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Emerald Jewel Wasp Hijacks Behavior and Reproduction in its Host to Benefit Progeny

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Publication Date

2021-08-23

Data Availability

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EMERALD JEWEL WASP HIJACKS BEHAVIOR AND REPRODUCTION IN ITS HOST TO
BENEFIT PROGENY

By

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A capstone project submitted for Graduation with University Honors

May 6, 2021

University Honors

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ABSTRACT

The parasitoid Emerald Jewel Wasp (*Ampulex compressa*) stings its host the American cockroach (*Periplaneta americana*) in two distinct areas of the central nervous system. In particular, the wasp injects venom into the sub-esophageal ganglion (SEG) and central complex (CX) of the brain, resulting in “hypokinesia”, characterized by the suppression of spontaneous locomotory activity and evoked escape responses. This allows the wasp to lead the stung cockroach into its burrow, where it deposits a single egg. More recently we have observed that stung animals exhibit an unusual reproductive abnormality: production of ootheca-less eggs. We hypothesize that disruption of host reproductive function may serve to conserve energy for the benefit of its progeny. I propose to artificially inject pure venom into the central complex of mated females to mimic the wasp sting and its physiological and behavioral effects. Virgin females will be staged ten days prior to post-ecdysis, mated, and then injected with venom directly from the venom sac of the wasp into their CX. Injected animals will then be tested for changes in locomotory behavior, including spontaneous walking and evoked escape responses as well as any signs of altered egg laying behavior. By pursuing these objectives, we hope to unveil biochemical strategies the wasp uses to subjugate its prey in order to maximize its reproductive success. Future directions of this project include determining the specific component(s) of venom that are responsible for these physiological and behavioral effects.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my faculty mentor, Dr. Michael Adams, for his never-ending encouragement, guidance, and support not only throughout this project but also throughout all the years I have been a part of his research team. In addition, I would like to express my appreciation and gratitude to my research partner Cebrina Nolan for being my mentor and teaching me the things I needed to know in order to thrive and grow. I would also like to thank my other research partner Adrianna Lopez for helping me maintain each insect colony, recording observational and experimental data, and dissecting the venom sac from female wasps. Lastly, I would like to acknowledge and thank every single person I have encountered throughout my time in research. It is because of everyone around me that has made me become the aspiring student I am today.

TABLE OF CONTENTS

Abstract.....	2
Acknowledgments.....	3
List of Figures	5
Introduction.....	6
Methods.....	10
Results.....	16
Discussion.....	22
References.....	26

LIST OF FIGURES

Figure 1.....	7
Figure 2.....	8
Figure 3.....	9
Figure 4.....	9
Figure 5.....	11
Figure 6.....	12
Figure 7.....	13
Figure 8.....	14
Figure 9.....	15
Figure 10.....	15
Figure 1A.....	16
Figure 2A.....	17
Figure 3A.....	18
Figure 3B.....	18
Figure 4A.....	19
Figure 5A.....	20
Figure 6A.....	20
Figure 7A.....	21

INTRODUCTION

The Emerald Jewel Wasp (*Ampulex compressa*) is a parasitoid wasp that hijacks the brain of its natural host, the American Cockroach (*Periplaneta americana*), and “zombifies” it. This allows the wasp to exploit the cockroach as a fresh food supply for its progeny. Parasitoids are organisms that fall under the larger umbrella of parasites, except for the fact that they utilize a host for the benefit of their lineage rather than for themselves. In order to gain a better understanding of how the Jewel Wasp subjugates its host, this project investigates whether artificial injection of venom into the brain of the cockroach can mimic the same behavioral and physiological effects caused by the natural wasp sting. A wasp sting into the brain of a cockroach results in a decrease in locomotory motion and egg production. By replicating these effects, the biochemical strategies that the wasp uses to subdue its prey and maximize its reproductive success will be unveiled. Although this project is specific to a certain field of study, conducting these experiments to investigate the objectives can expand our knowledge of how parasitoid species subjugate their hosts. Moreover, insects outnumber humans by a staggering ratio of two hundred million to one, making them the most dominant organisms on the planet. By understanding how insects live, evolve, and reproduce, humans can potentially improve their own lives by replicating the insects’ natural features in fields of science and technology.

In order to subjugate its host, the Jewel Wasp stings its cockroach host, inducing a lethargic state that decreases provoked movement and spontaneous movement (Williams 1942; Piek and Spanjer 1986). The Jewel Wasp stings the cockroach three times in three distinct regions known as the prothoracic ganglion, the sub-esophageal ganglion (SEG), and the central complex (CX) of the brain (Kaiser & Libersat, 2015). Within insects, a ganglion acts as a control

room for nerve cells that are responsible for controlling muscles and senses. Additionally, the central complex is a small high-density subregion within the brain. The first sting into the prothoracic ganglion paralyzes the two front legs of the cockroach with the purpose of making the next two subsequent stings easier for the wasp (Haspel, et al., 2003). The second and third sting into the SEG and CX induces hypokinesia, a lethargic sleep-like state that lasts for several weeks (Emanuel & Libersat, 2017; Kaiser & Libersat, 2015; Gal & Libersat, 2010; Haspel & Libersat 2003; Haspel, et al., 2003) (Figure 1). Previous research has determined that the venom



Fig. 1. *Ampulex compressa* stinging a cockroach in the suboesophageal ganglion (SEG) while using its mandibles to grip the roach's pronotum to subdue it. (From Arvidson et al., 2018)

from the wasp sting into the CX and SEG results in a decrease in escape response and spontaneous walking. This is consistent with reports that the CX and SEG play a significant role in coordinating locomotory motion within cockroaches (Kaiser & Libersat, 2015). Escape response provides a quantitative measurement of how much stimulus it takes to aggravate a cockroach until it runs away, while spontaneous walking is the measurement of how often a cockroach

naturally walks (Gal & Libersat, 2010). Since hypokinesia can potentially last for several weeks, it allows the wasp to lead the cockroach into its burrow and oviposit an egg on it (Arvidson, et al., 2019, 2018). The egg will go through a total of three larval instars that slowly eats its way into the abdomen of the cockroach (Fox et al., 2009). The developing larva consumes everything

but the gut and the central nervous system until it evolves into a pupa and finally emerges from the cockroach three months later (Arvidson, et al., 2018). An instar is a phase between molting stages during the development of insect larva (Arvidson, et al., 2018). Past researchers have successfully artificially injected drugs such as procaine and reserpine into the SEG and CX and have shown that venom injected into either of them is enough to induce hypokinesia (Emanuel & Libersat, 2017; Kaiser & Libersat, 2015; Gal & Libersat, 2010; Haspel & Libersat, 2003) (Figure 2).

