EXPLORING NEUROMODULATORY EFFECTS OF ADENOSINE DEAMINASE AND CORAZONIN IN EMERALD JEWEL WASP VENOM

By

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ABSTRACT:

The parasitoid jewel wasp *Ampulex compressa* induces hypokinesia (a sleep-like state) and reduced fecundity in its host, the American cockroach *Periplaneta americana*, through direct envenomation of its central nervous system (CNS). Over 250 venom components discovered through transcriptome analysis, I chose to investigate actions of adenosine deaminase (ADA) and corazonin (CRZ) on the host brain. To confirm presence of venom ADA, I applied venom to mammalian cells expressing cockroach adenosine receptor (AR) with fixed concentrations of adenosine (ADO). I investigated effects of ADA on insect locomotion by directly injecting it into the central complex (CX), known to be targeted by the wasp sting. I also attempted to interfere with adenosine signaling by silencing AR gene expression using the model organism *Drosophila melanogaster*. Recent studies of CRZ in ponerine ants suggested that this peptide suppresses fecundity. Therefore, Ampulex CRZ was injected into the CX and changes in fecundity was monitored. In particular, I measured relative gene expression of vitellogenin (VG) which are yolk proteins, in addition to measuring average protein content and average volumes of the ovarioles in stung, non-stung, and CRZ-injected females. Furthermore, egg-laying behaviors of envenomated and CRZ-injected gravid females were investigated. Results of my research indicate that decreased AR signaling has no effect on spontaneous movements or escape response. Thus, the role of venom ADA may be to alter the physiology of the host to make it optimal for larval consumption. Both stung and CRZ-injected gravid females displayed ootheca-less egg-laying behaviors and undeveloped ovaries. However, only envenomated females exhibited a decrease in VG gene expression. It is suggested that the role of CRZ in the venom may be to suppress ovary development and preserve energy within females. Results of my research elucidate alternate mechanisms of how venoms can inhibit reproductive abilities as well as provide further insight on the effects of ADO and CRZ in the CNS of arthropods.

ACKNOWLEDGEMENTS:

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

The emerald jewel wasp (*Ampulex compressa*) produces a "venom cocktail" that alters the internal biochemistry of its host, the American cockroach (*Periplaneta americana*), when injected into its cephalic ganglia [1]. This results in a behavioral change (hypokinesia, a sleep-like state), whereby avoidance of noxious stimuli is impeded. However, unlike most venoms, which induce paralysis, the cockroach retains most of its motor functions [1, 2]. Recent findings in the Adams lab demonstrate that the venom also obstructs the reproductive abilities of gravid female cockroaches by inhibiting formation the oothecal case (**Figure 1**). As a result, the stung, gravid, female oviposits its eggs without any protection, rendering the eggs vulnerable to desiccation and predation. This may be beneficial to the wasp by forcing the cockroach to retain most of its protein for wasp larval consumption. After analyzing the venom, over two-hundred protein components were identified. Most of the identified peptides are not associated with insect behavior modulation or found in other arthropod venoms [2]. I investigated the genetic and behavioral alterations brought about by two venom proteins, adenosine deaminase (ADA) and corazonin (CRZ), both which have not been identified in venoms before.

Figure 1: Picture taken of a gravid, stung, female ovipositing eggs without an ootheca.

The neuromodulator adenosine (ADO) and the enzyme that breaks it down, adenosine deaminase (ADA), are known to play significant roles in brain signaling and function [3]. Therapeutic modifications of adenosine receptor (AR) signaling are used for treatment of many brain injuries and abnormalities such as ischemia and Parkinson's disease [4, 5]. In addition, elevated levels of ADO in ADA-deficient mice and humans result in motor dysfunction, lower IQ, and alterations in brain morphology [6]. In addition, ADA's presence

in arthropod venoms or venom-like secretions is limited to its presence in the saliva of blood-feeding Diptera (e.g. *Lutzomyia longipalpis* and *Aedes aegypti*) [7, 8]. It is hypothesized that actions of this enzyme in saliva of Diptera is to create inosine for blood vessel dilation and reduction of platelet aggregation [7-9]. Despite the plethora of research studies on mechanisms of ADO and ADA in the mammalian CNS, their functions in the CNS of insects are not well understood. Understanding the effects of ADA and ADO in the insect CNS could provide fundamental knowledge of how this neurotransmitter signaling system regulates physiological behaviors in all animals.

CRZ is a highly conserved neuropeptide that is present among all insect orders except for Coleoptera; however, CRZ signaling results in a cascade of physiological changes that vary between species. Thus, no common functions of CRZ between insect species have been identified [10]. A more recent study using ponerine ants (*Harpegnathos saltator*) and fruit flies (*Drosophila melanogaster*) revealed that increased CRZ signaling in the brain reduces the expression of brain and fat body vitellogenin (VG) genes, which encode yolk proteins. When injected with CRZ peptide in the brain, ponerine gamergytes transformed into infertile workers. In addition, fruit flies highly expressing the CRZ peptide in the brain produced fewer eggs or expressed higher VG proteins when their CRZ receptor was knocked down [11]. Surprisingly, CRZ is highly expressed in the venom and activates insect CRZ receptors [2]. When stung by the jewel wasp, gravid female cockroaches lose their reproductive abilities similar to CRZ-injected ponerine ants. Thus, it is possible that venom CRZ is the main cause for this reproductive loss in stung, gravid cockroaches.

