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A Proteomic Analysis of Corydoras Sterbai Secretions and Tissues

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A PROTEOMIC ANALYSIS OF CORYDORAS STERBAI SECRETIONS AND TISSUES

By

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DEDICATION

This thesis is dedicated to my family and friends. Mom and Dad, thank you for supporting me all throughout my undergraduate education and backing my decision to go into higher education. To all my friends, thank you for listening to me and encouraging me to finish the program and my thesis. To my wonderful significant other, Taylor, for always supporting me, listening to my science rants, and being my better half.

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A PROTEOMIC ANALYSIS OF CORYDORAS STERBAI SECRETIONS AND TISSUES

Abstract

By Erik P. Wictor

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Defensive mechanisms vary widely in the animal kingdom ranging from physical defenses like spines to chemical defenses such as toxins. Toxins in these secretions and tissues can fluctuate from enzymes to lipids to uncharacterized chemicals. Next generation -omics technology and mass spectrometry are extremely important in analyzing these samples because of their ability to distinguish minute amounts of toxic substance within a complicated sample. The goal of this experiment was to look at secretions and tissues from *Corydoras sterbai*. All samples in this study were proteolyzed using a mixture of Trypsin and Lys-C, fractionated, and run through nanoLC-MS/MS analysis using an Orbitrap Fusion™ Tribrid™ mass spectrometer. Using guanidine hydrochloride as a denaturant, a total of 420 database peptide matches were discovered in the secretions and up to 777 database peptide matches among the tissues. Proteins of interest found in both the secretion and in the hypothesized gland include members of the prostaglandin synthesis pathway, phospholipases, and peroxiredoxins. It is theorized that *C. sterbai* uses its serrated rays in addition to these proteins to defend themselves from would be predators.

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CHAPTER 1: INTRODUCTION

Living long enough to be able to reproduce is considered the most important goal of an organism. Thus, organisms not at the top of the food chain have had to develop defenses to make sure that they don't become predated upon. These defenses come in a variety of forms such as teeth, claws, spines, coloration, and toxins. Up to this point, a vast percentage of all studied venoms have come from terrestrial organisms due to ease of capture of the organisms[1]. Aquatic organisms face the unique challenge that their secretions can be washed away in a stream or ocean. This has led to the incorrect line of thinking that there are a vastly greater numbers of toxic organisms on land versus water.

Catfish

Of some 33,100 species of fish that have been described, just over 3,000 of them are catfish[2,3]. These fish range in size from one centimeter to over two meters in length and have defense mechanisms that make them hard to catch and potentially impossible to eat; such mechanisms include toxins, sharp spines, and/or slimy secretions. C*orydoras sterbai*, as seen in Figure 1, is a fish whose maximum length is approximately seven centimeters on average standard length. that utilizes both sharp spines and what is thought to be a toxin[4]. Spines for this species are located on all but the caudal fin, as seen in Figure 2, while the secretion appears to come out just behind the pectoral fins due to a small opening. These small fish are typically found in Bolivia and central Brazil in streams and rivers where the water is soft and slightly acidic, with low levels of nitrates[5]. The river where they are most commonly found is the Rio Gauporé, which is found along the border between Brazil and Bolivia[5]. In nature, these fish travel in schools of six or more, so aquarium enthusiasts should have a minimum of six or more in the tank to make the fish are as comfortable as possible[5].

Figure 1. C. sterbai in a normal tank environment.

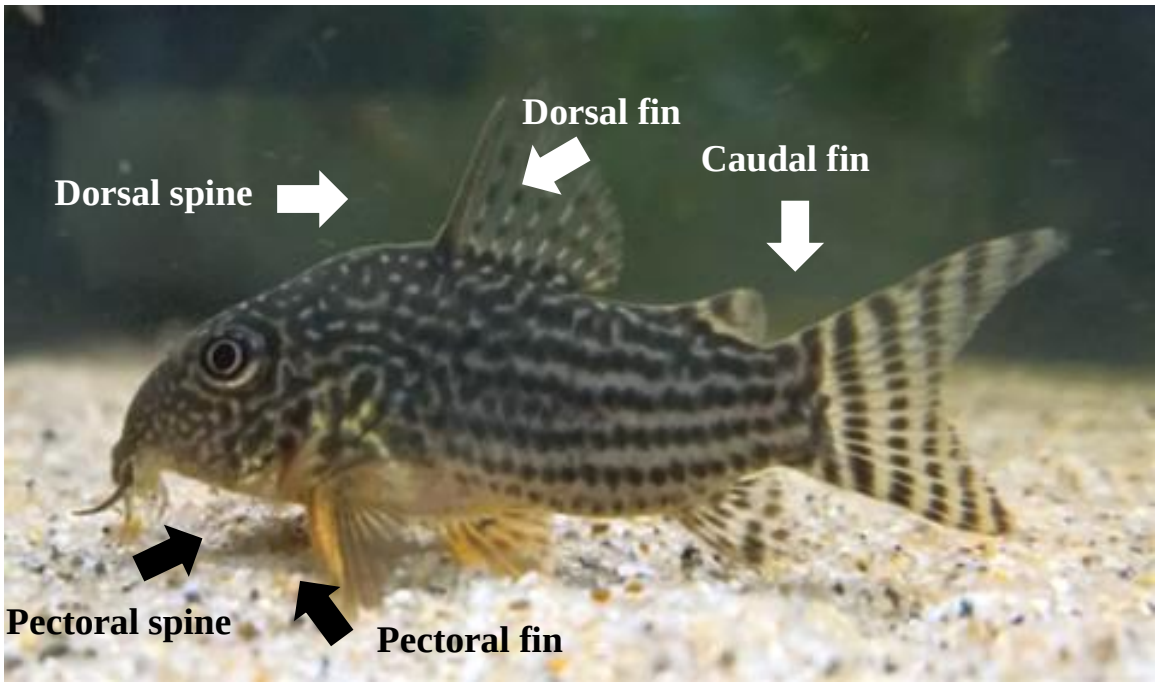

Figure 2. External anatomical labeling of C. *sterbai*.

Anatomy and Physiology of *C. sterbai*

Corydoras Defenses

All species in the *Corydoras* genus contain two thick sets of bony plates found along the side of the fish that give it protection along with several spines that it has on their body[4]. The armored plates are a passive mechanism of defense. The spines can be found on the leading edge of all fins, except the caudal fin (tail), in each pectoral fin, in the adipose tissue on their body, and the largest in front of their dorsal fin[5]. The poison is thought to be found in a gland next to barbs on the pectoral spine and is thought to flow down the spine, allowing the poison to potentially be absorbed into the predator through physical damage from the spines and or gills. The spines and toxin are a more active method of defense. The toxin appears to come out of an opening that is found just under the pectoral fin on either side of the fish.

Morphology and Identification of *C. sterbai*

Looking at the morphology of *C. sterbai* it is often confused with several other species of the same genus. One distinguishing feature are their yellow-gold pectoral fins. These brightly colored fins are a classic example of aposematic coloration for predators. They also have a dome-shaped head with white spots. This species has also been seen in an albino form[5]. *Corydoras haraldschultzi* is the exact opposite with light skin and dark spots, making it easy to confuse the two of them. In observing the fish, it is common to see *C. sterbai* swimming up to the surface and taking in a gulp or two of air. This allows the fish to have two methods of oxygen absorption. The lining of the digestive system of *C. sterbai* is highly vascularized which allows for the uptake of oxygen through the blood as well as through the gills which can be particularly useful if a stream starts to dry up[2]. The various physiological differences between the nine lineages of *Corydoras* can be found below in Figure 3.

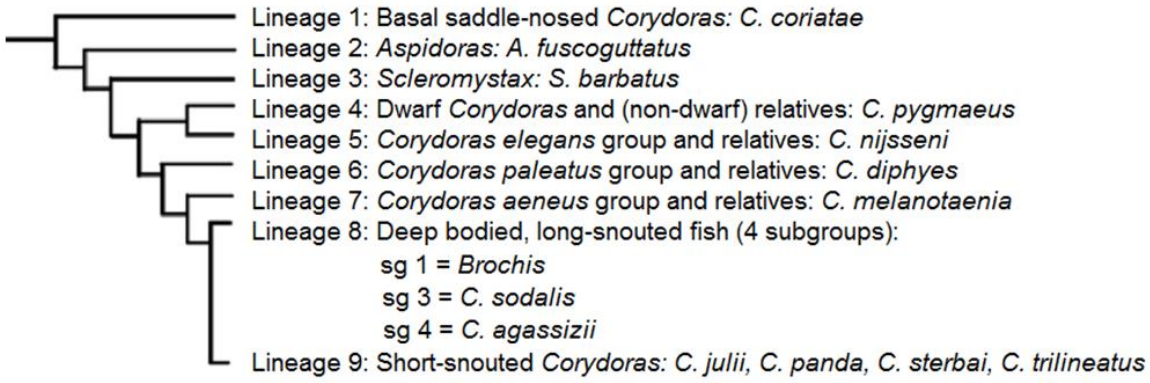

Figure 3. A representation of the nine lineages of *Corydoras* based upon Supplemental Figure 1 from Alexandrou *et. al* 2011. *C. sterbai* is found in Lineage 9.

