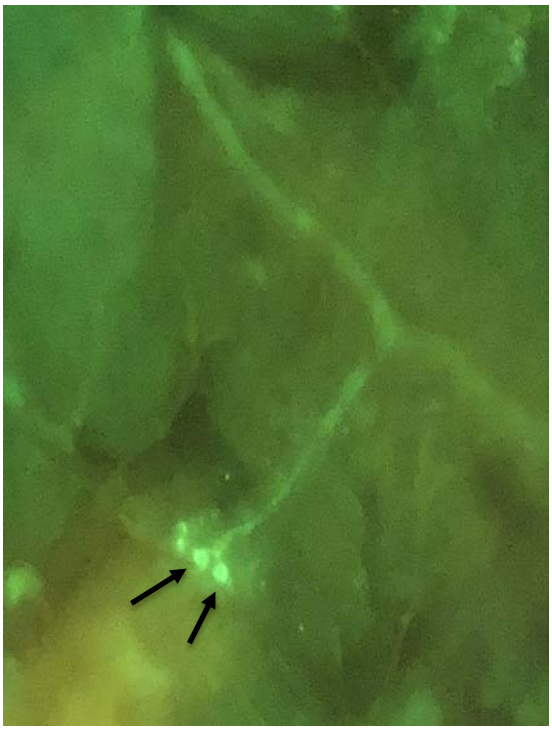

Figure 5: These two brightly fluorescent cells (arrows) are pyrokinin cells that are used as a control to show that the immunohistochemistry staining was effective and that the Inka cells should be visible with the staining. The two lines that come together are two axons coming together to attach to these neurons.

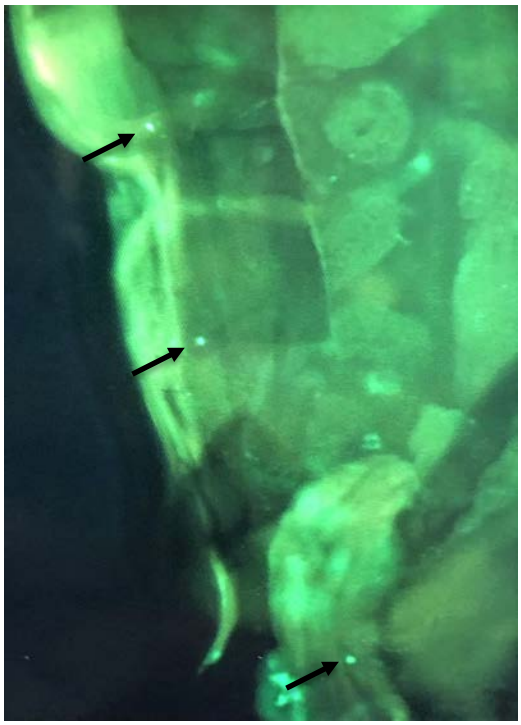

Figure 6: There are three segments (3, 4, 5) shown here of the *Aedes aegypti* mosquito (as shown in figure 4). Each segment contains an Inka cell that is located close to the body wall (arrows). The immunohistochemistry has allowed the antibody to go into the cell and the GFP tag to bind to this secondary goat anti-rabbit antibody in order for the Inka cell to be fluorescent.

DISCUSSION

An Inka cell map was found in the larva mosquito using immunohistochemistry and it was also found in the adult but not as obviously. The staining process was successful and showed

the location of these Inka cells in the mosquito. There is a cohesive map throughout the mosquito that show these cells along the tracheal tubes with a mirroring cell on the left and the right side of each segment. The cells are enlarged after a blood meal and allows the staining process to become more visible since the amount of steroid is increased. Although the cells were not disrupted from their original location the cells had to have slightly shifted after the dissection and the shaking in the staining process. Overall, the hypothesis was correct and the cells were in the segments following the central nervous system, and location of the Inka cells are known.

Aedes aeygpti mosquitos contain these Inka cells in an organized pattern that is now known to be along the tracheal tubes. The research showed demonstrated the location of these cells and this can be used for help with new experiments. Inka cells are important for many reasons including playing this large part in the molting process. Without these Inka cells, there would be no ETH released, which would cause the mosquito to stop the molting process [1]. There is a great impact of learning more about these Inka cells and lets us design future experiments that can be more accurate due to now knowing the location of these cells.