My research project was divided into three parts: 1) to identify a functional ADA in the venom, 2) to investigate the behavioral effects resulting from decreased ADO signaling in the CNS of insects, and 3) to study the reproductive consequences brought about by the venom and the venom CRZ peptide in the CNS of gravid females. Presence of ADA in the

venom was confirmed by applying venom mixed with fixed concentration of ADO to WTA11 cells expressing cockroach AR. Effects of decreased ADO signaling were investigated via direct injections of bacterial ADA into the central complex (CX) (area of CNS involved in locomotion) of 1-week old male cockroaches. Effects of ADA injections were assessed by recording spontaneous activity and escape response of ADA-injected males before and following treatment and by comparing their activity to denatured ADA-injected controls. In addition, the effects of decreased AR signaling was investigated by observing the activity of transgenic fruit flies (*Drosophila melanogaster*) with their AR silenced in the CX via RNA interference (RNAi). CRZ and venom effects on female reproduction were observed by recording ootheca-laying behaviors, measuring protein content and volumes of ovarioles, and by monitoring VG gene expression of CRZ-injected, stung, and non-treated females. The obtained results suggest that venom ADA and decreased AR signaling has no effect on cockroach locomotion and may play a role in altering the physiology of the insect. In addition, CRZ-injected and stung females possessed smaller ovarioles and had less protein in their ovaries compared to non-treated controls. The results of my research expose novel mechanisms of venoms in altering host behavior and physiology for ideal larval consumption. In addition, these mechanisms provide fundamental knowledge on the functions of the arthropod CNS.

METHODS

P. americana and *A. compressa* Husbandry:

Methods for wasp and cockroach have been previously described [2, 12]. Single female wasps were housed with three males in 40 cm (W) x 40 cm (L) x 52 cm (H) plexiglass cages. All wasps were reared in the laboratory vivarium at 26°C and 50–65% humidity on a 16:8 light:dark cycle. Water and honey were provided *ad libitum*. Individual adult female cockroaches were introduced into cages five times per week for parasitization. *P. americana*

were reared in 55-gallon trash cans and were provided water and kibble dog food *ad libitum*. Aluminum strips connected to a battery were attached near the top rims of the cans to provide a mild shock to roaches attempting to escape. For gene expression and behavior experiments, freshly ecdysed female and male cockroaches were collected daily and placed in the wasp vivarium for 1 week. Experiments on ovary development were done using virgin females collected and staged for 12 days in the vivarium.

Fly Strains and Genetics

Fly strains were reared at 18 °C and 50-60% humidity on a standard cornmeal–molasses medium with a 12-hr light–12-hr dark cycle. When collecting virgin flies, stocks were kept at 25 °C during the day and left in 18°C overnight. AR RNAi lines TRiP.JF02687 and TRiP.HMC03684 and Gal4 lines c119 and c819 for expression in the CX were purchased from the Bloomington Stock center in Indiana. Gal4 lines c547, c232, and c42 also providing tissue-specific expression in the CX were provided by Dr. Heberlein Ulrike. All Gal4 lines contain the Gal4 gene on the 3rd chromosome in exception to Gal4 c119 line which contains the Gal4 gene on the X chromosome. To enhance UAS-RNAi effects, the dicer-2 gene was crossed into 2nd chromosome of the RNAi fly lines; thus, the genotype of test flies are +/+; UAS-RNAi/UAS-Dicer-2; Gal4/+ while TRiPattP2 controls contain the same genotype as tests without the UAS-RNAi sequence on the 2nd chromosome. Dicer-2 and double balancer lines for crosses were provided by Dr. Naoki Yamanaka. Test and control crosses were kept at 25°C at 50-60% humidity and with a l2:12 hour light:dark cycle. 3-day old Gal4-UAS-RNAi virgin females were utilized for experiments monitoring their spontaneous activity. Gal4-UAS RNAi-dicer-2 test flies were compared to the activity of Gal4-TRiPattP2-dicer-2 controls. Methods for activity recordings have previously been described [13-15]. Locomotory assays were initiated at 6 PM and measured using the DAM2 drosophila activity

monitor (TriKinetics Inc, Waltham, MA). During experiments, flies were provided with enough food to last one week. The monitor and the flies were placed in an incubator set at 25°C and 50-60% relative humidity with a 12-12 light:dark cycle. (7 AM-7 PM). Activity data was collected and pooled for 3 days and analyzed using Graphpad Prism version 6 (La Jolla, CA).