Proteomics Overview

Proteomics at its core is a study of one or more proteins or peptides found in a sample. At the start, these proteins or peptides may have some structure, so they must be denatured. Denaturation is extremely important to make sure that the protease(s) used in the experiment has or have unfettered contact with all active sites. After proteolysis, only peptides should be left; intact proteins can prevent LC-MS analysis. Liquid chromatography coupled with mass spectrometry (LC-MS) is the next step in the project. LC-MS is an incredibly powerful tool that utilizes chromatography columns typically made of C18 chains to bind and then methodically elute as the gradient changes throughout the run. In this experiment, a tandem mass spectrometer was used which has the capability to first find the mass of all peptides in the sample based upon their time of flight and then it can fragment them into their amino acid components. Fragmentation is especially critical because then the amino acid sequence can be compared to a database using software to rebuild the fragmented peptide. Any matches in the database to any peptides help to either discover or confirm what was in the original sample. With this

information, scientists can then help to map out interaction networks and or perform other experiments. This workflow is best explained by Figure 4.

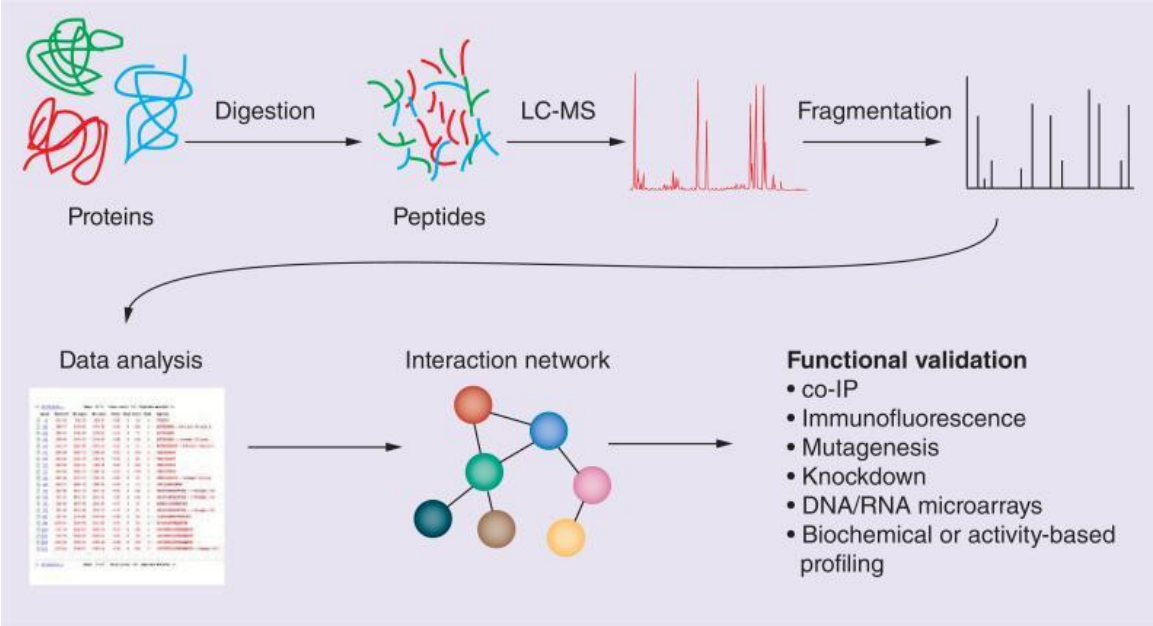

Figure 4. An overview of a proteomic workflow. Scientists begin with material in form the form of tissues, cells, or other material containing protein and end up with information that can be used in further studies.

Experimental Overview

The purpose of this thesis is to discover the components of the secretion that *C. sterbai* produces with the eventual hope is to analyze the secreted substances from more individuals of each of the nine lineages of *Corydoras*[6]. In time, this would give greater insight to how this genus has evolved their defensive mechanisms. In this experiment it is important to note that the cloudy secretions, tank water, and body tissues will be analyzed using proteomic techniques. The cloudy secretions and tank water were analyzed first as it was hypothesized that the fish would secrete a toxin that would match any of the other well characterized toxins found in aquatic species. After several attempts using different methods to analyze the secretions from

the fish and the tank water they were kept in, a careful dissection of one fish was done and six different types of tissues were collected. These tissues were ground up and then analyzed using the same methods and techniques as the secretions and tank water mentioned above.

CHAPTER 2: REVIEW OF THE LITERATURE

An Overview of Venoms and Toxins

Based upon previous research, organismal toxins can be proteinaceous in nature and cause a myriad of effects such as bleeding, inflammation, and pain[7]. This is because most organisms' toxins contain two or more bioactive compound groups such as neurotoxins, cytotoxins, and hemotoxins. The first step is to define a toxin. A toxin is a peptide, protein, or other molecule that can create an immune response in the target. Toxins are further dissected into two groups, venoms or poisons. As defined by Mebs (2002), a venomous animal must produce the venom using cells or in at least one gland, have a mechanism or excretion or extrusion of the venom, and have an apparatus to wound the prey or predator. A poison is also a toxin, but instead the target must absorb it in some fashion, such as through the skin, gut, or by inhalation through the lungs.

Neurotoxins

Neurotoxins destroy and/or inhibit the components of the nervous system on a molecular level. One way this occurs is by inhibiting the release of the neurotransmitter acetylcholine through destruction of SNARE proteins[9]. This can lead to muscle tetany and nervous system signaling impairment[10]. Another way neurotoxins can affect nerves is by binding to receptors[11].

Fasciculins. The first subgroup of neurotoxins is fasciculins, commonly found in green mamba snake venom, but can also be found in other species of mamba[10]. This subgroup of toxins causes intense muscle fasciculation or twitching by binding to the neuromuscular junctions and inhibiting, degrading, and or causing the acetylcholine to be hydrolyzed[10]. Intense muscle fasciculation can cause an organism to become paralyzed as all its muscles will flex,

rendering the prey helpless.

α-Neurotoxins. Another member of this group is the α-neurotoxin. This type of neurotoxin is similar to the fasculinins, but instead of affecting the neurotransmitter acetylcholine, it competitively binds to acetylcholine's receptor[12]. This can lead to suffocation as well as a heart attack as autonomic nervous signaling is disrupted. This toxin is found in two snake families, *Elapidae* and *Hydrophiidae*.

Tetrodotoxins. Tetrodotoxins (TTX) are a group of neurotoxins that have been found in numerous taxa across the phylogenetic kingdom. This particular kind of neurotoxin blocks the fast voltage-gated sodium channels in the neuromuscular and pulmonary systems eventually leading to death[13]. It is considered one of the most toxic substances in the world and is approximately 10,000 more time poisonous than cyanide[13].

Batrachotoxins. *Batrachotoxins* (BTX) are neurotoxins found only in beetles, birds, and frogs. They bind to similar channels like TTX and prevents depolarization of the fast voltage-gated sodium channels[14]. There are several forms of this toxic steroidal alkaloid with varying levels of toxicity[14].

Dendrotoxins. Dendrotoxins contain anywhere from 57 to 60 amino acids and are crosslinked by three disulfide bridges[11]. This collection of toxins deals with the voltage-gated potassium channel in neurons[15]. By binding to these receptors, this group of toxins affects the membrane resting potential of neurons and also they can bind to the Node of Ranvier in motor neurons, affecting cellular signaling[15]. These toxins have only been found in mamba snakes.

Cytotoxins

Another group of toxins that is commonly found in nature are cytotoxins. Cytotoxins are described as, "Substances that are toxic to cells; they may be involved in immunity or contained

in venoms."[16] These toxins can perform a variety of dangerous effects such as necrosis, apoptosis, and or a shutdown of mitosis.

Phospholipases. Pholspholipases (PLAs) are categorized into four main categories: secreted ($sPLA_2$), cytosolic ($cPLA_2$s), calcium-independent ($iPLA_2$s), and platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein associated (Lp)PLA_2s[17]. They specifically bind to the phospholipid bilayer in cellular membranes and then hydrolyze the fatty acid at the *sn-2* position of the membrane phospholipid[17]. This chemically turns the phospholipid bilayer into a lysophospholipid, which lyses the newly unstable cell membrane[18]. It should be noted that specifically PLA_2 is a major component of both old and new world snake venoms and that human pancreatic PLA_2 is similar to PLA_2 in old world snake venoms[17].

Hemotoxins. The next subgroup contains hemotoxins. These toxins destroy red blood cells and can also cause intense clotting in the victim, leading to rapid death. The presence of a positive charge on one of the loops in the structure of these toxins enables them to have an affinity for red blood cells[19]. Individuals referring to these kinds of toxins usually refer to the ones found in viper venom, but due to the wide definition of hemotoxin, a number of organisms all over the world can cause these issues[20].

