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Characterizing The Transport Of Parasitoid Wasp Genaspis Hookeri Venom Serca Into Host Drosophila Hemocytes

Christopher Lark
Illinois State University, lark.chris84@gmail.com

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CHARACTERIZING THE TRANSPORT OF PARASITOID WASP GENASPIS HOOKERI VENOM SERCA INTO HOST DROSOPHILA HEMOCYTES

CHRISTOPHER LARK

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The female endoparasitoid wasps introduce venom accompanied by an egg into the larval hosts' body cavity during oviposition. The venom contains a mixture of proteins that exploit the host's biological processes, such as, regulating gene expression, disrupting immune signaling pathways, or inducing immune cell death to give a decided advantage to the incipient wasp to develop unchallenged. *Drosophila* possesses a highly conserved innate immune response that recognizes and neutralizes foreign microbes and macroparasites. The venom of *Ganaspis hookeri* (strain G1) endoparasitoid wasp has co-evolved to suppresses the humoral phase *Drosophila's* innate immune response by inhibiting Ca^{2+} bursts needed to induce an immune response signaling pathway. The intracellular Ca^{2+} concentration of host plasmatocytes is altered via a venom specific homolog of the Sarco/endoplasmic reticulum calcium ATPase (SERCA) as a virulence factor. The venom SERCA (vSERCA) is able to significantly alter host intracellular Ca^{2+} homeostasis, disrupting cell signaling. SERCA is a multipass transmembrane protein, suggesting that vSERCA is likely packaged for transport through the venom into host plasmatocytes. Extracellular vesicles (EVs), or venosomes, to transport a variety of venom proteins and peptides. An excellent question is how vSERCA makes its way from the venom glands of G1 and into host plasmatocytes to affect Ca^{+2} levels. Here I will explore how G1 uses venosomes to package vSERCA and other key virulence factors for transport from the venom

gland, where they are produced, into host hemocytes. I characterize how G1 venom transports vSERCA and other important virulence factors necessary for regulating host signaling pathways essential for the Drosophila larval immune response, and the underlying biogenic mechanisms of venosome directed protein transport. These results are relevant to our understanding of the evolution of hymenopteran and related species.

KEYWORDS: *Ganaspis hookeri*, endoparasitoid, venom, SERCA, extracellular vesicle

CHARACTERIZING THE TRANSPORT OF PARASITOID WASP GENASPIS HOOKERI

VENOM SERCA INTO HOST DROSOPHILA HEMOCYTES

CHRISTOPHER LARK

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CHRISTOPHER LARK

COMMITTEE MEMBERS:

Nathan Mortimer, Chair

Tom Hammond

Ben Sadd

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CHAPTER I: INTRODUCTION

Hymenoptera is a diverse order of insects that includes bees, sawflies, ants, and wasps.[1–4] Parasitoid wasps make up a large superfamily of hymenopterans and obligately infect other arthropod species to allow for the development of their offspring. Female parasitoids use an ovipositor to lay their eggs during infection of their host and can be classified as ectoparasitoids that oviposit an egg onto the host, or endoparasitoids which infect the host by injecting an egg into its body cavity (hemocoel).[1,4–10]

The host hemocoel is a hostile environment for the developing endoparasitoid wasp egg, with hosts mounting a range of innate immune responses, both cellular and humoral, that are intended to kill the egg or arrest its development.[1,7–9,11]In response, endoparasitoids have evolved venom virulence factors that allow them to inhibit these defense responses by targeting host immune cells, or hemocytes, and preventing their function. The venom is introduced into the host along with the egg during infection and can exploit the host by modulating a variety of biological processes including regulating gene expression, disrupting hemocyte signaling pathways, or causing cell death of hemocytes, giving the advantage to the incipient wasp to develop unchallenged.[1–4] Species of endoparasitoid wasps have evolved diverse strategies to facilitate infection using a variety of virulence factors.[1,5–10]

Venom sequencing is an emerging area of research, and understanding the evolution and function of venom proteins is an exciting and significant field. Understanding the underlying mechanisms behind how venom factors are packaged and transported can provide a specialized perspective of interspecies coevolution, and a unique perspective into protein biogenesis and transport with implications for therapeutic applications. To develop a greater understanding of the composition and function of endoparasitoid venoms, it is essential to study their activity in

the host environment. The fruit fly, Drosophila melanogaster, is well established as a model system for studying signaling pathways that are conserved across species. D. melanogaster is infected by many species of parasitoid wasps and thus serves as an excellent model organism to explore the molecular mechanisms of parasitoid venom virulence.[1,7–9,12]

D. melanogaster uses a highly conserved innate immune system to recognize and respond to foreign microbes and macroparasites.[1,7–9] Flies mount two types of innate immune responses: the humoral response and cellular response. The humoral response is mainly targeted against invading microbial pathogens.[7–10] After entering the host's hemocoel, the space between the outer cuticle and inner organs, these pathogens first encounter the humoral immune response in the form of antimicrobial peptides (AMPs). AMPs are naturally occurring peptides that possess a broad range of antimicrobial activities.[8,13] Humoral factors are also involved in the cascade of enzymes that regulate melanization activity in the hemolymph.[7,8,13] Following the humoral response, the cellular immune response can be activated to neutralize pathogens via phagocytosis or encapsulation.[6–10,13] Due to hemocyte function often relying on humoral molecules to begin an immune activity, there is an overlap of humoral and cellular immunity.[6–8]

Encapsulation is a defense reaction that is activated against foreign bodies that are too large for phagocytosis, such as endoparasitoid wasp eggs and other macroparasites.[6–8,13] Instead of ingesting the foreign body, a number of specialized immune cells surround and adhere to the surface of the invading macroparasite to form a continuous layer. These specialized immune cells deposit melanin which hardens into a solid capsule to seal the pathogen, resulting in death.[1,7,8,10]

For successful parasitization and development of wasp larvae, endoparasitoids that infect D. melanogaster have evolved to modify and suppress the fly's immune response via venom

virulence factors. The venoms of different endoparasitoid species use a wide range of mechanisms to suppress the host immune response including causing the death of host immune cells, detoxifying the host's hemocoel and inhibiting host immune pathways.[1,6–8] The endoparasitoid Ganaspis hookeri (strain G1) uses the latter of these venom strategies. G1 utilizes a venom specific homolog of the Sarco/endoplasmic reticulum calcium ATPase (SERCA) as a virulence factor to inhibit host calcium (Ca2+) signaling.[7,8]

Ca2+ is a second messenger found ubiquitously across cell types and is integral to cell signaling.[12,14,15] Physical interactions with Ca2+ can regulate the conformation and charge of a subset of signaling proteins, which in turn alters cellular activity. In both insect and human cells, the cycling of Ca2+ levels in the cytoplasm is regulated by calcium-induced calcium release channels for Ca2+ release from intracellular stores, and SERCA for Ca2+ uptake. Typically, naïve circulating immune cells maintain a low cytoplasmic concentration of Ca2+.[10] After contact with a pathogen, immune receptors trigger an influx of calcium to activate immune signaling, like flipping a switch that turns on the lights in a room. When this calcium influx is genetically blocked, the cellular encapsulation response is inhibited.[7,8,10]

Several isoforms of the SERCA gene can be found in both insect and mammalian genomes, with most organisms encoding at least two isoforms.[7,8,16–19] Each isoform varies in its C-terminus, which affects the isoform's affinity for Ca2+.[7,8] The venom SERCA (vSERCA), which is found only in the abdomen of the wasp, represents a highly active isoform with a high affinity for Ca2+ and the ability to significantly alter intracellular Ca2+ homeostasis.[1,7,8] Following infection, vSERCA specifically targets host plasmatocytes, a hemocyte subtype that circulates in the hemolymph of fly larvae. Plasmatocytes rely on a burst of calcium to activate the cellular immune response, and vSERCA disrupts this intracellular calcium regulation in host

plasmatocytes.[7,8] This, in turn, prevents the encapsulation of the wasp egg, allowing for successful parasitization.