RNA Extraction and qPCR:

Table 1: List of Primers Used to Measure Relative VG Expression in Control and Treated Animals

Primer Name	Sequence and Position in cDNA
VG1 F	CTGCGTCCAGAGGATTCGAATTG (5216 to 5238 bp)
VG1 R	ATTATGCAAATCAGAATTAGAGCA (5324 to 5346 bp)
VG2 F	ATATTCCACCTCCGAGGCCACCAA (917 to 940 bp)
VG2 R	CTGCTATCTGAGTTACTTTCTAG (1015 to 1037 bp)
Actin F	TGACTGAGCGTGGTTACAGC (330 to 394 bp)
Actin R	CAGGAAGGAAGGTTGGAACA (534 to 553 bp)

Test subjects were stung or injected with synthetic *A. compressa* CRZ peptide on day 6 post-adult ecdysis. Fat body tissue was collected on the 7th day and stored immediately in -80°C. Total RNA was extracted using a Purelink RNA Minikit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Following RNA extraction, RNA samples were treated with DNAse using RNAse-Free DNase 1 (New England Biolabs, Ipswich, MA) and purified using pH 6 phenol/chloroform/isoamyl alcohol (25:24:1) (Thermofisher Scientific, Waltham, MA). The quality and concentration of RNA samples was determined by using a NanoDrop 2000 spectrophotometer (Thermofisher). A modified phenol/chloroform protocol was used for the second purification and is provided in the **supplemental information** section. To create cDNA, 500 ng of DNase-treated RNA was reverse transcribed using a Protoscript II First Strand cDNA kit and anchored oligoT primers (England BioLabs, Whitby, Ontario) according to the manufacturer's instructions. Primers for vitellogenin genes and the housekeeping gene have already been created and were used for expression analysis (**Table 1**) [16]. Primer efficiency was calculated, and melt curves were drawn using CFX Connect Real-Time PCR Detection System software (Bio-Rad). The efficiency for all primers were found to fall in the acceptable efficiency range of 94-105% and melt curves ensured that a single amplicon product was made. Quantitative PCR was carried out using this same software with amplification cycles set at 95 °C for 3 min, then 39 cycles of 95 °C for 15 sec, 55 °C for 20 seconds, and 72 °C for 20 seconds. Each reaction contained 1.2 µL of 1:10 diluted cDNA and 0.15 µL of 10 µM primers to make a total of 15 µL of reaction mix. Each reaction was assayed 3 times with the average cycle number used for analysis. Values for expression of vitellogenin genes in stung and CRZ-injected individuals were compared to that of the non-treated animals using the Pfaffl equation to provide their relative expression [17].

Ovary Volume Measurements

Methods for ovary dissections have been described [18]. Ovaries were dissected from 12-day old adult virgin females in phosphate-buffered saline as this was the peak of ovariole volume size before ovary formation. Treated females were stung or injected on the 5^{th} or 6^{th} day post-adult ecdysis. Data for ovary measurements were pooled as each did not display any significant difference from another. Following dissections, the length and width of 4 ovarioles were measured (2 from each ovary) using an ocular micrometer calibrated with a stage micrometer. Volumes of each ovariole were then calculated using the equation $2/3 \bullet \pi \bullet (\text{width}/2)^2 \bullet (\text{Length})$. Afterward, ovaries were stored in 100 mM Tris-HCl pH 8 for no more than 3 days in 4°C.

Crude Protein Extraction and Measurements

Methods for protein extraction of ootheca-less eggs, ovaries, and ootheca have previously been described [18]. Desired eggs or ovaries were homogenized in 100 mM Tris-HCl pH 8 and then centrifuged 3 times at 20,817 g for 10 minutes. The supernatant was used directly or stored no more than 3 days at 4 °C. The crude extract was measured using the Qubit protein assay kit and fluorometer (Thermofisher Scientific) according to the manufacturer's instructions.

Cloning of Cockroach AR receptor:

Methods for obtaining the cockroach adenosine receptor have been described [2]. Five male cockroach suboesophageal ganglia were dissected and total RNA was extracted using the Trizol method according to the manufacturer's instructions (Invitrogen Carlsbad, CA). cDNA was synthesized using the ProtoScript II First-Strand Synthesis kit (New England Biolabs) and mRNA was isolated using an oligo-dT primer. Primers to obtain the cockroach AR coding sequence were designed using an existing cephalic ganglion *Periplaneta* transcriptome. A Phusion Green high-fidelity polymerase (Thermofisher Scientific, Waltham,

MA) was used according to the manufacturer's instructions to amplify the coding sequence, which was then cloned into a pJet1.2 plasmid and sequenced for confirmation. The full coding sequence was then inserted into pcDNA3.1 for expression in WTA11 cells, which are Chinese hamster ovarian cells expressing aequorin.