Overview of Toxin Delivery Systems

Toxin delivery systems are complex and varied across numerous taxa. Organisms that have fangs contain common structures such as the compressor muscle, venom reservoir, primary venom duct, secondary venom duct, venom canal, and fang, spine, or stinger[21,22]. Toxins can also be found in saliva. Taxonomic groups such as *Solenodontiae, Desmodontiae,* and *Chiroptera* all contain organisms that produce venoms at different levels using modified salivary glands[23]. Common examples of the previously named families are the Cuban solenodon,

vampire bat, and bats in general. Other common structures that are used in envenomation include both stingers or hollow spines; these structures are found in scorpions and venomous fish, respectively[1,24]. In fact, spines themselves have evolved 11 different times within acanthomorphs and twice within catfish[1]. Spines in fish can be found close to the operculum, dorsal ray, and or in the caudal region[1]. Toxins can also be found on the epithelium of organisms. This is common to amphibians such as toads, frogs, and salamanders[25]. Studying the method of delivery is vital to finding out where the toxin is found in or on an organism. This not only helps to classify delivery systems but also helps when trying to remove the toxin from the organism to study it.

Glandular secretions.

Exocrine secretion mechanisms. Multicellular glands are glands that secrete a substance onto an epithelial surface use a specialized duct[26]. Examples of glands that do this are: sweat, salivary, mammary, and mucous glands[26]. These gland examples and more utilize three main types of secretion, merocrine, apocrine, and or holocrine[26].

Merocrine secretion. Merocrine secretion is a method by which a cell moves the secretory product into a secretory cell[27]. From there, the secretory product is released into a hollow gland lumen and then the product drains from the lumen to the surface[27].

Apocrine secretion. Cells going through apocrine secretion pinch off part of their cellular membrane to be removed from the cell[28]. Mammary glands in mammals employ this method of secretion[28]. Thus, proteins that are normally found in the cytoplasm would be found along with the hypothetical toxin. Also nuclear proteins have been found as part of the secretion by other researchers[28].

Holocrine secretions. Holocrine secretion involves the rupture of a mature cell's

membrane to secrete the product out of the cell and into the environment[29]. With this cataclysmic event, all protein products and other chemicals normally expressed in the cell should be found[29]. Using histological evidence, it appears that most fish venom glands use this method of secretion[1]. To analyze the proteins in these secretions, a powerful machine is needed.

Mass Spectrometry

Another key component of proteomics research that is being used today is the mass spectrometer. The definition of spectrometry as a scientific tool is: the measurement of electromagnetic radiation as a means of obtaining information about physical systems and their components[30]. The type of machine that will be used in this thesis research is an Orbitrap Fusion™ Tribrid™ Mass Spectrometer produced by Thermo-Fisher Scientific™. The sample is placed and kept chilled in a tray until it is ready for sampling. When ready, it is forced through narrow tubing at an extremely high pressure against a column[31]. The column contains spherically or asymmetrically shaped particles to help fractionate the proteins in solution, as different peptides will react in different ways when hitting the shaped particles[31]. The column used for this experiment contains a C_{18} resin that helps to bind peptides while removing contaminates such as salts and other solutes that can interfere with peptide analysis. The sample range works from one picogram all the way to thirty nanograms[32]. This research was done using the Electrospray Ionization (ESI) attachment. ESI can ionize numerous small peptides without destroying the larger parent protein[33]. By ionizing small parts of the larger molecule this can further the analytical powers of the mass spectrometer, especially if the mass spectrometer has a maximum size limit close to that of the sample[33]. Ionizing small peptides from the parent protein is beneficial to reconstructing the original amino acid sequence and can help to determine which peptides ionize more efficiently. Peptides then move into the Orbitrap™ for further analysis.

The Orbitrap™ works by attracting charged ions from the ESI attachment as they are injected into the chamber[34]. In order to make sure that the ions make it into the chamber, the voltage first has to be slightly lowered[34]. The ions then form an almost circular harmonic orbit around the inner electrode which is determined by their charge to mass ratio[34]. Once the desired orbital diameter has been achieved, then the analyzation of the ions can begin using Fourier transformations to determine the masses of the ions, which helps determine their compositions and to separate them for further analysis using different appendages[34].

After ionizing peptides into the gas phase, they must be systematically broken up into amino acids. Dissociation or the breaking of peptides into amino acids is performed using a method called Higher-Energy Collision Dissociation (HCD). Peptides then collide with nitrogen gas at high speeds inside the collision trap before they go into the Orbitrap™ to determine individual amino acid weight[35]. The weight of the individual amino acids is then summed and compared to the previously recorded mass of the peptide to check for accuracy. Data from the mass spectrometer can then be used in bioinformatic analysis with various software programs.

CHAPTER 3: METHODOLOGY

Fish Keeping

Eight *Corydoras sterbai*, 5.0 - 7.0 cm standard length, were purchased from a local Petco in Stockton, CA for research purposes. They were fed both flake food and live blood worms twice a week. All fish were housed in ten-gallon tanks that received 50-60% water changes weekly, with half deionized water and half tap water. Commercial dechlorinator (SeaChem Prime) was added to the tank water after it was changed to bind chlorines and other chemicals normally found in tap water. Tanks were kept at 27°C to simulate normal environmental conditions for the species.

Sample Preparation

Sample Collection

At the time of collection, 300 mL of fish water were taken out and divided equally among two sandwich-sized Ziploc® plastic bags. Five fish were then taken and placed in one bag at RT, to be stressed out. To stress the fish out, the bag was sealed and agitated by hand for up to 15 minutes so that the fish were not simply sitting there. After the fish showed clear signs of stress such as loss of muscular equilibrium, little to no movement, and or rapid breathing, they were removed and immediately placed back into their home tank water to recover. When the water also became cloudy, the fish were immediately taken out as well. Every time samples were collected all fish made a full recovery within ten minutes except one fish that died after being placed back in its home tank for recovery. The other bag was kept at RT during the stressing of the fish to serve as a control.

Lyophilization

The total volume was removed from each bag and equally divided into several conical tubes where they were immediately frozen using liquid nitrogen. They were then placed on a FreeZone 12 Liter Cascade Console Freeze Dry System and lyophilized to complete dryness. Dried samples were stored in -80°C freezers. Each one of the conical tubes from both the control and stressed sample had MQ H_2O washed down the sides of the tube to make sure all dried powder was collected. Then, all tubes were pooled into another fresh tube to be lyophilized again to dryness. Samples were reconstituted with 2mL Milli-Q water, briefly vortexed, and aliquoted.

Protein quantitation. All protein aliquots were subjected to a CB-X assay (G-Biosciences) to solubilize, clean up, and determine protein concentration. The kit was used according to manufacturer's instructions. The spectrophotometer used was a NanoDrop 2000c (ThermoFisher Scientific). All standards and unknowns were measured at 595 nm according to CB-X assay protocols and in a one milliliter cuvette.

SDS-PAGE

Reconstituted samples of both secretions and control varying in volume from 10 to 25 µL were diluted 1:1 with 2x Laemmli Sample Buffer (BIO-RAD). All reconstituted samples of secretion contained the same concentration of protein. ß-mercaptoethanol (BME) was also added to a final volume of 5% (v/v) to the Laemmli Sample Buffer to make sure disulfide bonds were reduced. The samples were boiled at 95°C for 10 minutes and then centrifuged at 5,000 rpm for one minute to pellet any undissolved proteins. The supernatant was run on both 7.5% Precast Mini-PROTEAN TGX Protein gels and 4%-20% Mini-PROTEAN TGX Precast Protein gels for 100 V for 75 to 90 minutes. Gels were stained with the Silver Stain Plus Kit (BIO-RAD)

according to manufacturer's protocol and imaged on a ChemiDoc XRS+ Imaging System (BIO-RAD). Clearly defined bands were cut out and stored at -80°C until destaining and in-gel digestion.

Sample Digestion, Desalting, and Peptide Recovery

Trypsin/Lys-C In-solution Digestion

Solubilizing the protein that was found in the secretions proved to be extremely difficult as well and took a couple of approaches to finally solubilize. For in-solution digestion, solubilization was performed using 8M guanidine hydrochloride with 10 mM dithiothreitol along with boiling at 95°C for both control and stress samples. Proteins were checked and briefly vortexed every 15 minutes. After 45 minutes all proteins were solubilized. After solubilization and reduction, proteins were alkylated using 200 mM iodoacetamide in 0.4 M Tris-Base buffered to a pH of 7.8 for an hour in the dark at RT. A quenching reaction using 200 mM 1,4-Dithiothreitol (DTT) in 0.4 M Tris-Base buffered to a pH of 7.8 for 45 minutes at RT was then performed to make sure alkylation was stopped. Both the control and stress sample were diluted to a volume of 1 mL to dilute the 8M guanidine hydrochloride to prevent Trypsin and Lys-C denaturation. Digestion was done using a Rapid Digestion-Trypsin/Lys-C Kit (Promega) according to manufacturer's instructions. A thermomixer was not available, so a Hybaid Hybridization Oven was used instead to mix the sample as it was heated. The reaction was stopped after 12 to 16 hours by adding glacial acetic acid to the digest solution until the pH was below 6. Each sample was lyophilized down to a volume of approximately 2 µL and stored at -80°C until the next step.