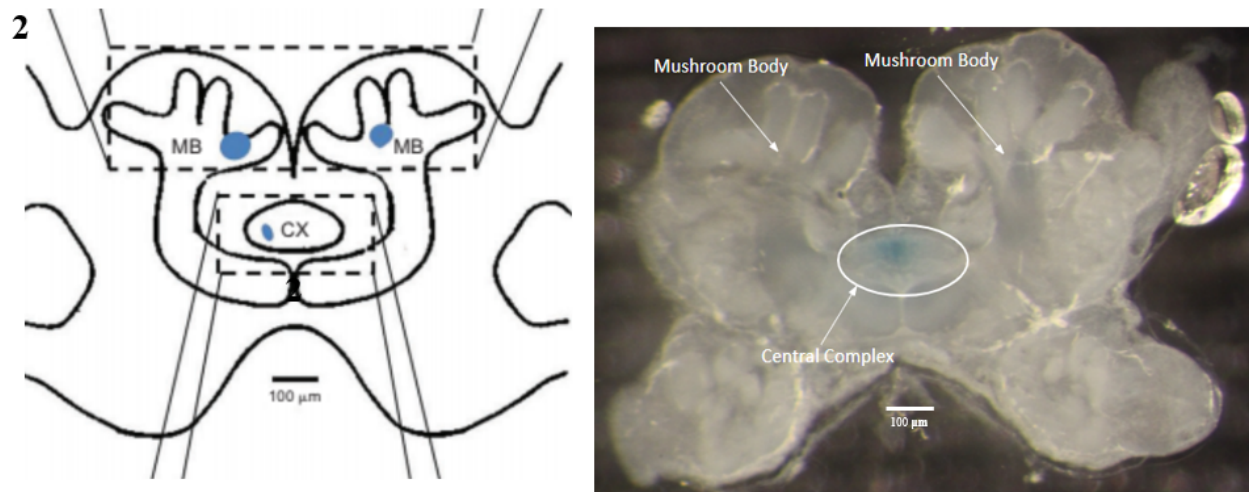


Fig. 2. The diagram on the left is from researchers Kaiser and Libersat indicating where the mushroom bodies and central complex are located within the cockroach brain. The photo on the right is an actual picture of a cockroach brain that was sectioned using a vibratome at 90 micrometers. Injection of 0.1% Janus Green creates the blue area marking in the injection site.

In addition to analyzing how wasp venom affects cockroach locomotion, the effects of venom on a female cockroach's reproductive cycle and organs will be studied. Cockroaches lay a protective shell that encloses their eggs in order to prevent them from drying out or being eaten by other

insects called an ootheca (Brunet, 1952) (Figure 3). Recently, an undergraduate colleague named Cebrina Nolan observed ootheca-less egg laying behavior in naturally stung female cockroaches. Ootheca formation is a five-day cycle in which yolk formation gradually increases from day one, peaks on days three and four, and is finally laid on day five (Bell, 1969) (Figure 3). It is hypothesized that the purpose of disruption in a host's reproductive system is to redirect nutrients and resources back into the abdomen of the cockroach for the wasp larva to consume.

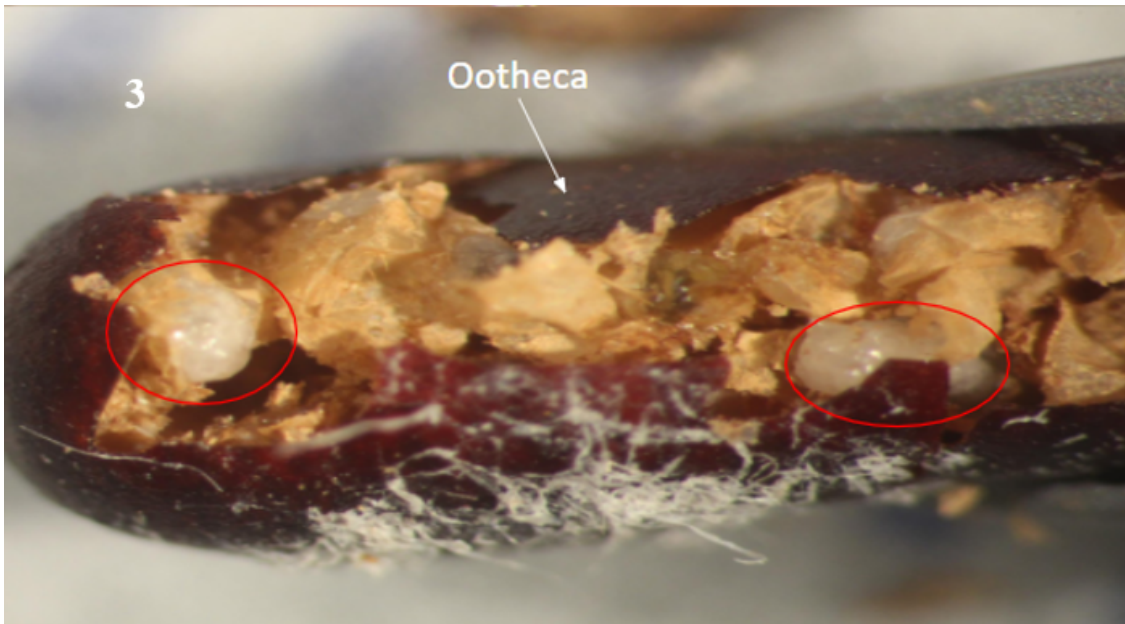


Fig. 3. An ootheca labeled with a white arrow along with individual eggs labelled in red circles.



Fig. 4. Picture of a female cockroach exhibiting ootheca-less egg behavior taken by Cebrina Nolan. Egg is circled in red.

A possible cause of ootheca-less egg behavior may be due to a neuropeptidergic neurohormone known as Corazonin (CRZ) that is present in all insects except for Coleoptera (Predel, et al., 2006). Although CRZ's functions vary between insect species, a study done on ponerine ants (*Harpegnathos saltator*) and fruit flies (*Drosophila melanogaster*) demonstrated that CRZ within the brain plays a role in the expression of brain and fat body vitellogenin genes. The vitellogenin gene encodes polypeptides that are precursors to yolk protein. Injections of CRZ peptide into the brains of ponerine gamergytes inhibited vitellogenin expression in the fat body and reduced ovary activation by reducing the number of mature oocytes. Additionally, the knockdown of CRZ receptors within the brains of fruit flies resulted in an increase in vitellogenin gene expression and egg deposition (Gospocic, et al., 2017). Interestingly, Corazonin is a component of Jewel Wasp venom (Ardivson, et al., 2019). It is possible that CRZ within the venom plays a key role in ootheca-less egg laying behavior because CRZ has been known to reduce reproductive abilities in ants and fruit flies.

METHODS

***P. americana* collection:**

Virgin female cockroaches that have been raised in a room at a temperature of 23°C and 50-65% humidity will be obtained from a 55-gallon trash can containing dog kibble and water (A virgin cockroach is indicated by a white or light brown coloration). Virgin females were staged ten days prior to post-ecdysis in separate plastic cages and were provided dog kibble and water as needed. Ten days after the initial collection, females will be mated by placing two healthy males into the cage and will be given 3-5 days to mate. Ootheca production will be monitored

and after three successful ootheca cycles (three ootheca laid) females will be ready for injection (Figure 5).