Luminescence Assays:

Recording of AR activation was executed using WTA11 cells. Methods for rearing and transfecting WTA11 cells have been previously described [2, 19-21]. WTA11 cells were maintained in DMEM:F12 media (Gibco/Thermofisher Scientific) supplemented with 10% FBS (Millipore Sigma, Burlington, MA), 1 x antibiotic/antimycotic, (Gibco/Thermofisher Scientific) and 250 μg/ml Zeocin. Cells were transfected with cockroach AR in the mammalian expression vector pcDNA3.1 using X-treme GENE 9 transfection reagent (Roche Diagnostics, Atlanta, GA). Cells were harvested with enzyme-free cell dissociation buffer (Gibco) and incubated with coelenterazine F (Nanolight Technology, Pinetop, AZ) for 2 h in suspension, protected from light. During the assay, 96-well plate were injected with an equal volume of cell suspension at a density of ~50,000 cells per well, and luminescence was recorded for 20 s post-injection on a LUMIstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany). Dose response curves were generated with GraphPad Prism's version 6 (La Jolla, CA) four-parameter nonlinear fit function normalized to the response of the highest dose (100% relative luminescence; % RLU).

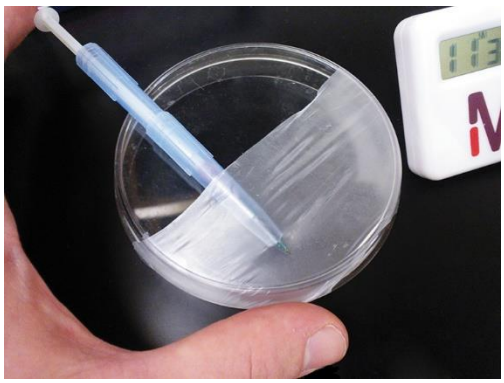

Figure 2: Picture was taken from Rajendrani Mukhopadhyay's "The cockroach hunter's spell" in *ASBMB Today*. A female jewel wasp is anesthetized and placed in a modified p1000 pipette tip. Afterward, she and the tip are placed on top of a cell plate and underneath a layer of parafilm. When she regains consciousness, her head is lightly tapped using a soft-ended plunger to provoke her to sting and release venom on the other side of the parafilm. The venom droplets are collected in pH 4 saline to prevent zymogen activation.

Obtaining Venom:

Methods for obtaining venom have already been developed [2, 22]. To identify a functioning ADA in the venom, female emerald jewel wasps were "milked" by provoking them to sting through a layer of parafilm **(Figure 2)**. The resulting venom droplets were collected in saline at a pH 4 (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 4 mM $CaCl_2$, 2 mM $MgCl_2$, pH adjusted using acetic acid) to prevent preliminary activation of enzymes. The collected venom was then separated into two aliquots, to which a fixed concentration of 1 μM of ADO in pH 7.2 cockroach saline was added. The first aliquot was incubated for 1 hour at a room temperature while the other was incubated at 98 ºC for 10 minutes to denature the enzyme. Afterward both aliquots were placed in -20 ºC until used for luminescence assays.

Injections into the Central Complex:

Methods for CX injections have been taken and modified from. [2, 23] Synthetic *A. compressa* corazonin peptide or *Streptococcus* ADA (Millipore Sigma, Darmstadt, Germany**)** (657.7 U) were dissolved in cockroach saline with 0.1% Janus Green B as a tracer. Injections were performed with a Drummond Nanoject II (Drummond Scientific, Broomall, PA) and microcapillaries beveled to 30 degrees and 20 μm diameter. Cockroaches were cold anesthetized on ice for 10-15 min prior to injection and immobilized dorsal side up and held down on a Peltier cold-plate set to 4 °C using modeling clay to minimize movements during surgery. A U-shaped pin was used to pin the neck and the clypeus to constrain blood-flow to the brain, thereby minimizing pulsation movements. Injections were then done below the cerebral hemispheres at a 90° angle with 36.8 nL of total volume injected. Following behavior assays, the brains from both control and test animals were dissected, fixed in 10%

PFA at 4 °C for 2 days. Dissected brains were vibratome-sectioned to confirm that the injection location was the CX.

ADA *P. americana* Behavior Assays

To investigating the effects of ADA on cockroach spontaneous locomotor activity, ADA from *Streptococcus thermocilus* was injected into the CX of 1-week old, virgin male cockroaches using a Drummond Nanojector. Behavior before and prior to the injection was recorded in 25 cm diameter arenas. Activity data was measured using EthoVision XT software (Noldus) and analyzed with Graphpad Prism version 6 (La Jolla, CA) software. The treated group received a 36.8 nL injection of 3% ADA in 0.1% Janus Green B in cockroach saline. The amount of *Streptococcus* ADA injected matches the activity of ADA in the venom 100-fold (calculations not shown). Controls were injected with the same volume and dilution of ADA that was incubated at 98 °C for 10 minutes to denature the enzyme. To measure the behavioral effects of both treatments, alterations in movement frequency, duration of movement, total distance moved, and average velocity were measured in thirty-minute bins before and following injections. A separate set of ADA and denatured ADA-injected cockroaches was used to investigate ADA effects on escape response. These assays were executed by placing cockroaches in a 24 cm diameter arena and measuring the distance they traveled after the tip of the abdomen was stimulated with a paint brush to elicit an escape response [24].