In-gel Digestion

Gel slices were thawed to RT and then were destained using ProteoSilver Destainer A and B (SigmaAldrich) as directed by the manufacturer. After destaining, the gel slices were washed twice with DI water, each time for five minutes. Then the slices were covered in a mixture of 25 mM NH_4HCO_3 and 50% Acetonitrile (ACN). The supernatant was removed and then the pieces were dried for 15 minutes to complete dryness using a Speed Vac (Thermo Scientific) using the medium heat setting. Reduction was performed using 10 mM DTT in 25 mM NH_4HCO_3. Alkylation was performed using 55 mM Iodoacetamide (IAA) in 25 mM NH_4HCO_3 at 56° C for 1 hour. All gel slices were digested with Trypsin Gold (Promega) for a minimum of four hours at 37° C. The supernatant was removed and then the gel slices were twice covered with a solution of 50% ACN/5% Trifluoroacetic acid (TFA) for an hour to further extract peptides. The initial supernatant and the next two supernatants for each slice of gel were combined separately in tubes and then dried using a Speed Vac (Thermo Scientific). Desalting and peptide recovery were done using a Pierce C18 Tip (Thermo Scientific) according to manufacturer's instructions for each slice. Each peptide elution volume was then lyophilized down to a volume of approximately 2 µL and stored at -80°C until the next step.

High pH Desalting and Fractionation

Originally, Pierce C18 Tips (Thermo Scientific) were used to bind digested peptides before ESI MS/MS. This technique yielded fewer worthy results than the future techniques, so it was omitted. Post-digestion, in-solution fragments were run through a Pierce High pH Fractionation Spin Column to make sure all peptides in solution were identified. The protocol was followed according to manufacturer specifications. The sample volume from the in-solution digest was over 1 mL, so several spin cycles were needed to make sure that the total sample

volume was bound to the column, after column conditioning. When the protocol was finished, elution fractions one and five, two and six, three and seven, and four and eight, were pooled to reduce runtime on the mass spectrometer and to reduce machine variability. After samples were pooled, they were lyophilized and then resuspended in 30µL 0.1% Formic acid in water. Before running the samples through the ESI, each sample was tested for the proper concentration of 0.15 µg/µL using the CB-X assay mentioned above.

Dissection

One *C. sterbai* was placed in a solution of 0.6 g/L of Benzocaine and sodium bicarbonate that was chilled to 4° C for a minimum of 15 minutes until all signs of vitality had stopped. The fish was then cut from the ventral side, posterior to the operculum, and then stopped until just about eye-level. Another incision was made the along ventral surface towards the tail for approximately 1.5 cm. A third cut was made starting at the end of the second cut and was made dorsally along the plates up to the level of the first cut, so that the flap of the plates could be pulled back. Tissue was taken from the flap of plates proximal to the opening found ventral and posterior to the pelvic fin. One pelvic fin was also removed from the fish to check the protein composition, as well as a snipping of the dorsal fin and tissue were also taken but not analyzed. A piece of scale was also taken for analysis. All tissues were immediately placed into 1.5 mL tubes, frozen with liquid nitrogen, and stored in a -80°C freezer. Figure below displays the cuts that were made to the fish.

Figure 5. General external anatomy of a fish. The red line on the fish was drawn to represent the flap of that was removed on both sides of the fish to expose what is hypothesized to be the toxin gland.

Tissue Homogenization

Post-dissection, samples were kept on ice before homogenization using a Bullet Blender Storm 24. Each tissue sample was put into a RINO tube filled with zirconium oxide coated steel balls with diameters from 0.9 to 2.0 millimeters. A ratio of 2:1:1 was used when comparing volumes of denaturing solution, sample, and homogenization beads. The denaturing solution used was 8 M guanidine hydrochloride with 10 mM DTT as a reducing agent. Samples were run on a power setting of 12 for two minutes, twice, with one minute of resting on ice in between to cool them down. Homogenized samples were then run through in-solution digestion and fractionation before MS/MS analysis.

Peptide Analysis

Chromatography

Each digested sample was subject to a method similar to *Larracas et al*. Differences from the method include HCD fragmentation instead of CID and the Orbitrap™ was used at both the MS^1 and MS^2 level to analyze peptide fragments and HCD collision results. Additionally, Solvents A and B were the same as[36]. Solvent B was used for the following times and concentrations: 2% for 0 to 5 minutes, 2% to 22% for 5 to 75 minutes, 22% to 38% for 75 to 100 minutes, 38% to 95% for 100 to 105 minutes, 95% for 105 to 110 minutes, 95% to 2% from 110 to 115 minutes, and 2% from 115 to 140 minutes. Each gradient run took 140 minutes using the same C18 column.

Mass Spectrometry

Mass spectrometry analysis was performed using an Orbitrap™ Tribrid™ mass spectrometer with an Easy-Spray ion source (Thermo Fisher Scientific) using similar methods to[36]. Differences included using a maximum inject time of 50 milliseconds with a quadrupole isolation window of 200-1400 *m/z*. At the MS^2 level a stepped energy collision was used with an HCD collision energy of 28% and a ± HCD collision energy of 3. Also, the maximum injection time was 150 milliseconds with the first mass at 100 *m/z*.

Database Construction and Proteomic Sequence Analysis

Protein and proteome sequences were downloaded from UniProt, T3DB, and CRAPome[37–39]. Sequences downloaded from Uniprot included data from both Swiss-Prot and TrEMBL. The databases downloaded on their respective dates are as follows: *E. coli* K12 on August 8th, 2017, *D. rario* on December 18th, 2017, prostaglandin on January 11th, 2018, and *Siluriformes* on August 16th, 2017. The entire T3DB database was downloaded on August 16th, 2017. Downloaded protein and proteome sequences were concatenated into one FASTA

database file to which peptide fragments were compared. Proteomic sequence analyses were performed using PEAKS Studio Version 8.5 (Bioinformatic Solutions Inc.). Database searches were performed using the following criterion: Parent Mass Error Tolerance of 2.5 ppm, Fragment Mass Error Tolerance of 0.02 Da, Precursor Mass Search Type: Monoisotopic, Enzyme: Trypsin and Lys-C, Max Missed Cleavages: 3, Non-specific Cleavage: 1, Fixed Modifications: Carbamidomethylation, Variable Modifications: Oxidation (M), Acetylation (N-terminus), Deamidation (NQ), Sodium adduct, Max variable PTM per peptide: 3, Searched Entry: 102737, FDR Estimation: Enabled, and De Novo Dependencies: 9. To filter out low quality peptides, a 1% FDR was used. In matching the peptides to the database, a base -10logP score of 20 was required along with a minimum of two unique peptides matching back to the same protein in the database to definitively say that a hit was confirmed.

CHAPTER 4: RESULTS

Because other organismal groups of venoms have been much more extensively studied, there are over 2000 venomous fishes that require extensive analyses to determine if they contain any bioactive peptides[1]. This study was done to identify any proteins in the secretion produced by *Corydoras sterbai* as well as tissues in the body.

Sample Collection

During sample collection *C. sterbai* always appeared to behave normally until the fifth minute. Sometimes as early as the fifth minute and then quickly after, individuals would appear to be stressed and the water would turn cloudy instantaneously as seen in Figure 6.

Figure 6. C. sterbai releasing hypothesized toxin. (Photo credit Ian Fuller)

As soon as the water turned cloudy, each fish struggled to maintain their vertical orientation and would slowly turn horizontal while starting to breathe at a much more rapid rate. All fish were immediately removed as soon as the water turned cloudy, to prevent death. Each time, all

fish were immediately returned to back to their tank and all fish always made a recovery except one adult that died.

SDS-PAGE Analysis

Figure 7 is an SDS-PAGE gel was run with both reconstituted venom samples and tank water samples. Gel slices were cut out at the following sizes: 150 kDa, 75 kDa, and 37 kDa, as those bands were the most visible. All proteins were digested in-gel with Trypsin Gold (Promega). Additionally, there seemed to be a faint band around 50 kDA, but this band was deemed too faint for analysis as seen in Figure 8.

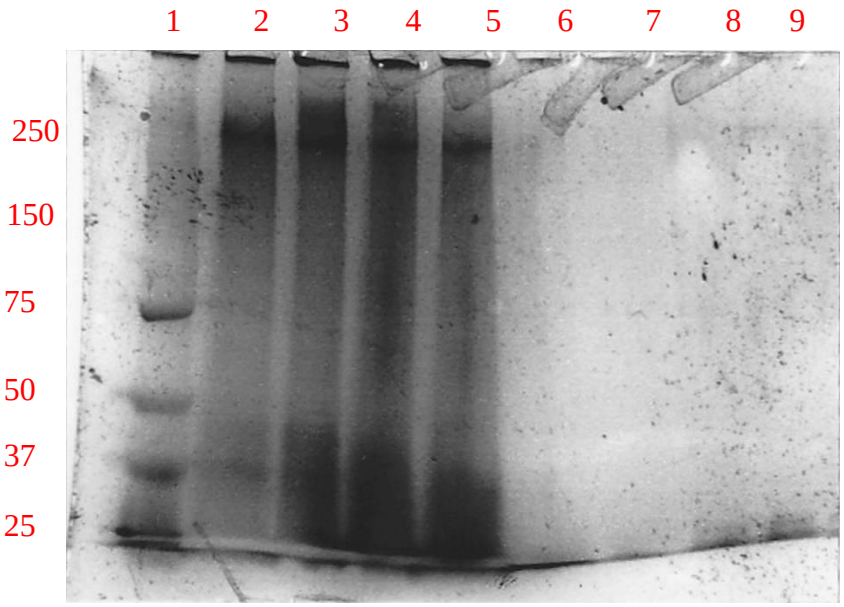

Figure 7. SDS-PAGE of hypothesized *C. sterbai* venom. Lane one contains protein standards. The sizes of the protein standards are as follows, 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa. Reconstituted venom samples are found in in lanes two through five and lyophilized fish water in lanes seven through ten. Lane six had sample buffer to prevent any bleed over or contamination between lanes.