Further experiments can be designed to follow how the mosquito oogenesis production is affected if the receptors that are able to respond to the ETH were knocked down. These receptors produced by cells in the CNS and in the corpora allata and without these receptors, certain functions like molting or sexual reproduction can be greatly reduced. The experiment can be carried about by using both a wild type mosquito, as a control, and a CRISPR-Cas9 knock-in mosquito line that has had a red fluorescent protein attached to the receptor. The mosquitos would be separated female and male at birth, to prevent mating. Then the male mosquitos would

Figure 7: These are two mosquitos that are being observed for the process of confirming a transfer of rhodamine in mating. The top mosquito is a successfully mated female that had a transfer of rhodamine. While the mosquito on the bottom is a control female that has not mated.

be fed a sugar diet with rhodamine which is a red fluorescent dye that can be consumed by the mosquito. Next would be to inject the males with the RNAi in order to knock down the expression of the ETH receptors in the mosquito. Then the females would be placed into the male's cage after the males have consumed this dye and been injected with RNAi. After mating the females would be screened to see if the dye was transferred over to them, this is to confirm mating between males and females. The reason the males consume this dye and females are checked for the transfer of rhodamine for successful mating is as a control since the males can be injured during the RNAi injection. This ETH functions as is an allatotropin signaling hormone that mediates oogenesis reproductive potential in males. This hypothesis will be tested by knockdown of the ETH receptor using RNA interference and assessment of its impact on fecundity following mating with control females.

The receptors of the ETH also need to be mapped in the mosquito and this can be done also using the same techniques as the Inka cell with immunohistochemistry. The receptors will have a red fluorescence after doing immunohistochemistry and using a confocal microscope it is possible to see even more of the location of the cells in a layered image. These receptors are for the hormones produced from the Inka cells, so it is important to know where the hormone is expressed and where the hormone is being received. With this information we can research

further to apply disease related experiments in order to learn more about this yellow fever mosquito.

Current research has discovered more about ETH shows Inka cells have been found by using immunohistochemistry in *Drosophila*, but is has not been researched in mosquitoes. In *Drosophila*, the ETH hormone has been found to work in a hormone cascade along with another hormone called Juvenile Hormone [2]. This hormone has to do with regulating mating experiences and mating in *Drosophila*. The Inka cell has a role in this Juvenile Hormonal signaling because the ETH that is regulated by the Inka cell can suppress the amount of JH being released [6]. Depending on other factors such as stress can affect whether the mosquito focuses on courtship or more on survival, therefore more ETH will be released to suppress this courtship behavior under stress. This research can also be conducted on mosquitos. Causing any type of heat stress to the mosquito before mating can most likely cause the same results.

REFERENCES

1. Ding, Yike (2020) Ecdysis Triggering Hormone: A Multifunctional Peptide Regulator of Reproductive Physiology in Yellow Fever Mosquito *Aedes aegypti* 6-8.
2. Meiselman MR, Kingan TG, Adams ME. Stress-induced reproductive arrest in Drosophila occurs through ETH deficiency-mediated suppression of oogenesis and ovulation. *BMC Biol*. 2018;16(1):18. Published 2018 Jan 30. doi:10.1186/s12915-018-0484-9
3. Roller, L., Zitnanová, I., Dai, L., Simo, L., Park, Y., Satake, H., Tanaka, Y., Adams, M. E., & Zitnan, D. (2010). Ecdysis triggering hormone signaling in arthropods. *Peptides*, *31*(3), 429–441. https://doi.org/10.1016/j.peptides.2009.11.022
4. Evans, B.R., Kotsakiozi, P., Costa-da-Silva, A.L. *et al.* Transgenic *Aedes aegypti* Mosquitoes Transfer Genes into a Natural Population. *Sci Rep* 9, 13047 (2019). https://doi.org/10.1038/s41598-019-49660-6
5. Nolan, Cebrina (2019) Exploring Neuromodulator Effects of Adenosine Deaminase and Corazonin in Jewel Wasp Venom, 16-19
6. Žitňan, D., Žitňanová, I., Spalovská, I., Takác, P., Park, Y. and Adams, M. E. (2003). Conservation of ecdysis-triggering hormone signalling in insects. *J. Exp. Biol.* 206,1275 - 1289