RESULTS

Identification of a functional ADA in the venom was carried out using WTA11 cells expressing the cockroach AR from the SEG and aequorin. Due to the AR receptor being a Gαq G protein-coupled receptor, AR leads to the release of Ca^{2+} mobilization and aequorin-mediated luminescence. Luminescence intensity was used as a measure of AR activation following application of ADO was applied. Successful transfection of the *P. americana* AR is validated by measurement of luminescence intensity with increases in ADO concentration (**Figure 3)**. ADO mixed with boiled venom resulted in similar levels of luminescence intensity as that of the control. However, incubation of the venom at RT with 0.8 µM of ADO resulted in luminescence intensity matching that of media containing no ADO (**Figure 3**). In addition, extended incubation periods of the venom with 1 µM led to decreasing luminescent intensity of the WTA11 cells (**Figure 4**). Thus, there is an ADA-like

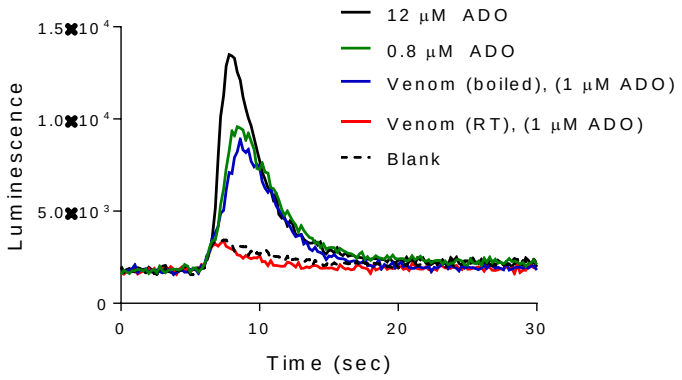

Figure 3: Luminometer assay illustrating loss of ADO signal when mixed with venom incubated at room temperature. WTA11 cells expressing a Gαq cockroach AR and coexpressing bioluminescent and Ca^{2+} binding aequorin were used to record AR activation. Denatured (boiled) venom failed to antagonize AR signaling. Active and RT incubated venom prevented AR signaling, implying that all ADO mixed with the venom has been depleted.

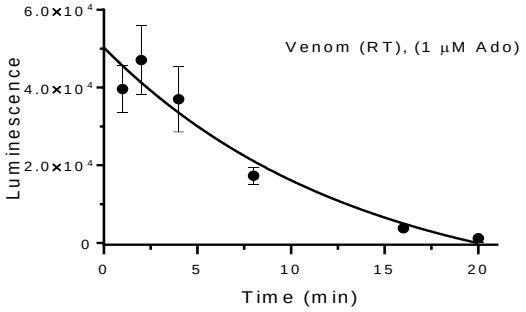

Figure 4: Luminometer assay using recombinant WTA11 cells illustrating progressive loss of ADO signal when 20 sting equivalents of venom are incubated with ADO at various time points.

enzyme is in the venom that breaks down the ADO molecule into a form that does not effectively bind to and activate the cockroach AR.

Disruption of ADO signaling: behavioral consequences for *D. melanogaster* and *P. americana*

Silencing of the AR in the CX of fruit flies resulted in no significant changes in spontaneous locomotory activity (**Figure 5**). Although the test cross containing the UAS-HMC03684 and Gal4 c547 displays a decrease in activity compared to its TRiPattP2 control, the difference did not reach significance using a Mann-Whitney unpaired t-test ($p = 0.0514$). In addition, ADA injections into the CX of 1-week post-ecdysis male cockroaches also resulted in no significant changes in any aspect of the animal's locomotory behavior, including distance traveled, frequency of movement, or locomotory velocity. Due to redundancy in patterns seen within each aspect listed, only the change in distance before and following injection is provided (**Figure 6**). In addition, responses to abdominal stimulation were not altered (data