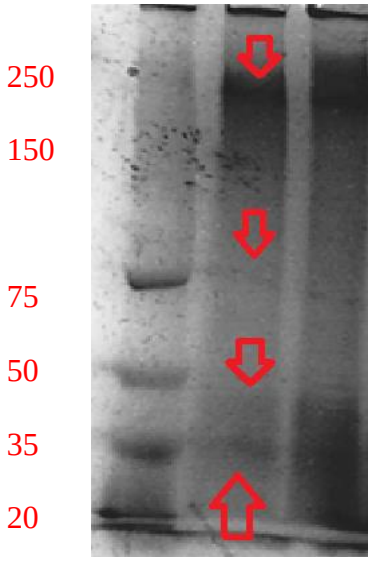

Figure 8. A subset of the SDS-PAGE gel in Figure 7. Each arrow above is pointing to a band that was considered for analysis. However only the 150 to 250 kDa band, the 75 kDa band, and 37 kDa bands were cut out and had their proteins analyzed.

Each of the three bands cut out had their proteins digested with trypsin and were analyzed as mentioned above. The twelve proteins from the 150 to 250 kDa band are listed in Table 1. Only two proteins were found in each of the two other bands that were analyzed and are listed below in Table 2 and Table 3.

Table 1
The Top 12 Most Confident Protein Database Matches from the 150 to 250 kDa Band as seen in Figure 8

Location of Protein in Cell	#Unique	Average Mass (kDa)	Description
Membrane/Blood	9	37.7	Ig alpha-1 chain C region
Nucleus/Cytoskeleton	8	41.8	Bactin2 protein OS=Danio rerio GN=actb2 PE=2 SV=1
Endoplasmic Reticulum	2	69.6	Prostaglandin-endoperoxide synthase 1 OS=Astyanax mexicanus PE=4 SV=1
Cytoplasm	5	68.8	Phospholipase A2, group IVAb (cytosolic, calcium-dependent) OS=Danio rerio GN=pla2g4ab PE=4 SV=1
Membrane/Endoplasmic Reticulum	2	56.2	Reticulon OS=Danio rerio GN=rtn4b PE=2 SV=1
Cytoplasm	3	21.9	Peroxiredoxin 2 OS=Danio rerio GN=prdx2 PE=1 SV=1
Endoplasmic Reticulum	3	10.3	Receptor expression-enhancing protein (Fragment) OS=Ictalurus punctatus PE=2 SV=1
Nucleus	2	9.9	Ubiquitin B OS=Danio rerio GN=ubb PE=4 SV=1
Cytoplasm	3	77.0	Arachidonate 12-lipoxygenase OS=Danio rerio GN=alox12 PE=1 SV=1
Mitochondria/Nucleus/Membrane	2	49.9	Adenylyl cyclase-associated protein OS=Ictalurus punctatus GN=CAP1 PE=2 SV=1

Table 2
The Only Two Protein Database Matches from the 37 kDa Band as seen in Figure 8

Location of Protein in Cell	#Unique	Average Mass (kDa)	Description
Cytoskeleton	3	41.8	Actin, cytoplasmic 1 OS=Danio rerio GN=actba PE=2 SV=2
Nucleus/Membrane	2	50.1	Elongation factor 1-alpha OS=Danio rerio GN=eef1a1l1 PE=2 SV=1

Table 3
The Two 75 kDa Band Database Matches from Table 4

Location of Protein in Cell	#Unique	Average Mass (kDa)	Description
Cytoskeleton		4	41.7 Actin, cytoplasmic 1
Cytoplasm/Exocytosed		2	16.0 Hemoglobin subunit beta

Table 4
Methods of Preparation of Peptides from the Same Sample Used in This Experiment and How They Improved Over Time

Origin of Peptides	PEAKS DB Matches	PEAKS SPIDER Matches
8M Urea Attempt 1 Control	1	1
8M Urea Attempt 1 Toxin	1	1
8M Urea Attempt 2 Control	1	1
8M Urea Attempt 2 Toxin	1	1
37 kDA In-Gel Band	7	19
75 kDA In-Gel Band	7	9
150 to 250 kDA In-Gel Band	23	38
8M Gdn HCl Attempt 3 Control	2	3
8M Gdn HCl Attempt 3 Toxin	420	751

In addition to having many more protein database hits for the water samples with the secretions in them as seen in Table 4 above, the reconstituted venom chromatograms are vastly different from the control water chromatograms. Each of the control chromatograms in Figure 9, Figure 10, Figure 11, and Figure 12 barely show any peptides compared the chromatograms from the stressed samples.

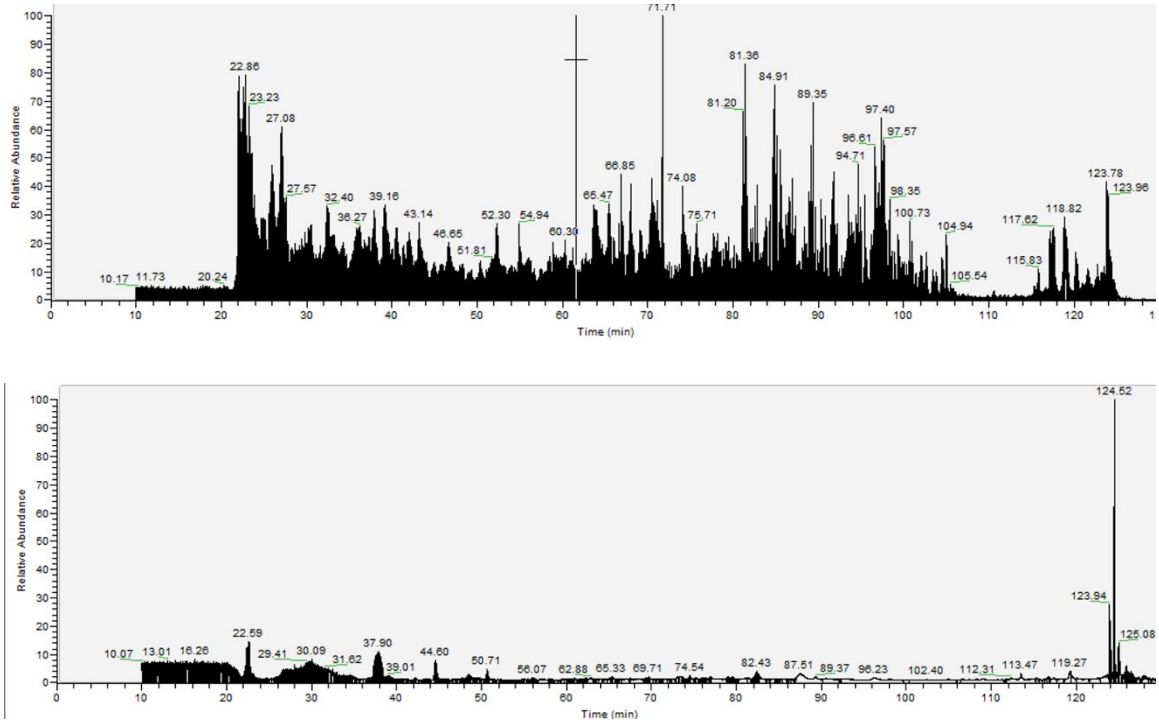

Figure 9. The two chromatograms above are both grouped fractions one and five. The top chromatogram is from the stressed samples while the bottom is from the control samples.