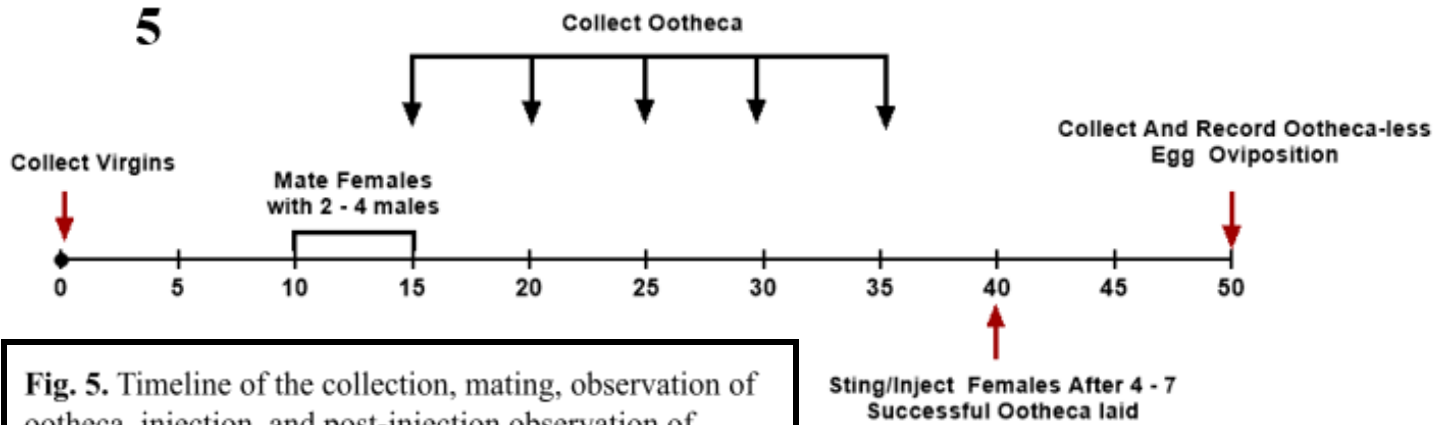
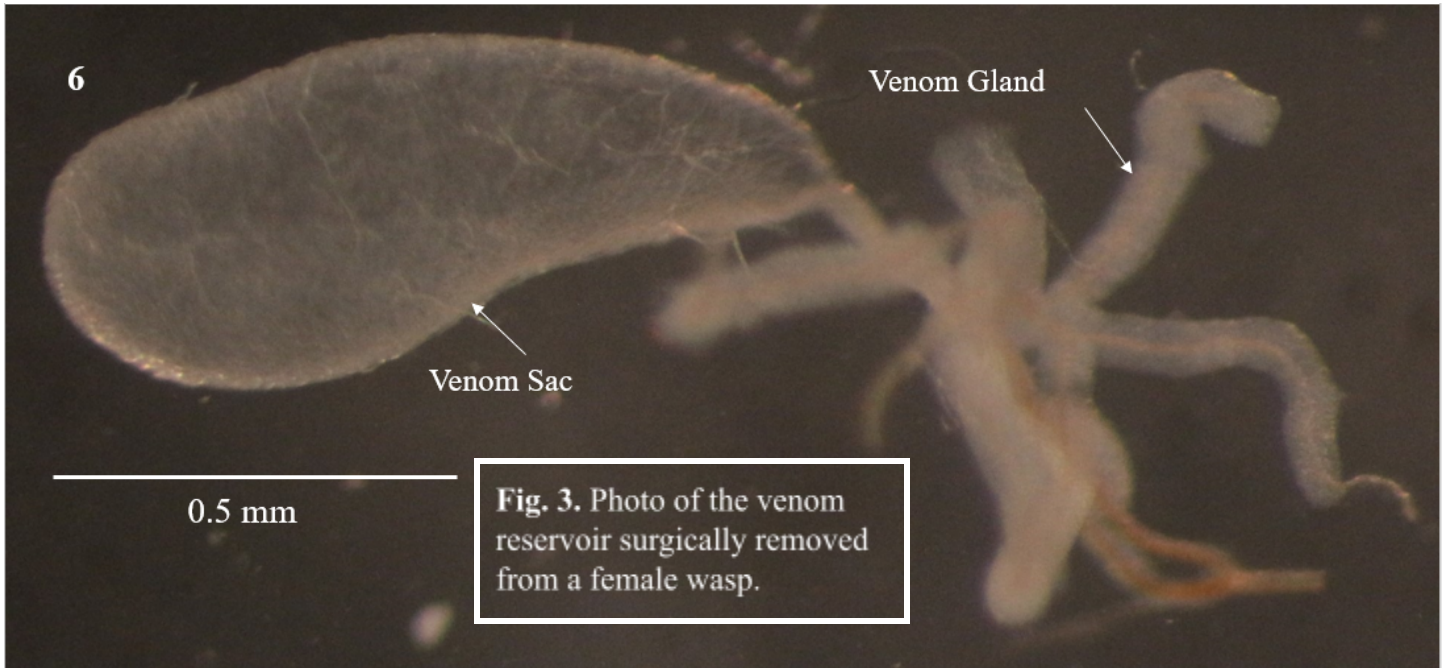


Fig. 5. Timeline of the collection, mating, observation of ootheca, injection, and post-injection observation of ootheca of female cockroaches

Venom Extraction:

One female wasp is collected from the main female bulk cage using a 50 ml conical tube and anesthetized in a plastic cup in a styrofoam container filled with ice for approximately 10-15 mins. All female wasps in the bulk cage are given honey and water as needed. All tools needed for the dissection are sterilized and prepared (such as small and large pair of dissecting scissors, two pairs of dissecting tweezers, 2 pins, dissecting dish, and Phosphate buffer solution - PBS) by wiping all metallics down with 70% ethanol and a Kim wipe. The legs, wings, and head of the wasp are removed using a large scissor and disposed of. The wasp is then placed ventral side up in the dissection dish with one pin through the thorax and below the pinched waist. The dissection dish is filled with PBS until the wasp is fully submerged. Using a stereomicroscope, the abdomen is slowly cut into sections to create flap-like incisions. Then the cuticle is pulled apart horizontally and cut, revealing the internal organs of the wasp. The venom sac lies

underneath the ovaries and is tangled within the venom gland (Figure 3). The ovaries are carefully removed and the venom sac is slowly untangled from the venom gland. A cut is made at the collecting tube to separate the venom sac from the venom tubules as well as the stinger.



Acquisition of Venom for Injection:

The venom sac is transferred from the dissection dish to a pre-made gel dish containing mineral oil. The venom sac is then poked with a pin/needle until all components of the sac are released, creating a spherical bubble. The remaining muscle fibers are removed. The dish containing the venom bubble is analyzed under an ocular micrometer that has been calibrated with a stage micrometer to determine the diameter of the bubble (Figure 4). All final measurements are converted to microns and the equation of a sphere is used to roughly estimate the volume of venom within the venom sac. Using a Drummond Nanoject II (Drummond Scientific, Broomall, PA) and microcapillaries beveled to have a 20 μ m diameter, 32.2 nL of venom directly from the spherical bubble was sucked up.

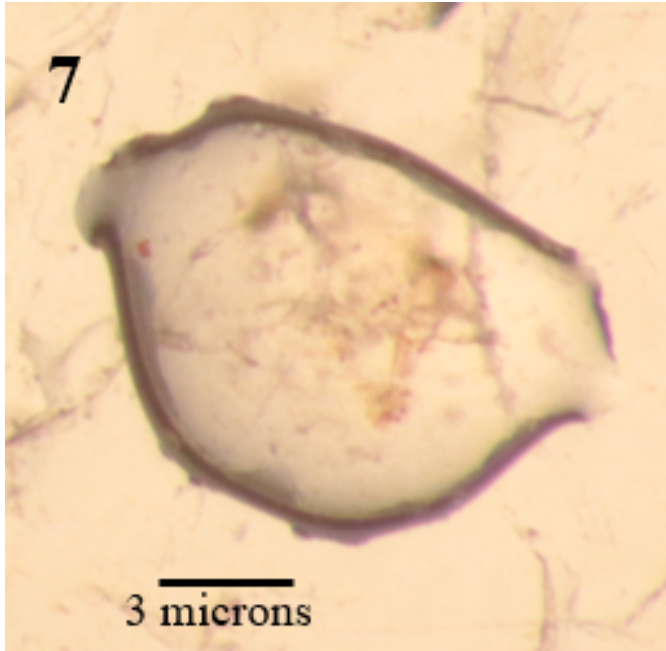


Fig. 7. Bubble of crude venom in mineral oil. The bubble of venom is treated like a sphere and therefore the volume of the bubble can be calculated using the radius of the sphere as well as the equation of the volume of a sphere ($\frac{4}{3}\pi r^3$).