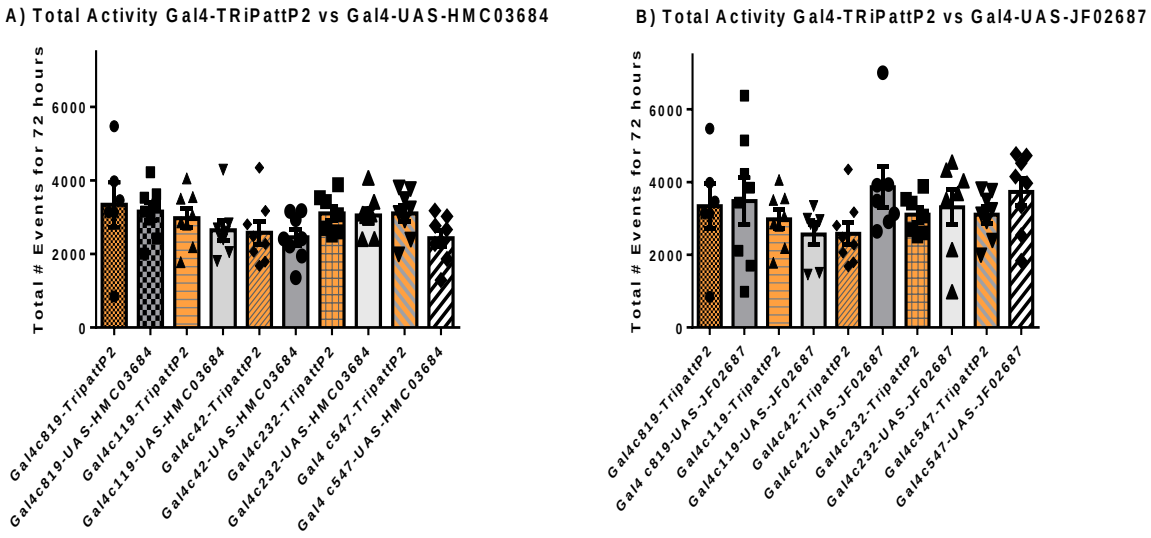

Figure 5: Figure 5a-b are pooled number of events collected from 3-day old virgin females for 72 hours using the DAM2 activity monitor (Trikinetics). Each fly cross has a n = 8. Orange bars are control flies containing the TRiPattP2 insert and the UAS-dicer-2 on the second chromosome and a Gal4 sequence on the 3rd or X chromosome. Grey bars are test crosses containing the UAS-RNAi and UAS-dicer-2 gene on the second chromosome and the Gal4 on the 3rd or X chromosome. A Mann-Whitney independent and non-parametric t-test with an $\alpha = 0.05$ was carried out between each control cross and its corresponding test cross. None resulted in a p value less than 0.05; however, the p-value for the activity between Gal4c547-UAS-HMC03684 and Gal4c547-TRiPattP2 in Figure 4a was 0.0514. This suggest that there may be an effect in silencing the AR gene in the CX, leading to a loss in activity, but additional replicates must be carried out.

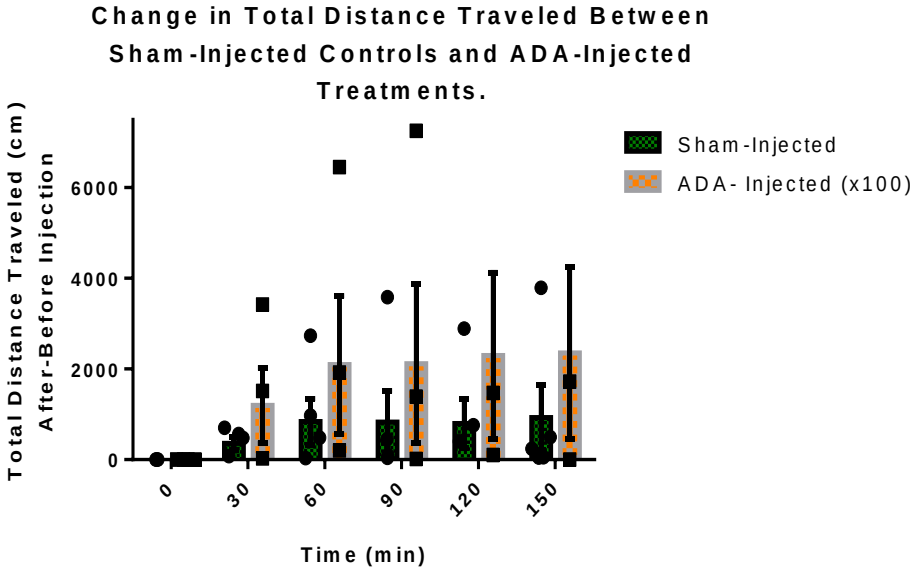

Figure 6: Spontaneous activity of 1-week old roaches was recorded using a handycam and measured using the Ethovison XT software (Noldus). Analysis of locomotory behavior was conducted using Prism version 6 (Graphpad, La Jolla, CA). Both treatments received a total of 38.6 nL of 3% *Streptococcus thermophilus* ADA (Millipore Sigma) that has been mixed with 0.1% Janus Green B in cockroach saline; however, denatured ADA-injected controls were injected with ADA enzyme that had incubated for 10 minutes at 98°C. A multiple comparison pair ANOVA test was carried out and no statistical difference was found between denatured ADA-injected (n = 5) and ADA-injected (n = 4) animals.

not shown).These results suggest that decreased AR signaling in the CX does not affect

spontaneous or evoked locomotory activity.