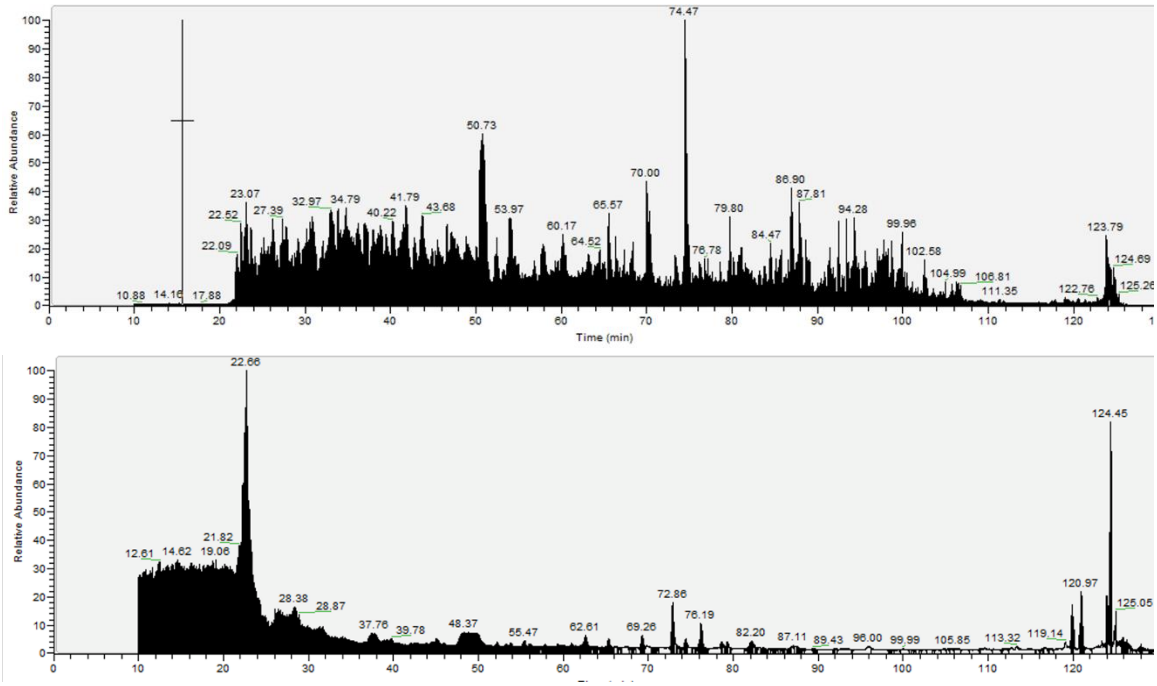

Figure 10. The two chromatograms above are both grouped fractions two and six. The top chromatogram is from the stressed samples while the bottom is from the control samples.

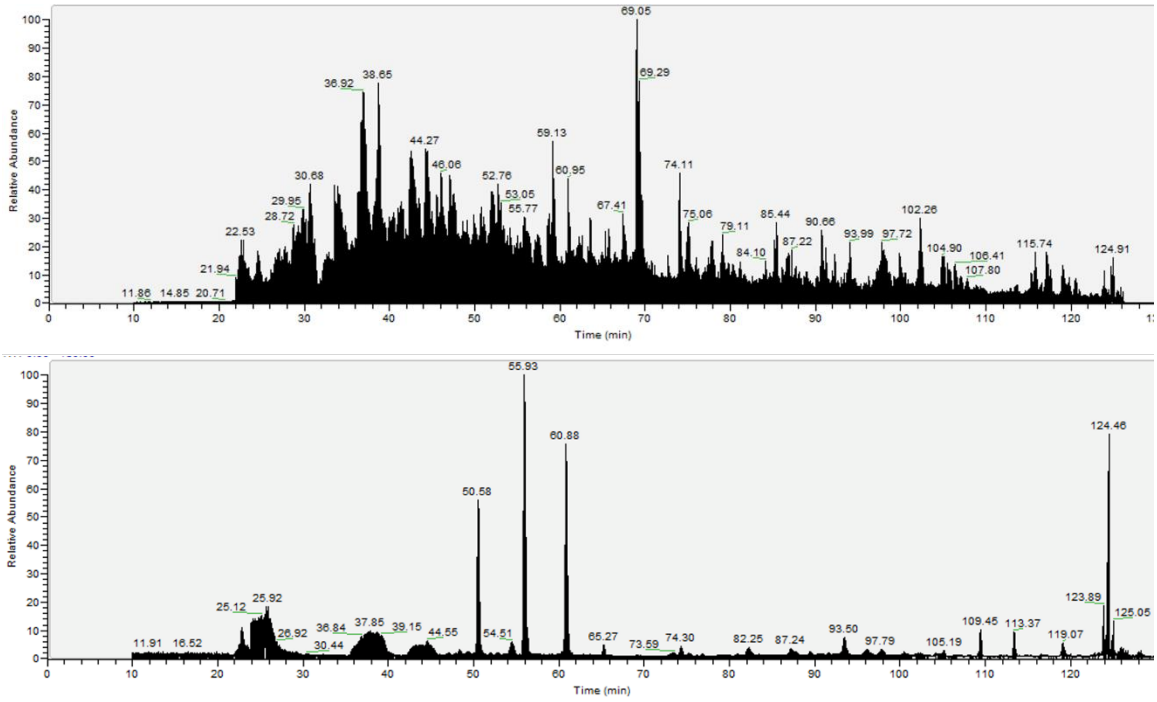

Figure 11. The two chromatograms above are both grouped fractions three and seven. The top chromatogram is from the stressed samples while the bottom is from the control samples.

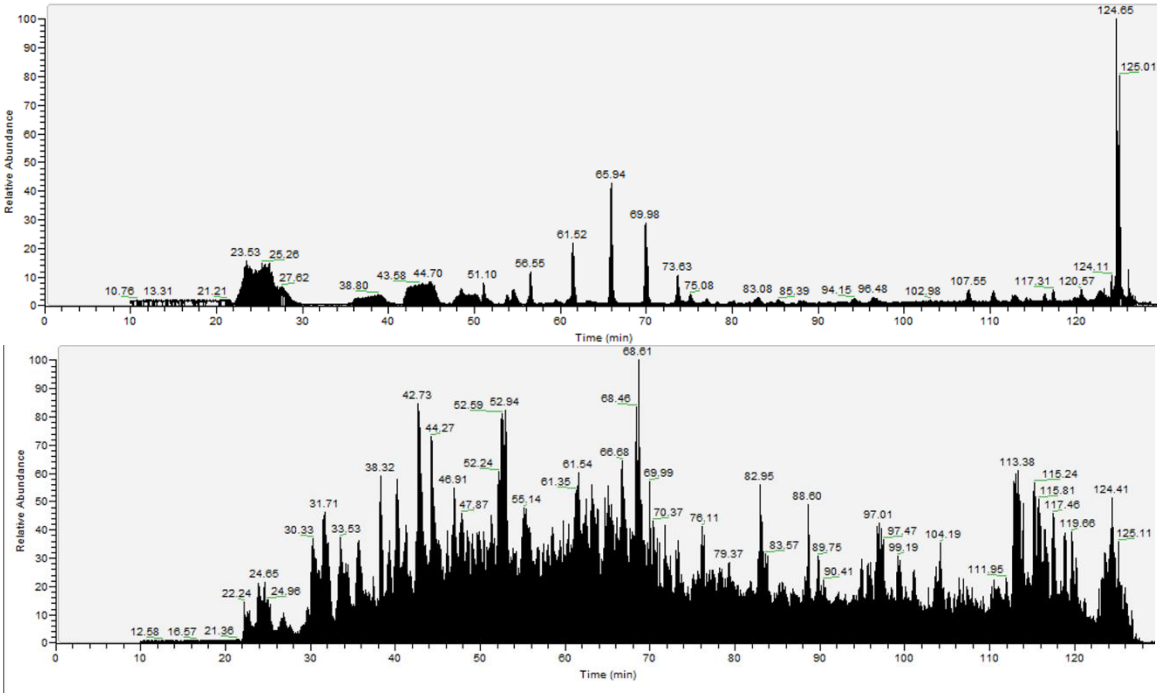

Figure 12. The two chromatograms above are both grouped fractions four and eight. The top chromatogram is from the stressed samples while the bottom is from the control samples.

Dissection

A dissection of *C. sterbai* was also performed during this experiment. One *C. sterbai* was euthanized using a solution of 0.6 g/L solution of benzocaine buffered with sodium bicarbonate at 0.6 g/L. Before the dissection, an opening posterior to the pectoral fin was documented as see in Figure 13. Additionally, an oval-shaped dark object containing what are thought to be shiny globules of a hydrophobic substance was discovered just ventral to the pectoral spice as seen in Figure 14.