Injection into the Central Complex:

Female cockroaches will be taken out from their cage and placed into small plastic cups. Female cockroaches will be anesthetized in plastic cups in a styrofoam container filled with ice for 10-15 mins (Arvidson, et al., 2018; Emanuel & Libersat, 2017). The cockroaches were set dorsal side up and held down with U-shaped pins at its neck and mouth while the rest of its body was held down with modeling clay (Kaiser & Libersat, 2015) all on a Peltier cold-plate set to 4°C. The U-shaped pin on the neck helped to restrict pulsation movements of the brain but was ineffective at stopping coagulation. Using a scalpel, a triangular incision (creating a flap that peeled posteriorly) was made on the cockroach head, revealing its brain. Using the Drummond Nanoject II, 32.2nL of venom was injected at a 90° angle right below the cerebral hemispheres (Figure 8). Disclaimer: No one knows how much venom the wasp injects into the central complex. This value is a rough estimate of how much venom is used since the total amount of venom within the sac was calculated to be 113 nL (equation of the volume of a sphere was used).

Similar steps were taken to inject cockroaches with corazonin and its scrambled peptide counterpart (control). 36.8 nL of corazonin and scrambled peptide dissolved in cockroach saline with 0.1% Janus Green B as a tracer were injected into the central complex of cockroaches. All cockroaches that were injected had their brains vibratome-sectioned to confirm a successful injection into the central complex.

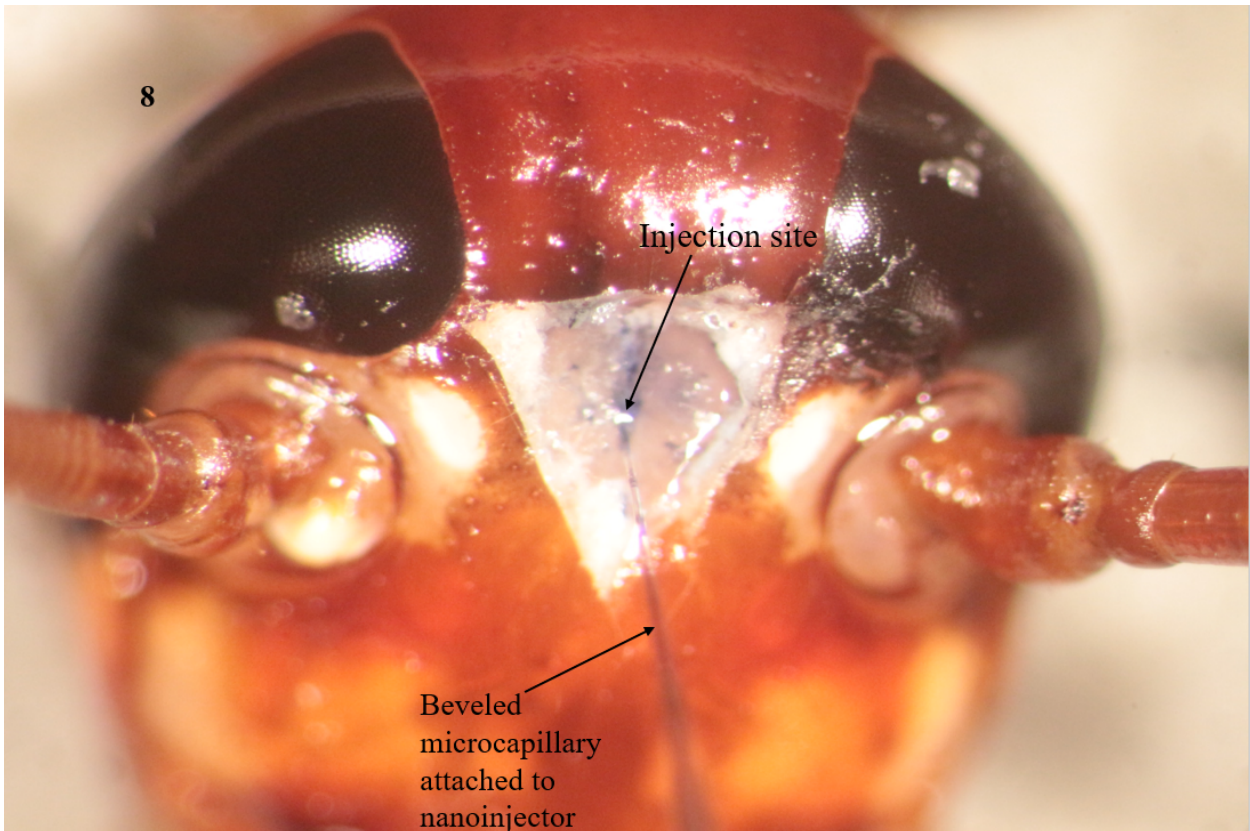


Fig. 8. Mock injection of 32.2nL of saline traced with 0.1% Janus Green using Drummond Nanoinject II and 20 μ m beveled microcapillary at a 90° angle right below the cerebral hemispheres.

Testing Escape Response and Spontaneous Walking:

After injection, cockroaches are placed into a 25 cm diameter arena and are allowed to acclimate for 15 mins. Escape response is measured by touching the antenna, cerci, and wings of

the cockroach with a small paint brush every minute for 10 mins (Figure 9). Spontaneous walking will be determined by splitting the circular arena (with tape) into four quadrants and recording how many quadrants the cockroach passes every minute for 30 mins (Weisel-Eichler & Libersat, 2002) (Figure 10).



Fig. 9. Escape Response analysis by touching the cerci of a cockroach via paintbrush.



Fig. 10. 25 cm diameter arena divided into four quadrants with red tape used in spontaneous walking experiments.

Observing ootheca-less egg laying behavior:

After escape response and spontaneous walking is recorded, all female cockroaches are returned to their original cages that have been cleansed, replenished with food/water, and have had the males taken out. Female cockroaches will be observed for ootheca-less egg laying behavior for the next few months and all resources are provided when necessary.

RESULTS

Spontaneous Walking and Escape Response Behavior

Spontaneous walking behavior was obtained by recording the number of times a cockroach passes a quadrant within a thirty minute time span. Due to COVID-19, the data for stung and control groups were not obtainable for this project. The median number of quadrants passed for a sample size of eight animals was 10 with a range of 60 (Figure 1A). The results were graphed based on previous results obtained by researchers Weisel-Eichler and Libersat that showed the effects of reserpine (a drug known to induce lethargy) on cockroaches. In their experiment, they tested four different groups and the number of times a cockroach passed a quadrant in 10 mins.