CRZ and Venom Effects on VG Expression and Ovary Development

Envenomation causes a significant decrease in expression of VG2 gene expression in the fat body of 7-day old virgin females that were stung on the 6th day post-adult ecdysis (**Figure 7a; Mann Whitney test**), while CRZ-injections failed to elicit this same response (**Figure 7b**). Despite the lack of significance seen in the gene expression data for CRZ-injected animals, significant differences in protein content and ovariole size for stung and CRZ-injected animals were detected (**Figure 7c-d**). The reason for this may be improvements in CRZ injection methods as data collected on ovariole volume and protein content were done later. Additional causes for this lack of decrease in gene expression work is that the effects of CRZ without the additional components within the venom may require more time to take effect, or that the injections themselves alter the animal's physiology in such a way that it allocates most of its energy to healing rather than vitellogenesis. In addition, controls injected with scrambled *A. compressa* CRZ that is unable to bind to CRZ receptors need to be carried

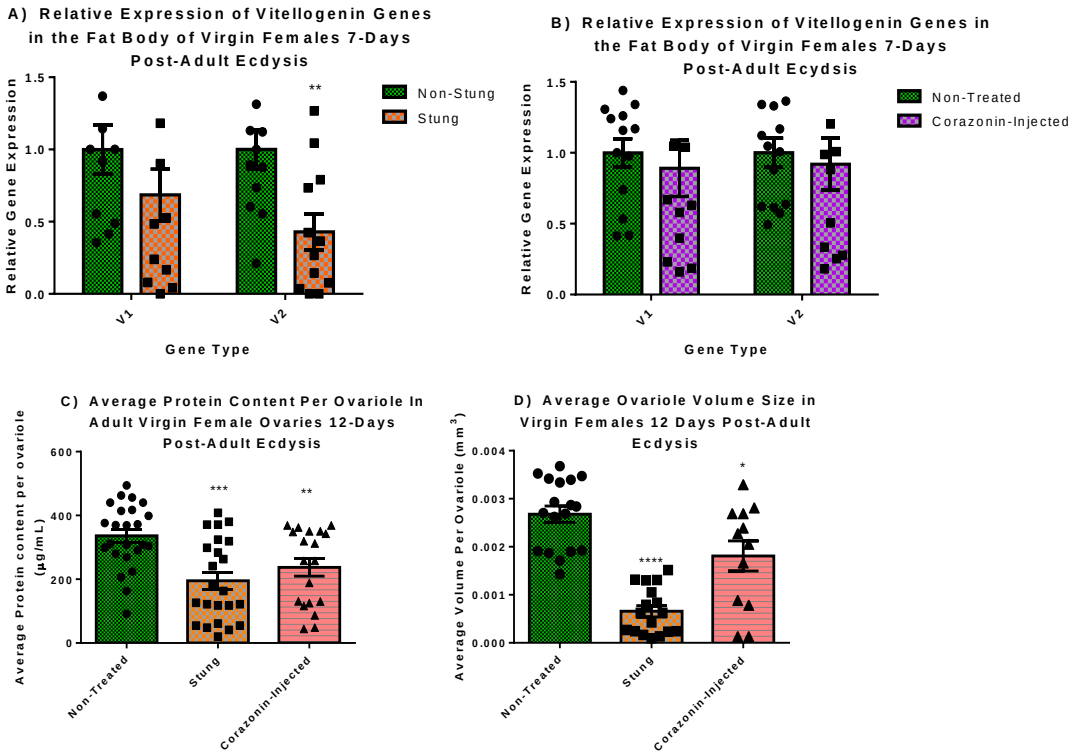

Figure 7: 7a) Gene expression of VG1 and VG2 in the fat body of stung (n =12) relative to non-stung individuals (n = 12) 7 days-post adult ecdysis. 7b) Gene expression of VG1 and VG2 in the fat body of CRZ-injected virgin females (n = 12) females relative to non-treated controls (n = 14) 7-days post-adult ecdysis. 7c) Protein quantification and ovariole volume sizes of ovaries within non-treated (n = 24), stung (n = 23), and CRZ-injected females (n = 20) 12-days post adult ecdysis. 7d) Average ovariole volume in stung (n = 17), CRZ-injected (n = 12), and non-treated individuals (n = 18). Females were stung or injected on the 5th or 6th day post-adult ecdysis and data for volume size and protein content were polled as no significant differences were observed between these two days. Ovariole volumes were obtained by measuring the length and width of each ovariole and applying them to this equation $2/3 \bullet \pi \bullet (\text{width}/2)^2 \bullet (\text{length})$. Each treatment was compared to its control counterpart using a Mann-Whitney independent and non-parametric t-test with an $\alpha = 0.05$. 7a-b) Only stung individuals were found to display a significant decrease in VG2 ($p = 0.0068$). 6c-d) However, both stung and CRZ-injected animals displayed a significant decrease in crude protein content and ovariole volume size with the p values for stung animals being 0.0004 and less than 0.001 respectively and for CRZ-injected animals being 0.0087 and 0.0212.

out to provide a clear understanding on the effects of CRZ on ovary development.