SERCA is a multipass transmembrane protein,[7,8] suggesting that vSERCA is unlikely to be freely secreted into the aqueous host environment, but instead may be packaged for transport through the venom. It has already been established that the venoms of endoparasitoids from the Ganaspis and Leptopilina genera contain extracellular vesicles (EVs) to transport a variety of venom proteins and peptides.[5,6,10,13] EVs are heterogeneous structures, which are bound by a lipid membrane and are shed from the cell's plasma membrane bilayer.[20–23] EVs can play a major role in altering a cell's internal environment and regulating cell signaling and cell motility.[5,20,22] Understanding the underlying biogenic mechanisms of protein trafficking directed by EVs has made research focused on EVs a hot topic as they present a variety of potential methods of therapeutic treatments.

One outstanding question is how vSERCA makes its way from the venom glands of G1 and into host plasmatocytes to affect $Ca+2$ levels. The system of delivering cell-specific packages of virulent proteins to regulate signaling pathways that occurs between endoparasitoids and their hosts is exciting but poorly understood. One of my goals is to begin understanding how the vSERCA venosomes are produced and to identify potential cargos that are necessary for regulating host cell function. My hypothesis is that G1 uses venom EVs (venosomes[10]) to package vSERCA and other key virulence factors for transport from the venom gland, where they are produced, into host hemocytes. The goal of my experiments is to test whether G1 uses venosomes as a method of transporting vSERCA and other important virulence factors necessary for regulating host signaling pathways essential for the Drosophila larval immune response.

CHAPTER II: RESULTS

Venom Protein Analysis

To test the hypothesis that vSERCA is being transported through the venom via a venosome, a sucrose gradient (**Fig. 1**) was used in combination with centrifugation to separate venom components by density. Venosomes tend to be denser than individual proteins, so I used this separation to test which fraction vSERCA was found in. I would expect to find individual peptides and proteins in the top layers of the sucrose gradient (i.e. fraction 1), and I would expect to find larger complexes, such as venosomes, in the lower portion of the gradient (i.e. fraction 5).

I first wanted to use sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to get an idea of the amount of protein in the venom sample, as well as to see how the proteins are distributed across the fractions. As previously mentioned, venosomes are larger complexes containing a variety of lipids and proteins. If G1 venom contains venosomes as venom transport factors, then I'd expect to see proteins in the lower (denser) fractions. I might also expect to see some venosome overlap from the fraction containing the largest quantity of vSERCA with the neighboring fractions because I wouldn't expect the sucrose gradient to perfectly isolate all of the vSERCA and subsequently all the venosomes containing vSERCA in one specific fraction.

To view proteins using SDS-PAGE, the proteins are denatured prior to being run through the gel matrix. However, it is likely that the charged lipid membrane of the venosomes would be resistant to the protein denaturation protocol and would instead need to be disrupted in a way that would allow their contents to be released. By substituting the typical sample preparation with a modified sonication protocol designed to disrupt venosomes, I expect to see a change in the result of the protein gel.

Once the fractions are run through an SDS-PAGE gel and transferred, the lower fractions have a marked number of bands. This suggests that the proteins are not likely to be separating by size but instead separating by density and that a number of dense complexes such as venosomes are present. Additionally, there is a noticeable difference in the results when comparing unsonicated samples (**Fig. 2a**) to sonicated samples (**Fig. 2b**). There is a clear increase in the number of bands (proteins) and in some cases a change in the intensity of the signal found in each fraction in the sonicated samples compared to the unsonicated samples. This difference is likely the result of the disruption of the charged lipid membrane, supporting the idea that many G1 venom proteins are transported in venosomes. There is also some lane distortion in Fig. 2b that is likely the result of the released lipids.

Next, I performed a Western blot with an anti-SERCA antibody to determine which fraction(s) vSERCA is found in. In the Western blot (**Fig. 2c**) a clear signal can be seen in lanes 2, 4, and 5 between ~100 kD and ~150kD. This is the expected size of vSERCA based on previous work8. Lanes 2, 4, and 5 also show signals at ~50 kD and ~100 kD. It is possible that the ~100 kD signal may be vSERCA on its own without any venosome associated complexes or post-translational modifications and has been previously observed in G1 venom.[8] The ~50 kD band is unexpected and may represent another isoform or cleavage product of SERCA not yet characterized. The signal from lanes 4 and 5 is likely showing the presence of vSERCA in a large complex such as a venosome. As previously mentioned, the perfect isolation of SERCA in any form, venom or otherwise, is unlikely given the protocol. I hypothesize that the vSERCA observed in fraction 2 likely represents vSERCA that was either not yet packaged at the time of venom collection, or was lost from venosomes that were disrupted during venom collection.

The streaking present throughout lanes 2-5 may be caused by either a high sample protein load or a large number of lipids. The amount of protein is unlikely to be the issue since I loaded the minimum amount necessary to ensure a consistently clear signal, so my prediction is that the lipid content caused this streaking. Lipids, which form the membrane of organelles like the endoplasmic reticulum or venosomes, have a polar (charged) hydrophilic head and a pair of nonpolar (uncharged) hydrophobic tails. The charged nature of lipids has been associated with disrupting sample movement in gel electrophoresis and disrupting the ability of molecular recognition assays such as mass spectrometry. The vertical streaking of the Western blot combined with the lane distortion in the protein gel suggests that the sonication of the venom fractions was enough to disrupt the lipid membranes of venosomes.

Mass Spectrometry

If vSERCA is being transported through venom as cargo inside venosomes, then it stands to reason that there may be other conserved proteins transported with vSERCA. These would likely include proteins that are essential for the proper function of vSERCA, proteins that may have virulence functions independent of vSERCA, and proteins that may play a structurally important role in venosomes. I wanted to identify these other potential proteins found in the vSERCA containing fractions to explore the possible mechanisms for the formation and function of virulent venosomes. To identify the proteins that co-localize with the vSERCA containing fraction, I submitted the fractions for mass spectrometry (MS). MS accurately measures the mass of peptides derived from the submitted fractions and maps them onto an established database of G1 proteins, and so can help me to identify what proteins are found in G1 venom, and more importantly, what proteins are co-localizing with SERCA.

Results from our MS analysis of unsonicated venom revealed a high concentration of charged lipids, supporting the idea that venosomes are present in venom fractions. If sonication allows the cargo to be released from association with charged lipids, I expect to find a variety of proteins represented in the MS analysis of sonicated venom fractions, including vSERCA, receptor proteins that may play important roles in immunity, and venosome structural proteins.