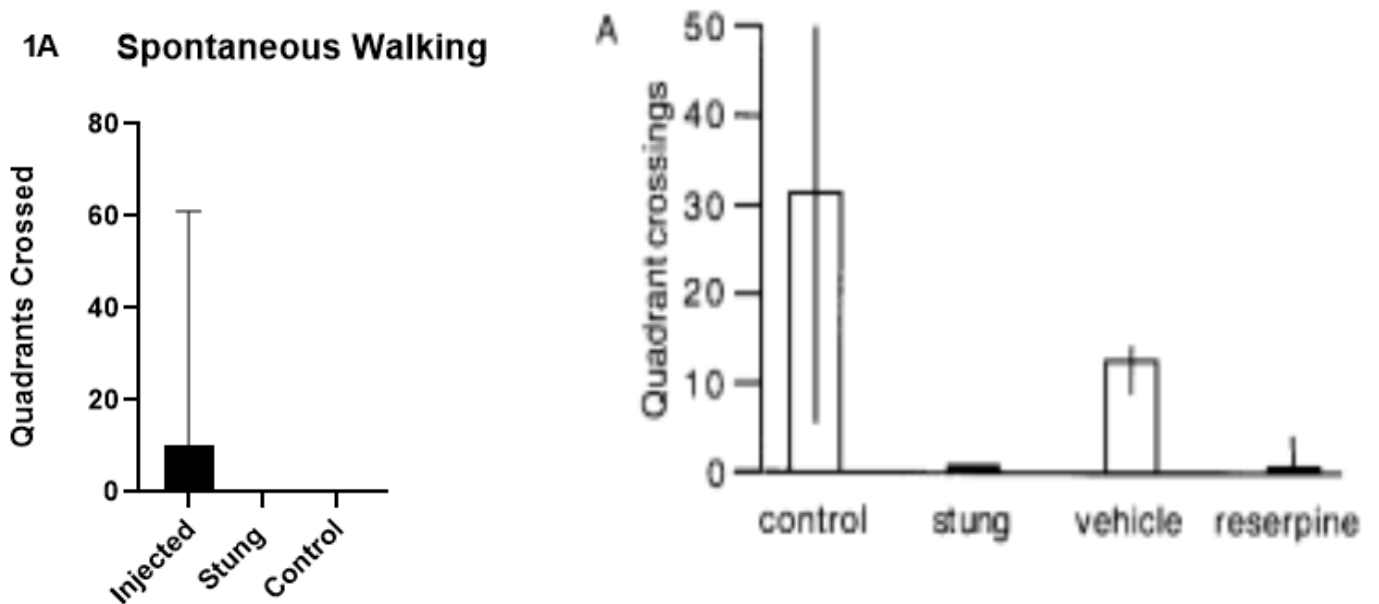


Fig. 1A (left). Crude Venom effects on spontaneous walking in cockroaches. Y-axis indicates number of times animal crossed quadrants in 30 mins. (Injected: $n = 8$, median = 10, range = 60). Due to COVID-19, stung and control data were not obtainable.

Fig. 1A (right). Data obtained by Weisel- Eichler and Libersat recording the effects of reserpine on spontaneous behavior. Y-axis indicates number of times animal crossed quadrants in 10 mins. ($P < 0.001$; Mann-Whitney U-test; stung: $n = 5$, controls: $n = 8$). Data shown are medians and range.

Escape Behavior was obtained in an arena by touching a cockroach with a paintbrush on its cerci or wings every minute for 10 minutes. The results of the experiment showed that there was a pattern of decreasing and increasing escape behavior within the first eight minutes after injection followed by a sharp increase in escape behavior at the end of ten minutes (Figure 2A). The results were graphed based on previous experiments done by Weisel-Eichler and Liberstat that showed the effects of flupentixol (an antipsychotic neuroleptic drug) over the course of sixty minutes.

2A Escape Response Behavior

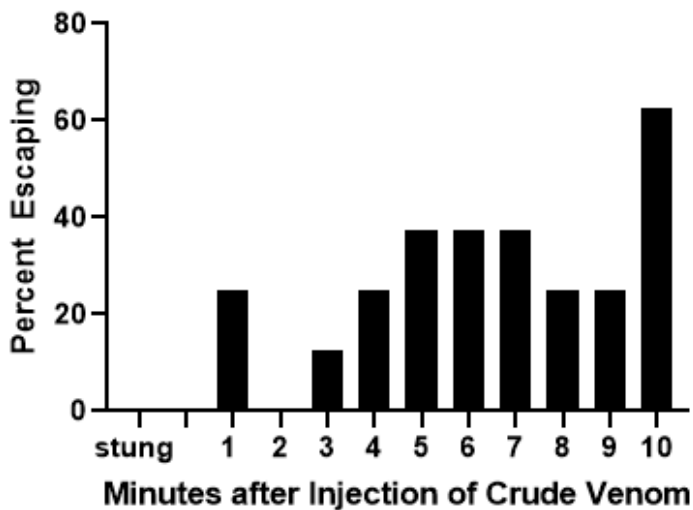
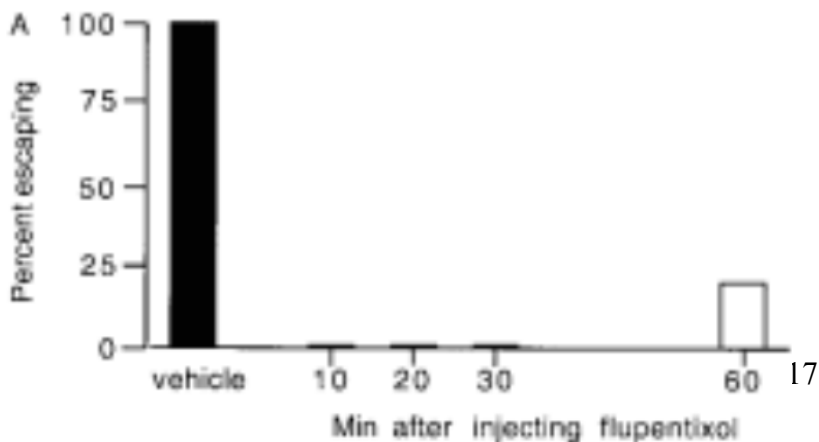


Fig. 2A (top). Crude Venom effects on escape behavior measured over a 10 min time span. Each minute cockroaches were touched on their cerci and wings with paintbrush. ($n = 8$). Stung data is unavailable due to COVID-19.

Fig. 2A (bottom). Data obtained from Weisel-Eichler and Liberstat determining the effects of flupentixol over a time range of 60 mins. ($P < 0.01$; Fisher's exact test; $n = 10$ each).



Observational data of ootheca-less egg laying behavior

Experimental data conducted in collaboration with Cebrina Nolan during 2019-2020 are shown throughout the results section. Observational data of ootheca-less egg laying behavior found in stung, corazonin-injected, and control individuals were recorded. The results showed that all stung cockroaches resulted in ootheca-less egg laying behavior compared to only 8.33% of the control group when injected with 36.8nL of scrambled peptide. When injected with 36.8 nL of corazonin dissolved in cockroach saline with 0.1% Janus Green B, the percentage of corazonin-injected individuals that exhibited ootheca-less egg laying behavior was 42.8% (Figure 3A). After the initial sting or injection the first occurrence of ootheca-less egg oviposition was observed and recorded. The stung group resulted in an average of nineteen days post-treatment to start laying ootheca-less eggs while the corazonin-injected group resulted in an average of twenty days post-treatment to start laying ootheca-less eggs (Figure 3B).