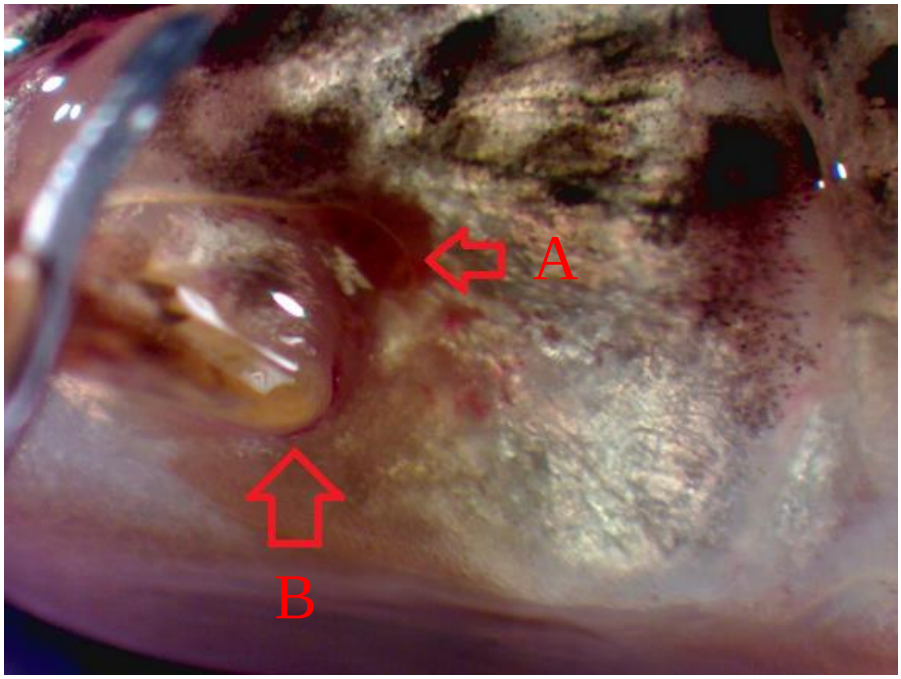

Figure 13. C. sterbai external anatomy. Here, *C. sterbai* has its pectoral fin snipped and lifted to expose an opening just behind the pectoral spine. Arrow A points to the opening behind the pectoral fin on the outside of the fish and Arrow B is the pectoral spine that has been lifted to expose the opening.

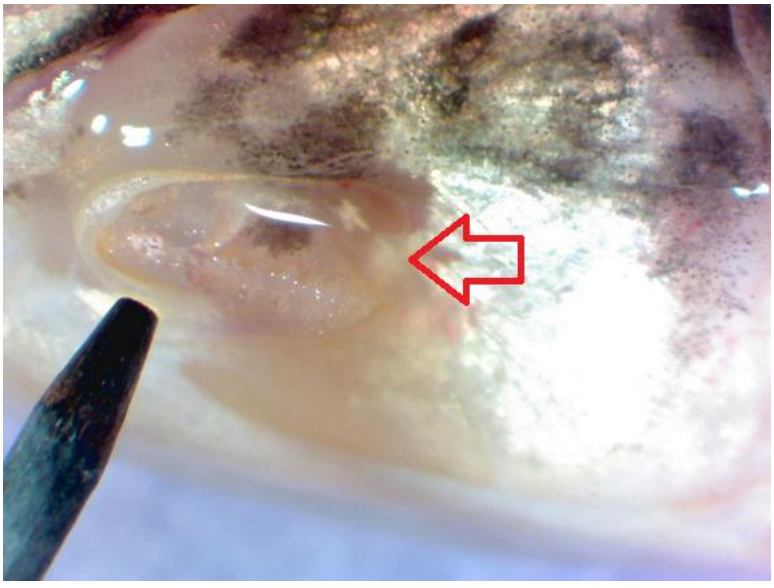

Figure 14. C. sterbai hypothesized venom gland. Close to the external ventral side of the fish and ventral to the pectoral spine is a compartment that looks like it contains small vesicles or granules.

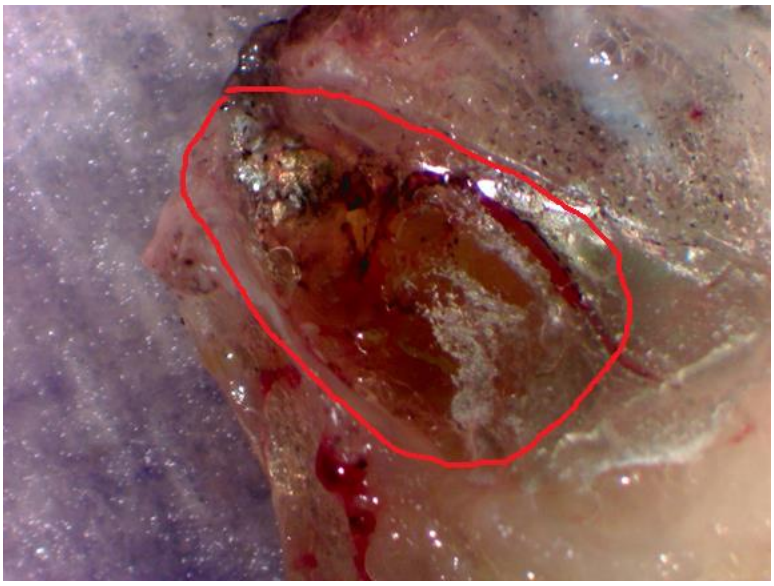

Figure 15. C. sterbai internal tissue dissection. The internal view of tissue underneath the opening found proximal to the pectoral spine. The red line denotes what is believed to be the gland containing the vesicles seen Figure 13 and Figure 14.

Table 5
The Number of Protein Database Matches Found for Each Tissue After MS/MS Analysis Using Proteomic Software

Tissue Type	**Number of Proteins Found**
Armor Plating	519
Connective Tissue	127
Dorsal Ray	116
Potential Toxin Gland	777
Skeletal Muscle	487
Pectoral Spine	127

After the dissection was performed, all tissues were ground up and analyzed for their protein composition at the time of death. The potential toxin gland had by far the greatest number of protein matches to the database. Proteins found in two or more of the tissues were removed from analysis in order to identify tissue-specific proteins and (hopefully) to better understand the unique processes in the gland. Looking at biological processes that these proteins are found in, the top five results based on number of proteins involved in that process are as follows: small GTPase mediated signal transduction, protein folding, intracellular sequestering of iron ions, iron ion transport, and cellular iron homeostasis. These proteins are unfortunately not unique to any process and are commonly found in most tissues throughout the body. Small GTPase mediated signal transduction is found within the ribosome which is where protein folding occurs[41]. Intracellular iron ions can pose a threat if they are not properly bound to proteins and solubilized[42]. The iron containing protein found in this study was ferritin and its subunits which can be found not only within most cells in the body, but also in the blood as well[42].

CHAPTER 5: DISCUSSION

The goal of this experiment was to determine if *C. sterbai* secreted a toxic substance as a predation defense mechanism. Toxin and venom research are incredibly important as they are and can be used in everyday research. Using several different methods of protein preparation, separation, and mass spectrometry analysis we have come to several conclusions.

After collecting the stressed water samples, *C. sterbai* does secrete a protein rich substance into the water. This conclusion is backed up by the increased peptide abundance seen in the control and stressed chromatograms as well as the number of database protein matches that are seen in Table 2. Figure 4 also displays the secretions coming out from the fish as it turns upside-down, clearly showing that has lost muscular equilibrium and is not behaving normally. The dark smears also found in the SDS-PAGE gels shows that much protein is found in the secretions as well.

In-solution tryptic digests of the secretion were performed first before attempting in-gel tryptic digests of the same starting material. Solubilization of the lyophilized proteins was attempted using 8M urea. This technique failed to solubilize all proteins, leaving a precipitate. Solubilization was then attempted using 8M guanidine hydrochloride and 10mM dithiothreitol along with heating at 95° C and periodic vortexing[36]. This new method completely dissolved proteins within 45 minutes. Desalting and peptide recovery were done initially with Thermo Fisher C18 Tips for each of the attempts to solubilize peptides with 8M Urea. This step was replaced with Pierce High-pH Fractionation Columns to reduce complexity in the sample when the solubilization step was replaced with 8M guanidine hydrochloride and 10 mM DTT. Samples were then pooled to reduce technical variability during runtime through our Orbitrap™

Tribrid™ mass spectrometer. Early attempts using 8M Urea as the part of the sample preparation provided very few database matches compared to the revised sample preparation seen in Table 2. Database matches using the revised sample preparation had greater than 400 protein matches for both the PEAKS DB and SPIDER search methods.

Within the PEAKS DB results for the stressed water samples using the latest method of preparation, several prostaglandin producing enzymes are continually at the top of the list based on PEAKS' confidence algorithms. The stringent parameters used in this experiment ensure that these enzymes are in the sample. Other organisms, such as the common honeybee, have high levels of prostaglandins in their venom[43]. Prostaglandins themselves are known vasodilators, markers of inflammation, and inhibitors of platelet aggregation at very low concentrations. This has led to the belief that *C. sterbai* could also secrete either high levels of prostaglandins into the environment as a protective mechanism or high levels of prostaglandin producing enzymes. Prostaglandins themselves are derived from long fatty-acid chains. Being hydrophobic, these molecules could be in the water and could easily be absorbed through phospholipid membranes in predatory organisms. If the prostaglandins are in the water, they could be absorbed through a phospholipid membrane. Prostaglandins would have an immediate effect in signaling localized pain. Absorption through the gills is documented with other chemicals such as the anesthetization method described above in this experiment.

In conjunction with prostaglandin producing enzymes, Phospholipase A2 (PLA2) was found in the secretion. PLA2's main function is to digest phospholipids which makes it extremely efficient at cutting eukaryotic cell membranes[44]. PLA2 has been found in the venoms of numerous taxa[44]. If prostaglandins are in this secretion, the inflammation that they signal

could bring extra blood to the site along to where the phospholipases are, causing even more damage to the predatory animal.