MS data demonstrated that there were a combined 521 proteins and peptides identified across fractions 1 through 5, with each fraction having between 230-320 proteins and peptides. Fraction 5, which is where the majority of vSERCA was identified, contained 290 other proteins. I next wanted to investigate the potential roles of this other potential venosome or cargo proteins, through functional annotation. Since G1 proteins have not been annotated, I mapped them onto their D. melanogaster homologs. Homologs of all but one of the 290 proteins found in fraction 5 were identified and used to annotate the venom proteins. Specifically, I used gene ontology (GO) term mapping to identify the cellular component, molecular function, and biological process associated with the proteins identified in fraction 5. Many of the proteins found in fraction 5 are homologous to proteins that serve as structural proteins, have ion binding domains, and are associated with functioning in metabolic and catabolic pathways. Cell component and molecular function analyses supported the identified pathways; for instance, the biological process term GO:0051238 identifies a pathway that sequesters metal ions (**Table 3**), while the cellular component term GO:0008043 (**Table 4**) suggests an intracellular ferritin complex, and the molecular function term GO:0008199 (**Table 5**) suggests proteins with ferric iron-binding ability.

SERCA Activity Assay

To verify that fraction 5 contains all of the proteins/complexes necessary to manipulate host Ca2+ signaling, I separately treated isolated D. melanogaster plasmatocytes with each venom fraction and sucrose control and assayed for changes in Ca2+ concentration. Plasmatocytes were incubated with the fluorescent Ca2+ sensor fluo-4 along with each fraction or control. Fluo-4 is a dosage-sensitive Ca2+ sensor, meaning that fluo-4 fluorescence intensity can be used as a readout of cytoplasmic Ca2+ levels[24]. I would expect to see a change in fluo-4 fluorescence intensity when the cells were treated with a fraction that modifies cytoplasmic Ca2+.

Plasmatocytes from each treatment were read in a fluorescent cytometer to measure fluo-4 fluorescence intensity. Fraction 5, which contained vSERCA, caused a marked decrease in fluorescence intensity relative to control (a decrease of 150 units of fluorescent intensity, **Fig. 3**), whereas the other fractions had only small effects on plasmatocyte Ca2+ concentration.

Immunohistochemistry and Imaging

A transmembrane protein such as SERCA does not function as a freely circulating molecule but instead resides within a membrane, specifically the sarcoplasmic or endoplasmic reticulum in the case of SERCA. SERCA is found in nearly all tissues and is particularly abundant in muscle, therefore, I would expect to find SERCA localized in muscle tissue of G1, including muscle found in the venom apparatus. I would also expect to find vSERCA localized inside the venom gland, where virulence factors would be transcribed, translated and packaged into venosomes, and the venom reservoir, where venom proteins are stored prior to infection. Although the tissue types and SERCA isoforms may vary in function, I expect similar patterns of localization in all of the tissues SERCA is present in. This expectation is based on what the

known shape of sarcoplasmic and endoplasmic reticulums generally have with a series of closely branching lumens. SERCA would be located within these branching lumen membranes.[25]

A SERCA antibody, which was predicted to recognize all SERCA isoforms, was used to identify where SERCA is localizing in the venom apparatus. I could then predict where vSERCA is being packaged for release into the venom. Within the venom apparatus, venom proteins are produced in the venom gland, secreted into the secretory duct through which they travel for storage in the venom reservoir, ready for injection into the host during infection. Therefore, I focused my imaging on the venom gland and venom reservoir to understand vSERCA localization. Due to the SERCA being a highly conserved protein typically found in myocytes, I imaged SERCA in the muscle surrounding the venom reservoir for comparison.

In the muscle tissue, SERCA is localized in a tight pattern within the striated cells as would be expected for a sarcoplasmic reticulum localized protein. Within the venom gland, vSERCA localizes in a similar pattern to that of the muscle tissue, suggesting that it is largely localized to the endoplasmic reticulum in this tissue. However, there are some notable differences in vSERCA staining. First, the staining within the venom gland ER is much more punctate and there are discrete cytoplasmic foci, suggesting that vSERCA may be exported from the ER. These puncta are more numerous near the secretory duct suggesting they may be transported out of the gland. Finally, there is an area of diffuse vSERCA staining at the proximal entrance to the venom reservoir, suggesting that vSERCA that has already been packaged is stored in this tissue.

CHAPTER III: DISCUSSION

The venoms of endoparasitoid wasps have evolved to enable the wasp to either disable the host's immune response or allow the invading egg to avoid activating the host immune response, following infection.[1,7,10,13] These different venom strategies result in a varying rate of successful parasitization. Ganaspis hookeri deploys the avoidance strategy by disabling the Ca2+ intracellular signaling pathway in circulating plasmatocytes. G1 uses venom Sarco/endoplasmic reticulum ATPase (vSERCA), a venom specific isoform of a well-conserved Ca2+ ion pump, to deregulate intracellular Ca+2 signaling in plasmatocytes. My findings support the idea that G1 uses venosomes to deliver virulence factors such as vSERCA to regulate the host immune response. These results lead to a model in which vSERCA is expressed in venom gland tissues where it is packaged into venosomes to be transported in the venom to target host plasmatocytes. I predict that following infection the venosome will fuse with the host cell's surface membrane and empty its cargo into the host cell, which would also allow transmembrane proteins, like vSERCA, to be incorporated into the membrane of the host cell. I would also expect to find other proteins and/or peptides in the cargo that may support vSERCA in its function or could disrupt intracellular signaling and/or metabolism.

As a starting point, G1 venom was purified and separated across five sucrose fractions to determine if vSERCA is being transported through the venom in venosomes. My Western blot results identified vSERCA in the densest fractions, suggesting that it is found in some sort of venosome or protein complex. I then used MS to identify any other well-conserved signal-regulating proteins that are co-localizing with vSERCA. An initial attempt to have the purified venom fractions analyzed with mass spectrometry was not successful because of an abundance of heavily charged lipids. The abundance of charged lipids supports the idea that venosomes are

present. Sonicating the fractions helped to release more venom proteins, which was evident in the increased number of bands present in the protein gel.

MS data demonstrated that vSERCA along with 290 other proteins were isolated in fraction 5, confirming the Western blot data showing vSERCA presence in this fraction. Fraction 5 also caused the most significant decrease in Ca2+ levels in the fluo-4 fluorescent assay. There were SERCA signal bands in lane 4 of the Western blot, which I attribute to some residual venosomes that hadn't finished traveling through the sucrose gradient during centrifugation. A signal is also present in lane 2 of the Western blot and may be the result of vSERCA that is free of any venosomes, and may have arisen from venosomes that lysed during one of the steps of the purification process prior to the fractions being separated and sonicated.

Drosophila homologs of all but one of the 291 proteins found in fraction 5 were identified and annotated. To identify the possible functions of the proteins that co-localize with vSERCA, I used gene ontology (GO). Many of the proteins found in fraction 5 are homologous to structural proteins, ion binding proteins, and are associated with metabolic and catabolic pathways. Cell composition and molecular function supported the identified pathways. The results of the GO terms query present a pattern of functional proteins found in the vSERCA venom fraction that can be categorized as contractile filaments such as myosin filaments which can be used for transport of intercellular cargo, metabolic components like nucleotide-binding proteins that can bind ATP and GTP, catabolic components like proteasome core complexes that have the ability to degrade proteins[26–28], binding metal ions, like ferric ion binding proteins to be used as nutrients for cells[19,29]; and signaling, such as Toll-7, which plays a role in activating the innate immune response[11,12,28,29]. I predict that these cellular pathways would be affected by the proteins found in fraction 5 following infection.