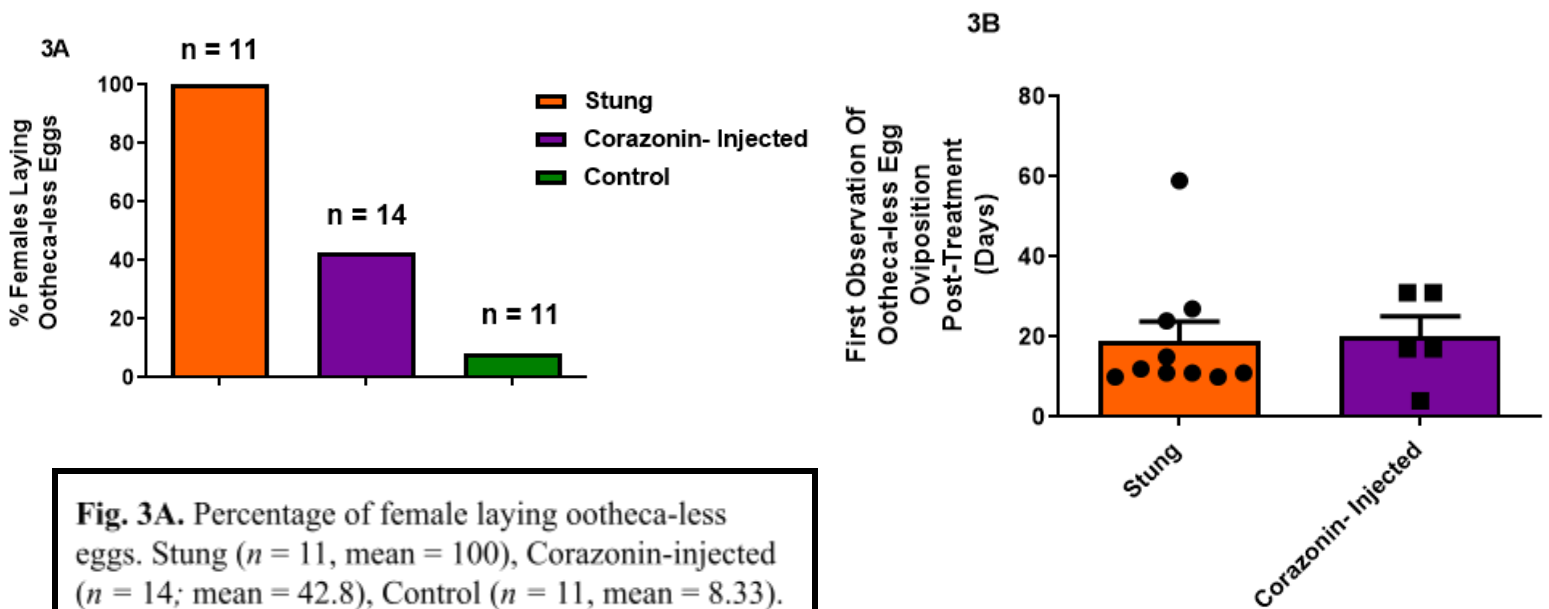
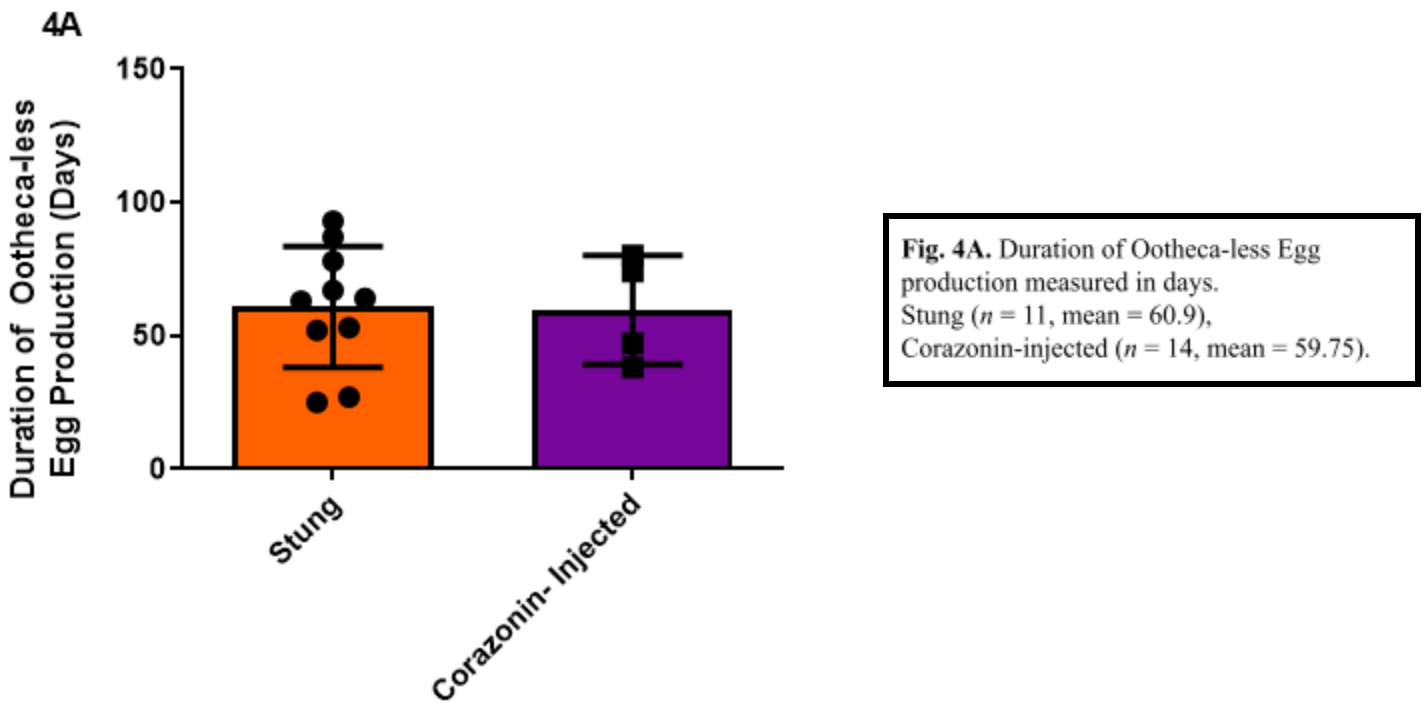