Surprisingly, gravid, stung individuals resumed vitellogenesis 2 weeks following the sting, as the ootheca-less eggs laid contain the same amount of protein as that of ootheca in non-treated controls (**Figure 8**). However, the number of eggs produced by each individual is far less than that of non-treated individuals, as the maximum number produced by a stung individual is 9 and the maximum amount that can be contained within an ootheca is 16 (data not shown). This, behavior continues for around 2 months until the animal regains the ability

to form an ootheca (data not shown). Even then, the ootheca laid by individuals are smaller than that of controls until after the oviposition of their 3rd ootheca following treatment (data not shown). Some CRZ-injected gravid individuals were able to display the same ootheca-less egg oviposition observed with stung animals; although, this behavior is observed 1 month following injection while normal ootheca are laid between that time (data not shown)

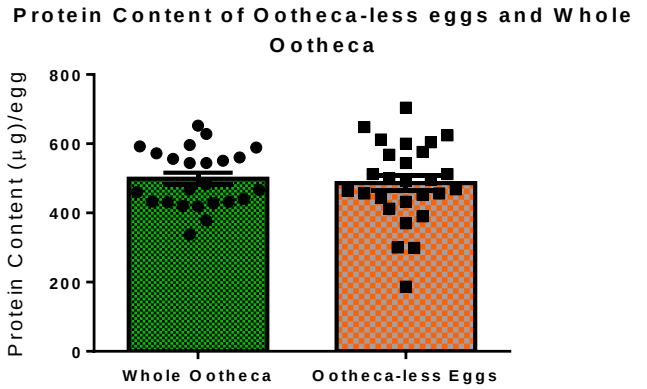

Figure 8: Quantification of crude protein per egg in ootheca-less eggs (n = 27) and whole ootheca (n = 24). A Mann-Whitney unpaired t-test was carried out using Graphpad Prism version 6 (La Jolla, CA) and revealed there is no significant difference between protein content in stung and non-stung animals (p = 0.9441). This suggests that the sting delays VG expression temporarily and that stung animals can regain the ability to develop eggs.

CONCLUSION

Behavioral effects induced by venom ADA were investigated using transgenic flies with their AR in the CX silenced and 1-week old male cockroaches that were injected with 3% *Streptococcus thermophilus* ADA in their CX. Both experiments resulted in no significant changes in spontaneous locomotory behavior nor changes in escape response when compared to their controls. Thus, the venom ADA may result in physiological changes that make the host optimal for larval consumption rather than play a role in suppressing locomotory behaviors. However, both the sting and CX *A. compressa* CRZ-injections resulted in decreased ovariole size and ovary protein content in 12-day old virgin females. In addition, both stung and CRZ-injected individuals display ootheca-less egg ovipositions behaviors; however, females injected with CRZ display this behavior 1-2 months following injection and lay ootheca while stung females oviposit ootheca-less eggs 2 weeks following injections and do not lay ootheca. In addition, only stung females displayed a significant decrease in VG gene expression while CRZ-injected

females did not. This suggests that increased CRZ signaling in the CX causes a down regulation in pathways responsible for VG intake into the ovaries and genes responsible for ootheca formation. This may be beneficial to the larva of the wasp by preventing the female from using up metabolic resources or ovipositing a potential nutritious resource for the larva. To prove this hypothesis, improvements in injection technique and additional replicates on VG expression in CRZ-injected females and females injected with scrambled CRZ peptide will be carried out. My research provides fundamental information on alternate signaling pathways venoms use to manipulate the physiology of the host. In addition, my work reveals alternate functions of CRZ in the CNS of arthropods, in particular to CRZ inhibiting ovary development in female cockroaches.

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SUPPLEMENTAL INFORMATION

RNA Purification Protocol

RNA Pellet Formation

1. Add RNase-free 5 M $C_2H_7NO_2$ (ammonium acetate) and RNase-free water to samples so that the volume of ammonium acetate added is 10% of the resulting total volume (i.e. 100 µL sample + 20 µL ammonium acetate + 80 µL water = 200 µL total volume)
2. Add equal volume of RNase-free phenol: chloroform: isoamyl alcohol to your samples then let sit for 1 minute at room temperature. Centrifuge at 12000 g for 15 minutes at 4°C.
3. Remove the aqueous layer and add x2 the volume of 100% RNase-free ethanol that has been chilled to -20 °C. Store samples in -80 °C for at least 30 minutes.

RNA cleanup

1. Centrifuge samples at 12000 g for 15 minutes at 4 °C.
2. Decant ethanol and remove the rest using a pipette.
3. Add 1000 µL of RNase-free 80% ethanol that has been chilled to -20 °C. Centrifuge samples at 12000 g for 15 minutes.
4. Decant ethanol from pellet and remove the rest using a pipette.
5. Dry pellet on ice in a fume hood for 30 minutes to 1 hour.
6. Elute using RNase-free water and quantify.