G1 venom doesn't destroy host plasmatocytes, it only prevents them from activating the cellular immune response by blocking Ca2+ signaling[8,15]. However, this most likely isn't the only change that takes place within the plasmatocytes, and it's not the only physiological change that takes place post-infection. Previous work has shown that once infected, the amount of time prior to pupation is markedly shortened, suggesting metabolic pathways are modified[1–4]. It is interesting that at least one type of G1 venosome essentially hijacks the hemocytes responsible for recognizing infection and signaling the timely immune response to neutralize the venom and wasp egg. In this way, G1 venom prevents the potential life-saving signal from being sent, leaving the host's existing cellular machinery to be held hostage. The plasmatocyte is like the patrolling security guard taken hostage by a vSERCA venosome and is tied up and prevented from sounding the alarm, while a crew of system hackers come in and take control of an otherwise secured network.

Thinking about the host-parasite relationship, the parasite ideally wants its host to be as healthy and robust as possible. The host condition is vital to the survival of the developing wasp. The protein Pyruvate dehydrogenase E1 alpha (Pdha) is linked to pyruvate activity[30,31], which is present in fraction 5 and is involved in sucrose synthesis. It is well established that sucrose is an essential metabolic molecule[32–35]. By increasing the metabolism, the rate of host development also increases allowing the host to grow at an accelerated rate, allowing the developing wasp to maximize vital resources for its own development. If it helps, think about the witch in Hansel and Gretel, and how she wanted to fatten the children up before she ate them. In my scenario, the fly larvae represent the children, and the wasp larvae are the witch. Increasing the fly larvae's growth output requires an increase in energy input at the cellular level and provides increased benefit for the developing wasp.

Cells use components such as actin filaments like capulet (capt) (**Table 2**) and myosin molecules like mhc (**Table 2**) in a way similar to a railroad for intracellular molecular transport, where actin acts as the transport rail and myosin the transport vehicle. The protein capt is an actin-binding protein that regulates actin filament assembly, and the protein mhc is a motor protein that provides contractile force along actin filaments[36]. Regulating contractile filament associated proteins may change the volume of intracellular molecules being transported throughout the cell to reflect the altered metabolism. With the cell doing all this extra work, it would undoubtedly need to increase nutrient intake as well, and binding metal ions vital for cell function would help maintain cellular nutritional needs. Transitional ion binding has been shown to be a key factor in regulating virulence factors of Staphylococcus aureus[37] and may be similarly important in the regulation of venom molecules, or perhaps the altered host cells need the ability to regulate these molecules autonomously[29]. Metal ion binding also participates in buffering the effects of oxidative stress.

Cells are only so durable with a finite amount of processes they can carry out before they are no longer able to properly function[38], similar to a machine with moving parts. Those parts must be well maintained to extend the length of time that machine lasts and how much work it can complete efficiently over its lifetime, just like oil changes and other scheduled

maintenance and parts replacement allows a machine to operate efficiently and not break down. A cell is similar in that, as cellular output increases, so too do the wear and tear on the cellular mechanism. Oxidation-reduction reactions and catabolic pathways are essential for regulating cellular turnover by degrading worn-out cellular components at a rate fast enough to keep up with the increased cellular function, allowing for newly transcribed cellular components to replace the old.

Maybe the most interesting protein found in fraction 5 was Toll-like venom protein, which is homologous to Drosophila Toll but with a smaller Leucine-rich repeat (LRR) domain[1]. This Toll-like venom protein may be essential for allowing the venosome to gain access to Drosophila hemocytes. Drosophila Toll-like receptors (TLR) are transmembrane proteins required for receiving signals, which are transduced to activate downstream NFκB transcription factors and immune signaling[11,39,40]. The Toll-like venom homolog has conserved LRRs but lacks transmembrane or TIR domains[1]. It would be worth exploring how altering the Toll-like venom receptor affects the venosomes ability to infiltrate Drosophila plasmatocytes, or if there is a change in immune response post-infection.

In other wasp models, the venom contains a diversity of venosome types with varying sizes and shapes. Although the function of these different types of venosomes has not fully been described to date, it would be interesting to explore the idea that G1 venom contains different types of venosomes, and if so, what different cargos and functions are associated with each specific venosome type. A next step could be to get an electronic microscopy scan of G1 venom to visually quantify the venosomes and determine if venosomes all look the same or if they differ in size and shape like in other wasp venoms. If there are other types of venosome, then maybe there are also other cell types that are targeted or can be targeted by the venosomes in G1 venom, or perhaps there are more specialized venosomes to more specifically regulate different aspects of the host's intracellular environment. By understanding the principle underlying the function of venosomes, researchers may gain insight into potential therapeutic applications in medicines. Utilizing viral vectors has been a powerful tool in the application of gene therapy. Venosomes' ability to transport cell restructuring cargo to specific cell types could lend to current gene therapy methods used today.

The long-term goal of this research is to apply this understanding towards diseases caused by inhibited Ca2+ cycling, such as heart failure, and towards novel tissue-specific therapeutics to target diseases[4,22,41,42] such as cancers that show dysregulated cell signaling. The data collected on G1 venom properties to date are a good starting point for future research into understanding the biogenesis of venosomes, possibly contributing to the emerging field of exosome-mediated therapies[22,42]; and understanding cell altering venosomes and the function of virulent proteins may be a window into effectively manipulating cell production and lifespan. With improvements and modifications to the venom protein purification process, and several more replicates of sucrose gradients, the process of generating venom fractions that dependably separate venom components into consistent layers can be improved. An improved purification protocol would reduce variability in samples, and increase assay accuracy. The research completed so far is an important step towards understanding venosomes and mapping G1 venom protein function.

CHAPTER IV: MATERIALS AND METHODS

Biological Material

The Ganaspis hookeri (strain G1) wasps were caught in Homestead, Florida in 2008. All parasitoids were reared at about 25°C on Drosophila yakuba. After emergence, adults were kept at 25°C on agar medium with honey on the cotton tube stopper. D. melanogaster (w1118 strain) were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). The D. yakuba R strain was obtained from the San Diego Species Center (San Diego, CA). All Drosophila were reared on a standard medium (10% cornmeal, 10% yeast, agar, and nipagine) at 25°C.

Wasp Infections

Wasp infections were performed as described[1–4]. Three female wasps were allowed to attack second instar fly larvae in 5 mL plastic vials, filled with Drosophila medium at 25°C.

Venom Protein Isolation

50-100 female wasps were dissected into 100 ul 1x PBS, augmented with 0.37 mg of Complete Mini Protease Inhibitor Cocktail (Roche). Venom gland tissues are collected on ice, then manually homogenized in non-lysing conditions. Then the cells are pelleted by centrifugation at 10,000 g for 5 minutes, and the venom supernatant is removed and stored in a 1.5mL Eppendorf tube at 4°C.

Venom Protein Purification

The 10-50% sucrose gradient is underlaid in a 600 600uL tube. 100uL of venom isolate is added to the sucrose gradient[43]. The gradient is centrifuged at 130,000 rcf for 12 hours at 4°C. Five equal fractions are removed and placed in separate 1.5mL Eppendorf tubes.