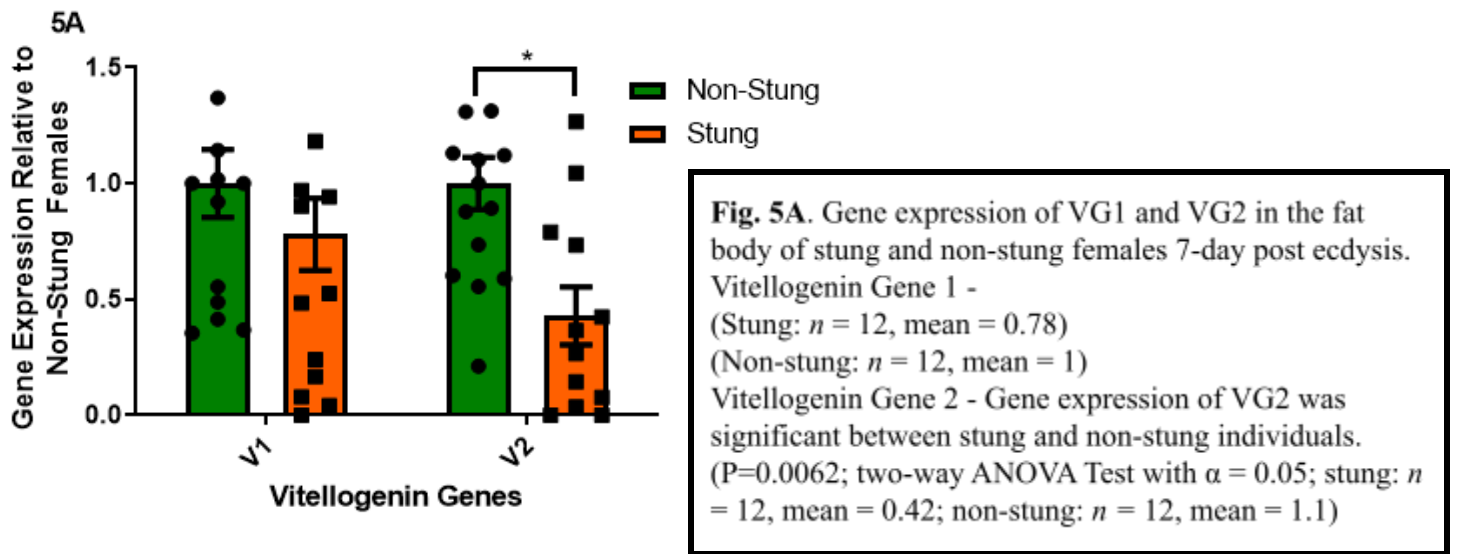
Fig. 3A. Percentage of female laying ootheca-less eggs. Stung ($n = 11$, mean = 100), Corazonin-injected ($n = 14$; mean = 42.8), Control ($n = 11$, mean = 8.33). **Fig. 3B.** First Observation of ootheca-less egg oviposition after treatment. Stung ($n = 11$, mean = 19). Corazonin-injected ($n = 14$, mean = 20).

Stung and corazonin individuals were observed months after the sting/injections to observe the possible total duration of ootheca-less egg production. The stung group reported an average of 61 days of ootheca-less egg production after the initial sting. Meanwhile, the corazonin-injected group reported an average of 60 days of ootheca-less egg laying behavior after the initial injection (Figure 4A).



Venom and Corazonin Effects on Vitellogenin gene expression

The effects of venom on the expression of VG2 gene expression between stung and non-stung cockroaches were analyzed. Envenomation in stung cockroaches resulted in a significant decrease of VG2 gene expression compared to non-stung cockroaches ($P=0.0062$) while the difference in VG1 gene expression between stung and non-stung was not significant (Figure 5A).



When comparing the effects of venom from a natural sting to the artificial injection of corazonin, corazonin treated cockroaches also exhibited a significant decrease of VG2 gene expression compared to non-treated individuals. Similarly, there was no significant difference in VG1 gene expression found in non-treated vs corazonin-injected cockroaches as previously seen in non-stung vs stung (Figure 6A).

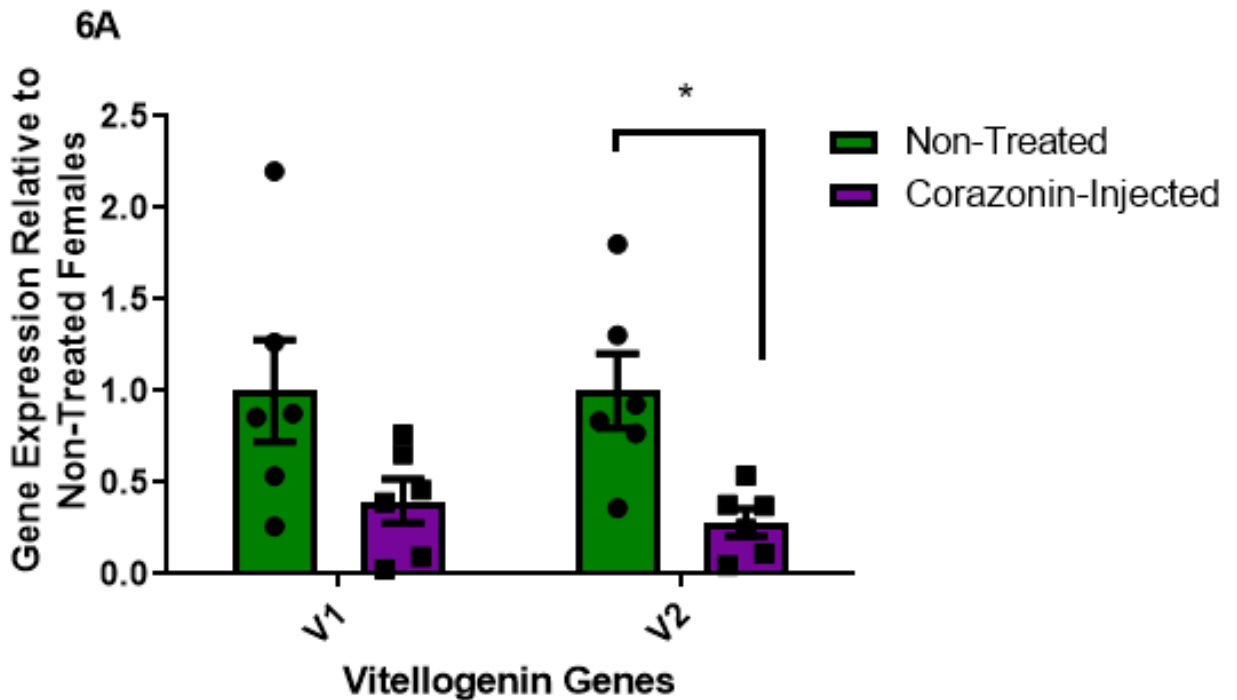


Fig. 6A. Gene Expression in the fat body of non-treated and corazonin-injected females 7-days post ecdysis. Vitellogenin Gene 1 - (Non-treated: $n = 12$, mean = 1). (Corazonin-injected: $n = 14$, mean = 0.39). Vitellogenin Gene 2 - Gene expression was significant between non-treated and corazonin-injected individuals. ($P=0.0048$, two-way ANOVA with $\alpha = 0.05$; non-treated: $n = 12$, mean = 1; corazonin-injected: $n = 14$, mean = 0.28).

Results from the expression of vitellogenin genes in the fat body of 7-day old virgin females that were stung on the 6th day post-adult ecdysis between corazonin-injected and scrambled peptide individuals proved to be interesting. Although insignificant, scrambled peptide-injections seemed to have a larger effect on decreasing VG gene expression compared to the injection of corazonin (Figure 7A). The scrambled peptide used in this experiment was a scrambled version of the corazonin peptide which was ordered from a lab company. One would expect to see the opposite effect take place since the scrambled peptide served as a control and should not have had any effects on VG gene expression. Corazonin should have had a bigger effect on VG gene expression instead of scrambled peptide. A possible error during the experiment was that corazonin and scrambled peptide were accidentally switched during the injection process leading to these results.