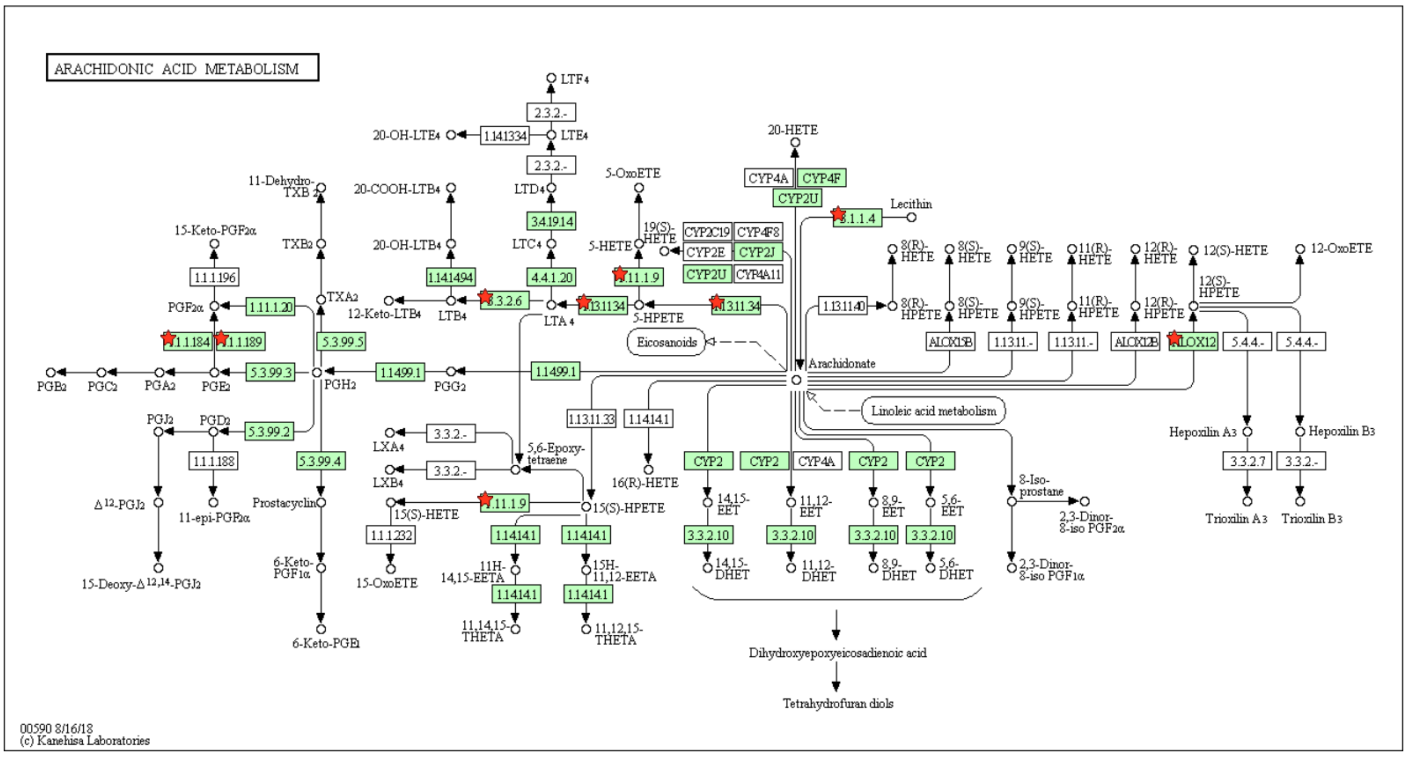

Figure 16. The KEGG pathway analysis of arachidonic acid metabolism. This figure above illustrates the number of prostaglandin synthesizing enzymes in various pathways that were found in the sample.

Along with prostaglandin producing enzymes and PLA2, Peroxiredoxin 2, 4, and 6 were found in the lyophilized secretion. Peroxiredoxins have been known to work in conjunction with PLA2[46].Peroxiredoxin 6 specifically is a bifunctional enzyme and can hydrolyze phospholipids using PLA2[44].

Looking at the hypothesized non-toxic proteins that were found in the secretion, proteins from all parts of the cell are shown in Table 1, Table 2, and Table 3. Previous research has shown that fish most likely secrete toxins using holocrine secretion secrete these proteins as well, which explains why nuclear and cytoplasmic proteins both were seen[47].

After removing overlapping proteins found in the other six tissues, the remaining 309 proteins remaining in the dissected hypothesized toxin gland were then input into the DAIVD Bioinformatics Resource 6.8.

With the evidence in results and discussion, it is hypothesized that the several prostaglandins producing enzymes, phospholipases, and peroxiredoxins *C. sterbai* secretes, could work together. This concoction would rapidly affect cellular membranes and could cause rapid edemas, swelling, and pain in the affected area(s). This defensive mechanism seems to only be secreted under stressful conditions and likely is fast-acting as members of this genus quickly invert and appear lifeless during collection soon after the water turns cloudy.

Preparing and separating proteins using the SDS-PAGE gels as seen above did not yield as many results as in-solution digestion due as bands were cut out instead of analyzing the whole secretion or ground up tissue. Excising certain bands influenced the theoretical sizes of proteins that were studied versus using the High pH Fractionation Column to separate out all proteins allowed for many more proteins to be studied. Because of the stringent parameters, the 75 kDa and 37 kDa bands only had two proteins each using the database search method in PEAKS 8.5. In the 150 to 250 kDa band there were many more protein matches, but only the top ten included some of the groups of hypothesized toxic proteins as mentioned above.

The sizes of the bands that were cut out in this study often did not contain proteins that were of that size. As seen above, most of the lane was a smear indicating degradation of proteins or proteolysis could have happened before the sample could be frozen. The dark stains at the top of the gel and poor overall resolution could be an indication of this issue[47].

If parts of proteins bonded together after they were proteolyzed with trypsin, it would give the illusion that the whole protein had migrated to that part of the gel. An additional issue

was whether all the proteins in solution were completely denatured through heating while in loading buffer. Some early on SDS-PAGE solubilization attempts contained precipitated proteins at the bottom after boiling and centrifugation indicating that not all proteins were denatured and solubilized before loading. This could not have completely denatured all proteins. Actin and hemoglobin were two proteins that were found in bands close to their expected size.

Within this study, there were limitations that were imposed by both the techniques, machines, and software used. Undoubtedly, peptide loss could have happened while preparing samples for the mass spectrometer. The freezing and thawing of samples could create breakages in proteins while could then affect proteolysis[48]. Additionally, peptide loss could have occurred during the binding of the column for fractionation as well as the grinding of the tissues as not all the tissue may have been ground up and some peptides may not have bound to the column. When using the quadrupole mass analyzer, the mass to charge ratio used destroyed peptides outside of the desired range preventing a fuller coverage with the database. With the software used, PEAKS 8.5, the settings used were very stringent. Both very small Parent Mass Error Tolerance and Fragment Mass Error Tolerances help to ensure that the peptides that were found were very accurate, but small variances could have prevented peptides from being accepted into the dataset. Additionally, an ALC score of 95 percent was used, which is extremely strict on top of the -10logP value of at least 20. All these stringent settings together made sure that our peptides were very real, but this also does remove other peptides that could have been real as well. Lastly, the mass spectrometer used along with the software used was not able to tell us about the abundance of peptides found in samples. This would have been extremely useful to help see if the levels of hypothesized toxic proteins found in the secretions and tissues were

much higher than what is found in other tissues, this could indicate that those proteins might be components of the toxin.

Further research could be conducted in a variety of areas. One of the first steps would be to sequence the genome of *C. sterbai* this would allow for direct gene comparisons with known toxic or venomous organisms. This first step would immediately categorize the species as toxic. After looking at the genome, a transcriptome of the tissues that were dissected also could have been done. The transcriptome is much more useful with a previously sequenced genome. Furthermore, a sequenced genome and transcriptome would greatly benefit any future proteomic study on this species. Predicted peptides from the genome could be assembled into a database and then compared to what was found in tissues and in secretions. This could ensure that the mass spectrometer and PEAKS 8.5 software used in this experiment can more accurately match. More accurate peptides could then be compared to bioinformatic databases or to any uploaded database in PEAKS 8.5. Finally, a quantification of the levels of proteins found in both the tissues and secretions would be extremely useful. The quantification data could reveal if the levels of enzymes that make prostaglandins in what is believed to be the gland, to be much higher than that is what is found in all five of the other tissue samples. Additionally, if the genomic data shows that *C. sterbai* does produce toxins that are like other organisms, the levels of those proteins could be studied as well.

In addition to sequencing nucleic material, an analysis of lipids in both the secretions and in the tissues could be done using the mass spectrometer used in this research. Hopefully the physical number of prostaglandins could be measured and then compared to what is found in other venoms to see if the dosage could be enough to cause pain and inflammation in a predator.

The number of prostaglandins could also be compared to other tissues found in *C. sterbai* as well as other organisms to see if the levels are out of the ordinary.

Future research on this organism is extremely important as the number of aquatic toxic or venomous organisms is dwarfed by that or known terrestrial venomous organisms.

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