Venom Sonification

In order to disrupt the venosome membrane and ensure proteins potentially held as cargo within venosomes are released, each fraction of purified venom samples is sonicated. From each fraction, a 1:1 ratio of purified venom samples are then placed in a sonicating water bath for 20 minutes at room temperature. Next, the samples are centrifuged at 10,000g for 10 minutes at 4°C. Then, 10μl of DTT for every 40μl of 2x sample buffer is added and incubated for 10 minutes at 70°C[43].

SDS-PAGE Gel Electrophoresis

30 ul of each of the 5 sonicated sucrose gradient fractions is applied in an individual well of 7.5% polyacrylamide SDS-PAGE agarose gel, then ran at 40 mA per gel for ~50 minutes, or when the dye runs off the gel.

Protein Stain

Following SDS-PAGE, the gel is incubated in Gold Biotechnology Blazin' Bright luminescent UV protein-dye solution for 5 minutes. The UV signal will show stained bands where proteins are present and will be visualized with a digital camera and accompanying software

Western Blot

The proteins are transferred from the SDS-PAGE gel to a PVDF membrane. After the transfer, incubate the membrane in milk block (1x Tris-Buffered Saline (TBS) + 5% powder milk + 2% bovine serum albumin) for 1 hour to reduce non-specific binding. Wash three times in TBST (1X TBS + 0.2% Tween 20). Then, incubated with the primary antibody in milk block (.1 ml anti-rabbit SERCA + .9 ml milk block), and secondary antibody (.1 ml anti-rabbit HRP + .9 ml milk block). The secondary antibody aids in the detection of proteins by binding to the

primary antibody. The secondary antibody is incubated with an enhanced chemiluminescent

(ECL) to produce a signal that is visualized with a digital camera and accompanying software.

Sample Preparation for MS Analysis

Thirty-five microliters each of sucrose gradient fractions in SDS sample buffer (2% SDS,

10% glycerol, 50 mM Tris, 0.5X PBS, trace bromophenol blue) were separated onto a

NuPAGE™ 10% Bis-Tris Protein gel (Cat.# NP0315BOX, Invitrogen) at 200V constant for

10min. The gel was stained using a Colloidal Blue Staining Kit (Cat.#LC6025, Invitrogen)

following the manufacturer's instruction.

Each gel lane was excised and digested overnight at 37°C with Pierce™ Trypsin

Protease, MS Grade (Cat.#90058, Thermo Scientific) as per manufacturer's instruction. Digests

were reconstituted in 0.1%FA in 5:95 ACN:ddH2O at ~0.1ug/uL.

Mass Spec. nLC-ESI-MS2 Analysis and Database Searches

Peptide digests (8µL each) were injected onto a 1260 Infinity nHPLC stack (Agilent 11

Technologies), and separated using a 100 micron I.D. x 12.5 cm pulled tip C-18 column (Jupiter

C-18 300 Å, 5 microns, Phenomenex). This system runs in-line with a Thermo Orbitrap Velos

Pro-hybrid mass spectrometer, equipped with a nano-electrospray source (Thermo Fisher

Scientific), and all data were collected in CID mode. The nHPLC was configured with binary

mobile phases that included solvent A (0.1% FA in ddH2O), and solvent B (0.1% FA in 15%

ddH2O / 85% ACN), programmed as follows; 10min @ 5%B (2µL/ min, load), 90min @ 5% -

40%B (linear: 0.5nL/ min, analyze), 5min @ 70%B (2µL/ min, wash), 10min @ 0%B (2µL/

min, equilibrate). Following each parent ion scan (300 -1200m/z @ 60k resolution),

fragmentation data (MS2) was collected on the topmost intense 15 ions. For data-dependent

scans, charge state screening and dynamic exclusion were enabled with a repeat count of 2,

repeat duration of the 30s, and exclusion duration of the 90s

The XCalibur RAW files were collected in profile mode, centroided and converted to MzXMLusing ReAdW v. 3.5.1. The data were searched using G1 protein sequences and SEQUEST, which was set for two maximum missed cleavages, a precursor mass window of 20ppm, trypsin digestion, variable modification C @ 57.0293, and M @ 15.9949.

Mass Spec. Peptide Filtering, Grouping, and Quantification

The list of peptide IDs generated based on SEQUEST (Thermo Fisher Scientific) search results were filtered using Scaffold (Protein Sciences, Portland Orgegon). Scaffold filters and groups all peptides to generate and retain only high confidence IDs while also generating normalized spectral counts (N-SC's) across all samples for the purpose of relative quantification. The filter cut-off values were set with minimum peptide length of >5 AA's, with no MH+1 charge states, with peptide probabilities of >80% C.I., and an FDR <1.0%. Scaffold incorporates the two most common methods for statistical validation of large proteome datasets, the false discovery rate (FDR) and protein probability [17-19]. Relative quantification across experiments was then performed via spectral counting [20, 21], and when relevant, spectral count abundances were then normalized between samples [22]. Using quantitative analysis, I determined that fraction 5 contained the most vSERCA and would be the fraction I focused on.

Ex vivo SERCA Activity Assay

Healthy third instar larvae are washed in 1x PBS and are dissected into wells of a diagnostic slide (Tekdon, Inc.) containing 30μL of 1x PBS. Three wells are used with 5 larvae bled into each well for at least 5 minutes each to allow hemocytes time to adhere to the slide. The PBS is removed and either experimental buffer (Fluo-4 + venom fraction) or control buffer (Fluo-4 + PBS containing an amount of sucrose equivalent to the concentration found in the

venom fraction) is added. Fluo-4 AM from Thermo Fisher Scientific is a calcium indicator that

increases in fluorescence upon binding Ca2+. Samples were allowed to incubate in the dark for

15 minutes before being placed in an Invitrogen Tali Image-Based Cytometer, where changes in

cell fluorescence could be measured. The fluorescence intensity of cells is proportional to

intracellular Ca2+ levels.

Statistics

All analyses were performed in RStudio version 1.1.463. Using the "multcomp"[44],

"lme4" [45], "lmerTest"[46], "effects"[47], "dplyr"[48] packages. Mixed linear models, including replicate

as a random effect, were used to test for differences of fluo-4 fluorescence levels between venom

fraction and the control replicate groups. The model treats a replicate as a random effect while

averaging the result by taking differences between replicates into account. The resulting whisker

graph shows the distribution of fluorescent intensity data through quartiles. This disregards

random effects in the final statistics.

Immunohistochemistry

5-10 venom glands were dissects into wells of a diagnostic slide containing 30µL PBS.

The PBS was replaced with 2% formaldehyde in PBS and left to fix for 15-20 minutes. The

tissues were washed with PBS at least 4 times and washed once with PBT (39.6 ml PBS + 400 ul

20% Triton). The venom glands were then transferred to a 1.5 ml Eppendorf tube and allowed to

gently rock for one hour in a block solution of PBTG (9.9 ml PBT + 100 ul 4% goat serum).

Then, the block was replaced with SERCA Alexa 488 diluted at ~1:1000 in PBTG as the primary

antibody probe and allowed to incubate on a rocker at room temp for 5 hrs, or at 4°C overnight.

The sample was washed 3-4 times with PBTG block for a total of at least 1 hour. The phalloidin

633, a green fluorescent dye that stains actin, is diluted at 1:1000 in PBGT as the secondary

antibody probe and allowed to incubate on a rocker at room temp for 5 hrs or at 4°C overnight wrapped in foil, then washed 3-4 times with PBTG and let the sample sit in last wash wrapped in foil until mounted. The samples were then viewed with a Leica SP8 confocal microscope.