Relative Expression of Vitellogenin Genes in Fat Body of Virgin Females Seven Days Post Adult Ecdysis

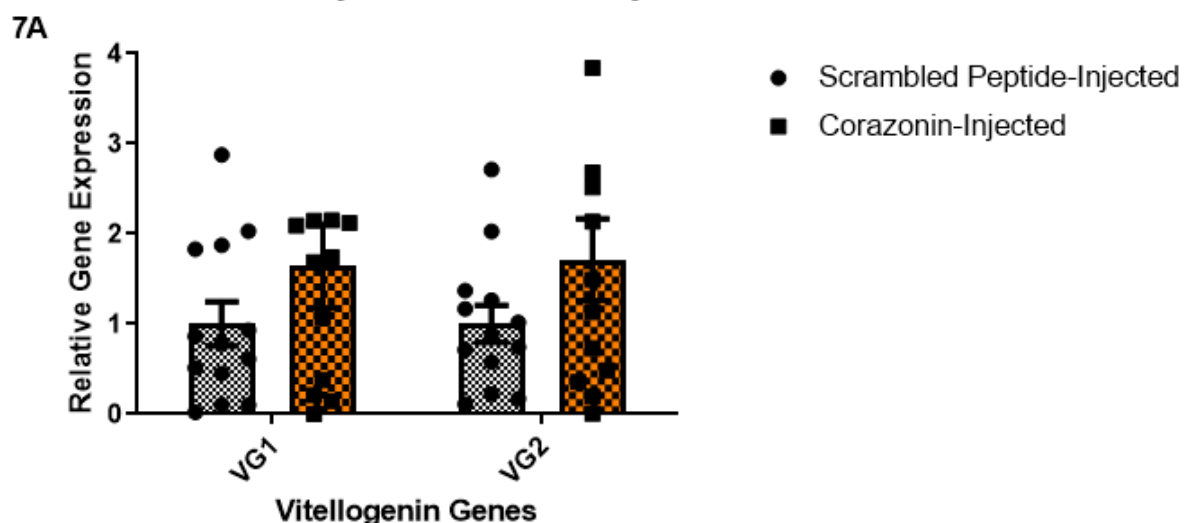


Fig. 7A. Gene expression of VG1 and VG2 in the fat body of scrambled-peptide injected and corazonin-injected females 7-days post ecdysis. Vitellogenin Gene 1 - (Scrambled-peptide Injected: $n = 8$, mean = 1). (Corazonin-Injected: $n = 12$, mean = 1.64). Vitellogenin Gene 2 - (Scrambled-peptide Injected: $n = 8$, mean = 1). (Corazonin-Injected: $n = 12$, mean = 1.71). Two-way ANOVA tests were conducted that produced no significant differences of gene expression between scrambled peptide and corazonin injected cockroaches. In fact, the data of scrambled and corazonin injected seemed to have opposite effects on vitellogenin gene expression since scrambled-peptide acted as the control and would have little to no effect on VG gene expression while corazonin should have had a more impactful role in decreasing VG gene expression.

Discussion

The purpose of this project was to uncover the biochemical strategies the Emerald Jewel Wasp utilizes in order to subjugate its host in order to maximize its reproductive success. By artificially injecting venom obtained from the venom sac into the central complexes of cockroaches, altered locomotory and reproductive behavior was observed. The experimental techniques used to analyze the venom's effect on cockroach locomotion were heavily influenced by previous work done by scientists Weisel-Eichler and Liberstat. It is important to note that their experiments revolved around injecting lethargy inducing drugs into the hemolymph of cockroaches while this project revolves around injecting crude venom into the CX. By using similar methods, spontaneous walking and escape behavior data on artificially injected venom was collected, however, due to COVID-19 data on stung and control groups were not obtainable. According to the spontaneous walking data, it would seem that artificially injected venom to the CX seemed to have a similar effect to a natural stung roach or reserpine injected roach. However, since there are no control and stung groups, no statistical tests were implemented. Moreover, escape response data regarding crude venom injections into the CX seemed to not have a significant impact on escape behavior compared to flupentixol injected roaches. This discrepancy may be explained by the difference in injection location. It is possible that injection directly into cockroach hemolymph has a larger impact on locomotory behavior compared to the central

complex since insects have an open circulatory system. Injection into the hemolymph may allow certain chemicals or neurotransmitters better access to the neuronal systems responsible for spontaneous walking and escape behavior compared to injections into the CX. Nevertheless, artificial injection of crude venom into the CX did decrease escape response in the earlier half of the ten minute range and spike in increased escape response behavior may be attributed to the cockroach slowly recovering.

Due to COVID-19, data presented in this project regarding ootheca-less egg laying behavior were obtained around 2019 in collaboration with Cebrina Nolan. According to the data, corazonin-injected individuals did exhibit ootheca-less egg production but not to the extent of naturally stung cockroaches. Moreover, the mean values of first observed ootheca-less egg behavior post-treatment as well as duration of ootheca-less egg production between stung and corazonin-injected individuals were very similar. These results may indicate that corazonin may play a role in reproduction in cockroaches. Corazonin effects on relative vitellogenin gene expression, specifically vitellogenin gene 2, in both non-stung vs stung and non-treated vs corazonin-injected proved to be significant. Therefore, corazonin may play a role in decreasing vitellogenin expression (VG2) and ultimately decreasing egg yolk production in cockroaches, which can explain why stung roaches exhibit ootheca-less egg laying behavior. On the other hand, there was no significant difference in vitellogenin gene expression of VG1 in both non-stung vs stung and non-treated vs corazonin-injected. This may be due to the fact that these injections/experiments were conducted back in 2019 when central complex injections were not yet refined and still had problems such as the pulsation and coagulation of a cockroach's brain making it difficult to hit the CX. Results of relative vitellogenin gene expression in both VG1

and VG2 proved to be interesting, not because there were no significant differences between scrambled peptide-injected vs corazonin-injected individuals, but because of the impact on gene expression each one had. Scrambled peptide-injections served as the control group in this experiment yet seemed to have a larger impact on decreasing vitellogenin gene expression in both VG1 and VG2. Likewise, corazonin-injections did not seem to have any impact on this experiment with an average gene expression mean of 1.69 compared to an average gene expression mean of 0.335 in the non-treated vs corazonin-injected experiment. This phenomenon may be attributed to a mix-up error during injections (i.e scrambled peptide-injected individuals may actually be corazonin-injected individuals but were labeled incorrectly or the scrambled peptide and corazonin samples were accidentally switched during the injection process).

It is important to note that in nature, the wasp will sting the cockroach in both the central complex and the suboesophageal ganglion but the experiments conducted in this project only revolved around CX injections. In the future, further research and experiments need to be done on just injecting the SEG with crude venom as well as injecting both the SEG and CX at the same time and recording the effects. It is stated in the literature that injections only into the CX and SEG can result in altered locomotory behavior (hypokinesia), however, those experiments used lethargic/numbing inducing drugs such as procaine and not pure venom obtained directly from the venom sac (Kaiser & Libersat, 2015). As for corazonin affecting vitellogenin gene expression, there could simply be other proteins or enzymes that aid corazonin in reducing egg yolk production or reducing ovary activation. Although corazonin does have an impact on vitellogenin gene expression, that impact was found within ant and fruit flies. When it comes to cockroaches there are possibly other components of the venom that help induce ootheca-less egg

laying behavior. By studying how the Emerald Jewel Wasp subjugates its host for the benefit of its progeny, new and exciting research on the mechanisms of how parasites and parasitoids are able to subdue their prey can be better understood.

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