Bioinformatics

The venom proteins identified by MS as co-localizing in fraction 5 with vSERCA were assembled into a FASTA sequence database. Because the G1 genome is not well annotated, BLAST was used to identify the homologous proteins in D. melanogaster, a species with a highly annotated genome. I used the command line BLAST (version 2.7.1) with a custom made BLAST database including all identified protein sequences from the D. melanogaster genome (from http://flybase.org, genome release 6.31).

The Flybase36 gene IDs were then used for gene ontology (GO) term mapping through Panther (http://geneontology.org/)[49,50]. The biological process, molecular function, and cellular component databases were individually searched and the D. melanogaster genome was used as the background gene set for enrichment analysis. GO terms with a corrected enrichment p-value < 0.05 are considered significantly enriched in the sample.

Fig. 1 Sucrose Gradient
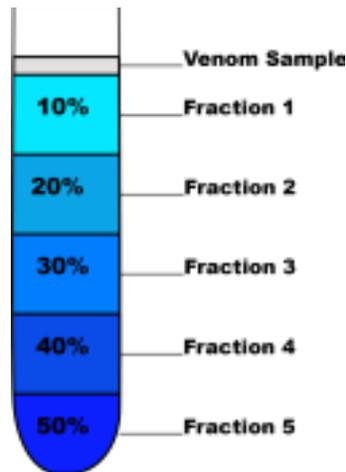
The Sucrose Gradient A 10-50% sucrose gradient is established and after centrifugation at 130,000 rcf for 12 hours at 4°C, five equal layers are separated into individual 1.5mL Eppendorf tubes as corresponding fractions.

Fraction 1 At a concentration of 10% sucrose and 90% PBS, this is the least dense layer of the gradient. This layer is expected to have individual venom proteins and peptides, as well as proteins and peptides that may have initially been a part of complexes that were disrupted during the purification or centrifugation processes.

Fraction 2 At a concentration of 20% sucrose, this layer is expected to have some overlapping venom proteins and peptides from fraction 1 and possibly less dense protein complexes.

Fraction 3 At a concentration of 30% sucrose, this this is where I'd expect to start seeing vensomes and other large protein complexes.

Fraction 4 At a concentration of 40% sucrose this layer is expected to have venosomes and other large complexes that are overlapping and possibly more dense than fraction 3.

Fraction 5 At a 1:1 concentration of sucrose to PBS this layer is the densest of the gradient. I would expect to see some overlapping venosomes and complexes from fraction 4 along with the most dense venosomes and venom protein complexes. However, I would not expect to see any pelleting because most of the venom content is expected to have reached its equilibrium point before reaching the bottom of the centrifuge tube.
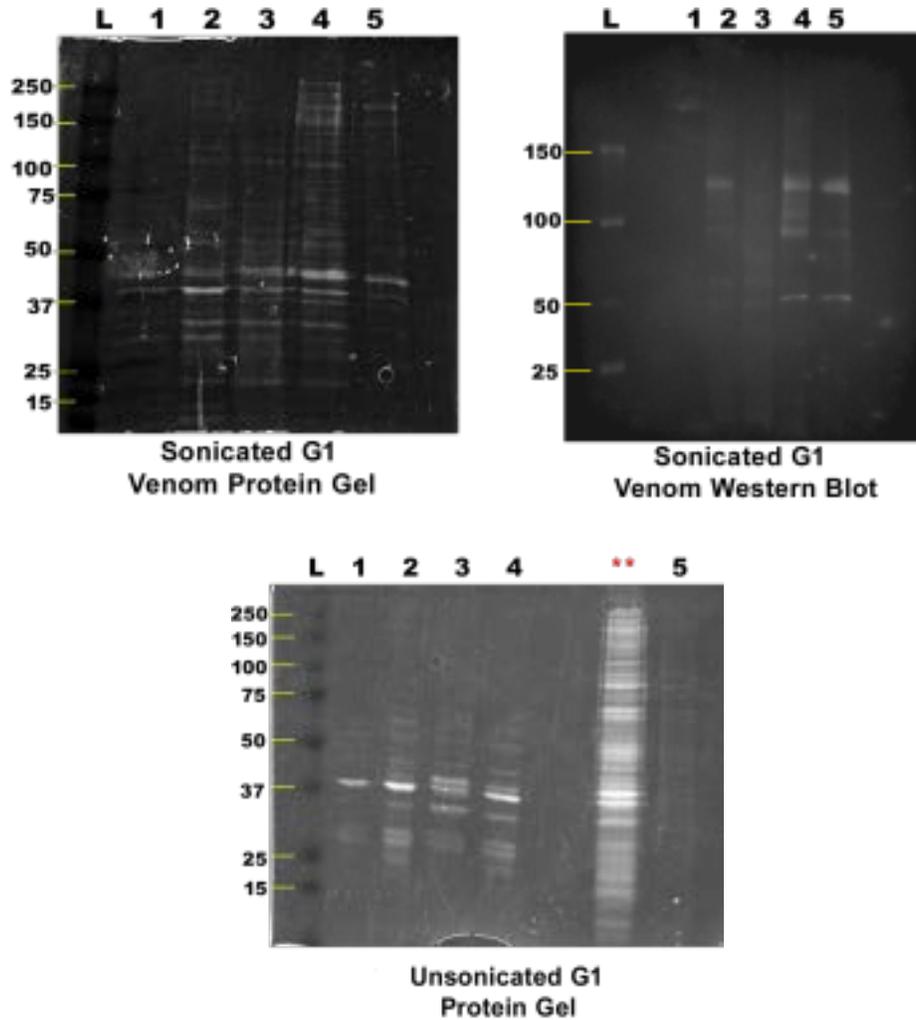
Fig. 2 Protein Gel and Western Blots

Figure 2. SDS-PAGE protein analysis of fractionated G1 venom. For each panel, lane L represents the ladder and is labeled by size. Each lane contains the corresponding sucrose venom fraction (ie. lane 1 contains fraction 1). (a) SDS-PAGE of unsonicated purified venom stained with Blazin' Bright UV protein stain. The brighter the signal, the more protein that was detected. The lane marked ** is a contaminated sample that was not analyzed further. (b) SDS-PAGE of sonicated purified venom stained with Blazin' Bright UV protein stain. (c) The same samples as B were analyzed by Western blot probed with anti-SERCA.

Fig. 3 Venom Fractions 1-5 Calcium Assay Statistic Plots
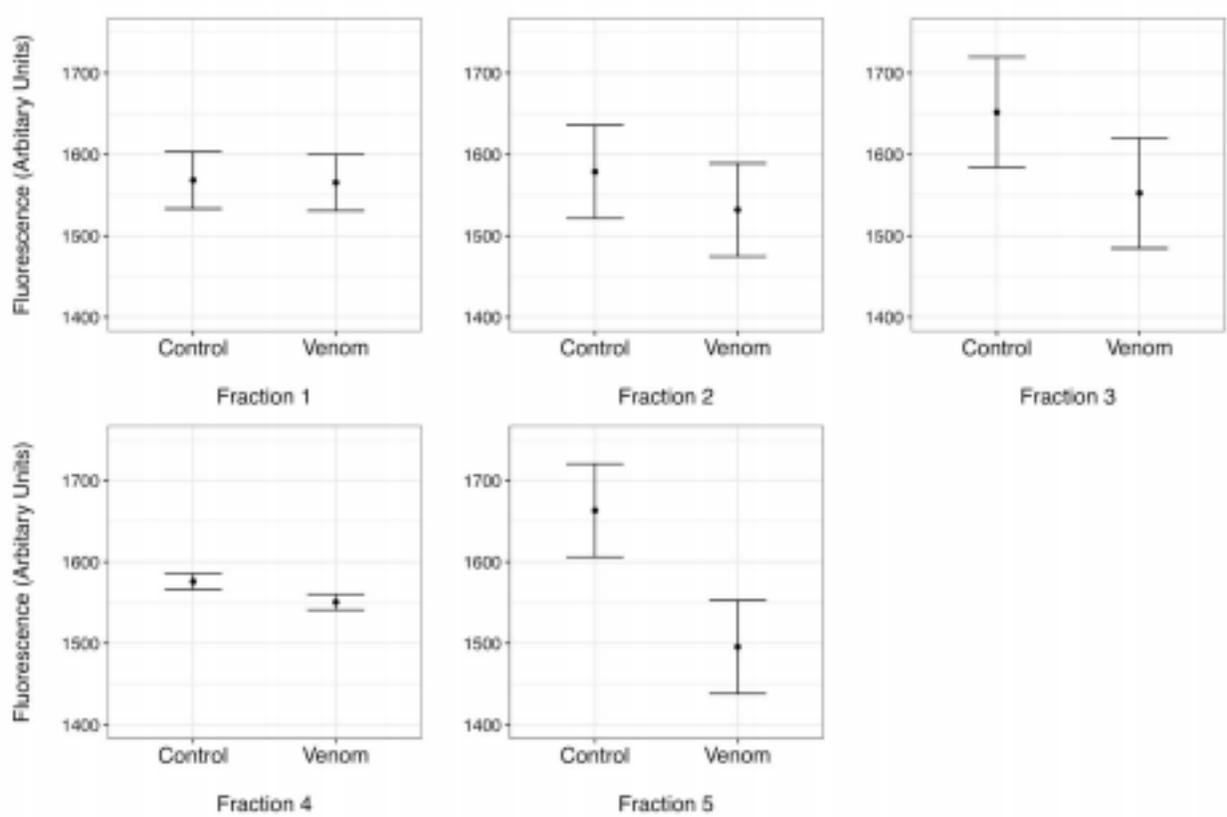
Invitrogen Tali Image-Based Cytometer was used to measure the change in fluorescent intensity of Drosophila second instar larvae hemolymph samples that were incubated with calcium indicator Fluo-4 AM from Thermo Fisher Scientific. A linear modeling code was used in R to generate the whisker graphs that reflect the change in fluorescent intensity. The graphs show the fluorescent intensity +/- the standard error.

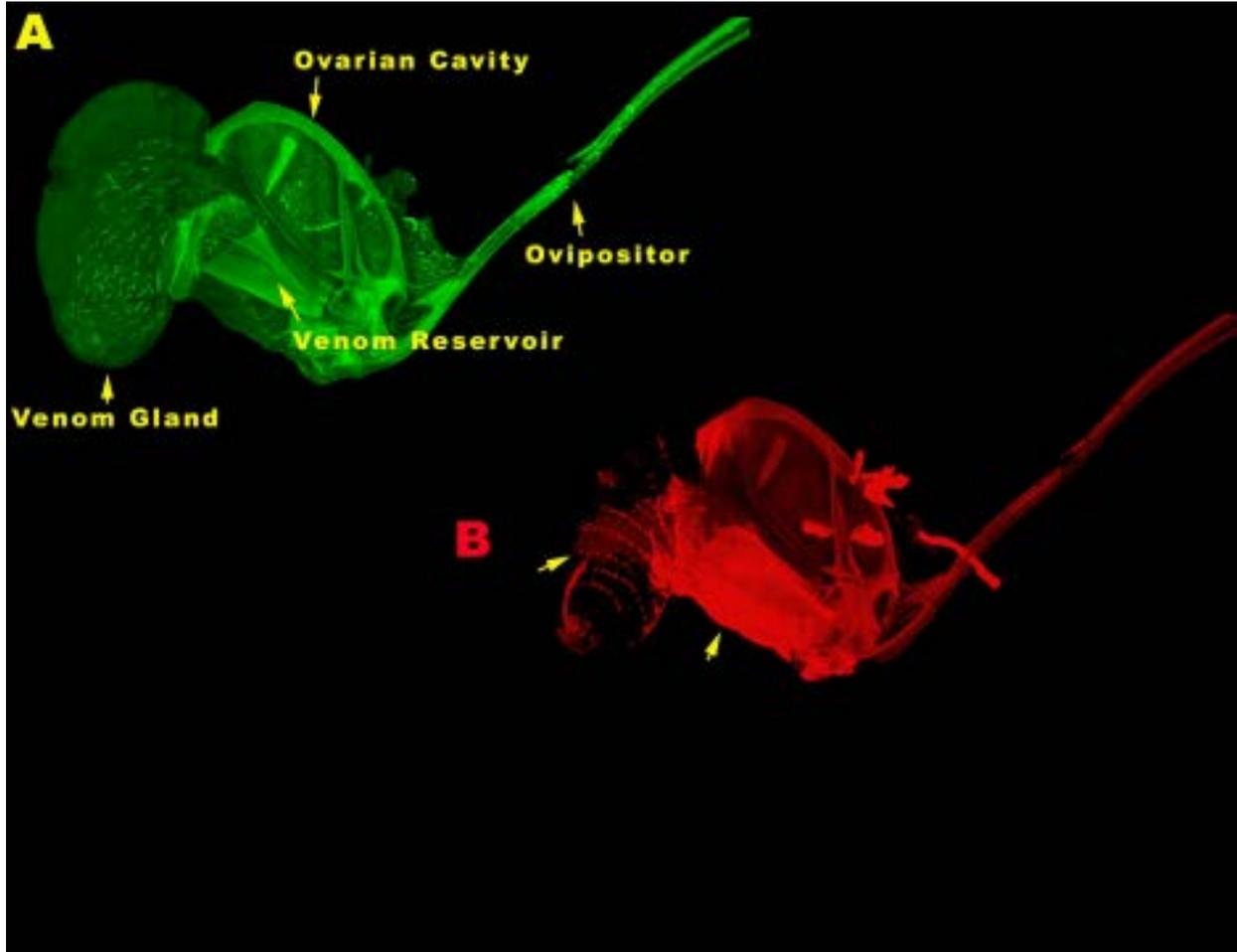Fig. 4 G1 Venom Apparatus Confocal Images

Figure 4. Confocal imaging of SERCA localization in the venom apparatus. Images were taken simultaneously using a 10x objective and 3.99 digital zoom. (**A**) Image of the whole G1 venom apparatus stained with anti-SERCA, and with structures labeled. (**B**) Image of the whole G1 venom apparatus labelled with phalloidin.

Fig. 5 G1 Venom Gland Confocal Images
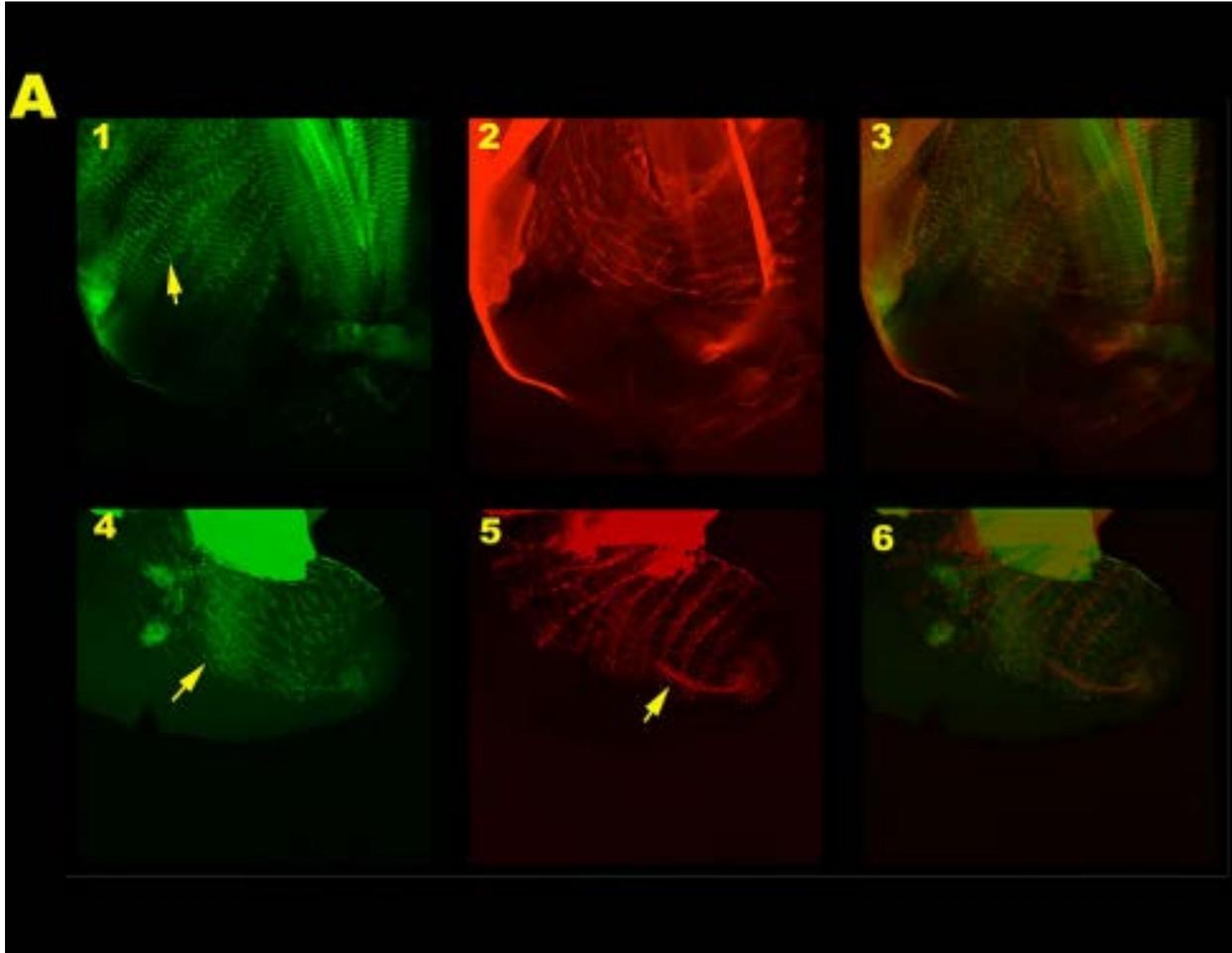
Figure 5. Confocal imaging of SERCA localization in the venom reservoir. Images taken using a 10x objective and 3.99 digital zoom of anti-SERCA (green) localized in the sarcoplasmic reticulum (**A1**), actin structures labeled with phalloidin (red) on the venom reservoir (**A2**), and a composite of the two images (**A3**). (**A4-C6**) Images taken using a 10x objective and 2.77 digital zoom of anti-SERCA (green) localized in the venom gland and venom reservoir (reservoir is indicated by the arrow) (**A4**), the winding system of tunnel-like actin structures (labelled with phalloidin, red) with the arrow indicating the secretory duct (**A5**) and a composite of the two images (**A6**).

Table 1 Mass Spectrometry Fraction Quantification

Mass Spectrometry Fraction Quantification	
Fraction #	# of Proteins Found in Fraction
1	230
2	311
3	314
4	320
5	291
Total	521

Table 2 Mass Spectrometry Analysis Fraction 5 Preview

Mass Spectrometry Analysis Fraction 5 Preview		
Accession ID	**Flybase ID**	**Gene Symbol**
comp1045	FBgn0263006	SERCA
comp1567	FBgn0028325	Pdha
comp4522	FBgn0035371	AhcyL1
comp288	FBgn0030449	Fer3HCH
comp3360	FBgn0261458	capt
comp16783	FBgn0022709	Adk1
comp2164	FBgn0040064	yip2
comp4656	FBgn0003255	rk
comp45454	FBgn0264695	Mhc
comp3177	FBgn0035917	Zasp66
comp12432	FBgn0010482	l(2)01289
comp6889	FBgn0034476	Toll-7
comp882	FBgn0278608	Dsp1
comp2103	FBgn0023023	CRMP
comp1291	FBgn0028685	Rpt4
comp303	FBgn0026415	Idgf4

Table 3 Biological Process GO Terms

Biological Process GO Terms		
GO ID	**Biological Process**	**P-Value**
GO:0051238	sequestering of metal ion	2.55E-02
GO:0010499	proteasomal ubiquitin-independent protein catabolic process	1.03E-04
GO:0008063	Toll signaling pathway	8.77E-04
GO:0009063	cellular amino acid catabolic process	1.38E-02
GO:0007623	circadian rhythm	6.23E-03
GO:0048511	rhythmic process	6.08E-03
GO:0055114	oxidation-reduction process	1.33E-06
GO:0007166	cell surface receptor signaling pathway	4.99E-02
GO:0032989	cellular component morphogenesis	3.79E-02
GO:0044238	primary metabolic process	1.04E-02

Table 4 Cell Composition GO Terms

Cell Composition GO Terms		
GO ID	**Cell Component**	**P-Value**
GO:0008043	intracellular ferritin complex	9.24E-03
GO:0032982	myosin filament	1.78E-02
GO:0005839	proteasome core complex	1.76E-05
GO:0030018	Z disc	4.35E-02
GO:0015629	actin cytoskeleton	1.10E-03
GO:0043233	organelle lumen	4.29E-02
GO:0031974	membrane-enclosed lumen	4.01E-02

Table 5 Molecular Function GO Terms

Molecular Function GO Terms		
GO ID	**Molecular Function**	**P-Value**
GO:0004030	aldehyde dehydrogenase [NAD(P)+] activity	3.52E-02
GO:0008199	ferric iron binding	3.06E-02
GO:0051371	muscle alpha-actinin binding	3.69E-03
GO:0016620	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	5.44E-03
GO:0005524	ATP binding	1.27E-02
GO:0000166	nucleotide binding	7.01E-03
GO:0036094	small molecule binding	1.67E-02
GO:0003824	catalytic activity	1.20E-05

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