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Sex and Starvation Influences Latrotoxin Expression in the Brown Widow Spider

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in
Biology

By
Mattie Harris

Under the mentorship of Dr. J. Scott Harrison

ABSTRACT

Widow spiders (genus *Latrodectus*) are well-known for their potent venom. Seven latrotoxin proteins constitute the main components of widow spider venom. The vertebrate specific (α -latrotoxin) and insect specific (α -latroinsectotoxin) latrotoxins have been well-characterized with respect to structure and function. Regulation of latrotoxin gene expression is not well understood but sex and feeding could be factors influencing production. In this study, I used quantitative qPCR to (1) characterize the expression patterns of both the insect and vertebrate specific latrotoxins in male and female brown widow spiders (*Latrodectus geometricus*) to characterize sex-biased expression and to (2) study expression patterns when female spiders are fed an insect and when fed a vertebrate relative to a starved condition. Sex-biased expression was strong in both genes, with an average of 30-fold higher expression in females. During the feeding experiment, α -latroinsectotoxin was upregulated upon insect feeding and α -latrotoxin expression did not change regardless of the condition.

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Introduction

The production of toxins in plants and animals is ecologically and metabolically expensive (McCue, 2006, Ibanez, et al., 2012). One study completed on three North American pit viper snake species found that there was an increase of 11% in the snake's resting metabolic rates following venom extraction (McCue, 2006). Plant toxin production and storage can be shown to compete with energy and nutrients being used for growth of the plant (Ibanez et al., 2012). Selective use and regulated production are mechanisms to control these potential costs. Spiders in the genus *Latrodectus* (widow spiders) produce a venom consisting of a complex mixture of proteins and other small molecules (Cooper et al., 2015). Unlike other species, widow spider toxins are not solely located in venom glands. Venom can also be found in their legs, abdomen, and egg sacs (Yan and Wang, 2015). Latrotoxins are a group of protein neurotoxins serving as the main active component of widow spider venom. It is estimated that only 85% of widow spider bites to humans are envenomating. This means that around 15% of all widow spider bites are dry bites, which could give insight into the cost of venom production and use (Peterson, 2006). Latrotoxins are taxa-specific in their effects. Seven latrotoxins have been identified in the following categories: crustacean specific α -latrocrustatoxins, insect-specific α , β , δ , ϵ , λ -latroinsectotoxins, and vertebrate specific α -latrotoxins (Rohou et al. 2007). The structure and effects of many latrotoxins proteins have been characterized (Magazanik et al., 1992). However, information on the regulation of latrotoxin gene expression, and in fact the regulation and control of spider venom composition and production as a whole, is sparse (Cooper et al., 2015). The main focus of

this study is to investigate the role of sex and feeding as factors influencing production of latrotoxins.

Sex-biased genes are a category of genes found in both males and females that are expressed differently, or in only one sex. Sexual dimorphisms arise within almost identical genomes in part through sex-biased gene expression (Mank et al., 2008). Both sexual selection and natural selection are seen to act upon sex-biased genes due to their roles in reproduction, physiology, and behavior in species. The gonads have shown expression patterns with the greatest sex-biased variability when compared to other organs (Mank et al., 2008). Evolutionary studies performed on fruit flies, *Drosophila melanogaster*, indicate that male-biased genes evolve much faster compared to female-biased genes (Zhang et al., 2004). A similar study done in embryonic chickens found that around 18% of genes in any given tissue were sex-biased (Mank et al., 2008). The expression of these sex-biased genes can be studied to give insight into the factors that influence sex differences within a given species including sexual dimorphisms, physiological, and ecological differences.

Sexual dimorphisms are differences in morphology, behavior, and physiology between males and females. These sexual dimorphisms are seen universally in nature (McLean et al., 2018). Among terrestrial animals, spiders exhibit considerable sexual size dimorphism (Cordellier et al., 2020). At hatching, most spider species appear to be monomorphic. The development of widow spiders varies in rate and duration between males and females. Female widow spiders take an average of 17 to 18 weeks to mature while their male counterparts take 7.5 weeks (Forster and Kingsford, 1983). The average male lifespan is four to ten weeks, while the females are known to live two years

(Mahmoudi et al., 2008). The divergence in attributes between males and females begins around the third instar. At this point, the juvenile spiders exhibit differing pedipalp sizes between the sexes. In adults, there is generally a considerable overall size difference between males and females (Mahmoudi et al., 2008). Female spiders can be up to 20 times larger than males (Peterson, 2006). At sexual maturity, the pedipalps of male spiders transition from feeding appendages to reproductive organs. Sperm is stored at the end of the pedipalps after being produced in the testis (Cordellier et al., 2020). In sexually mature females, the pedipalps continue to function as sensory and feeding organs and are not used directly in reproduction. In many spider species, males stop feeding or live off prey captured by females following sexual maturity (Cordellier et al., 2020). One recent study conducted showed that widow spiders express toxin genes equally before reaching sexual maturity (Torres et al., 2021). Given these life history differences, latrotoxin genes might be a good candidate to study sex-biased expression.

Prey encounter or prey type may also serve as a signal for expression regulation as venom is important for prey capture. Studies have shown that predatory venom use in spiders is modulated by both prey size and fight intensity displayed by prey (Cooper et al., 2015). In a study involving scorpions, it was concluded that venom composition and amount varied depending on the prey and predator species (Evans et al., 2019). The presence of taxa-specific toxins and sexual dimorphisms in widow spiders led me to ask these following questions (1) is there is sex-biased expression in the latrotoxins of adult brown widow spiders and (2) does the expression of α -latrotoxin and α -latroinsectotoxin changes when given insect or vertebrate prey. I predicted that female spiders would upregulate all toxin gene production when compared to males. For the feeding study, I

predicted that α -latrotoxin would be upregulated when fed a vertebrate and α -latroinsectotoxin would be upregulated when fed an invertebrate.

Methods

(a) Widow Spider Maintenance and Spiderling Rearing

The lab sustains a breeding stock of brown widow spiders collected across Georgia and California. The spiders that are collected from the wild lay eggs sacs from sperm that has been stored from previous mating. As the sacs are produced, the individual eggs are extracted from the egg sac and placed in a petri dish in an incubator set at 26.5 °C until hatching. A bucket of water is placed in the incubator with the spiders and eggs to keep the environment humid. Following hatching, each individual spiderling is placed into its own plastic container and is allowed to mature while surviving off its yolk sac for around one week. After this time, the spiderlings are fed one to two fruit flies weekly until they reach maturity and can be fed as adults. The lab-reared adult spiders were used in this study.

The adult spiders are enclosed in individual 2 oz plastic cages and are lab-reared under controlled conditions in an incubator at 26.5 °C with a rotation of 12-hour light and dark cycles. Adult female spiders are fed a mealworm (*Tenebrio molitor*) biweekly, from the colony maintained in the lab. Adult male spiders are fed two to three fruit flies (*Drosophila melanogaster*) weekly from flightless colonies in the lab.

(b) Sex-Biased Expression

Upon reaching maturity, both male and female, lab-reared spiders were collected for analysis 4 to 7 days after being fed. Both males (n=7) and females (n=8) were placed in the freezer for less than 5 minutes. For the RNA extraction, the entire male sample was used. The females were split longitudinally, and half of each female was used in the RNA extraction. This was done to keep the tissue sample under 100 mg and to get a sample of all organs to be consistent with the male RNA extraction.

(c) Fed versus Starved Expression

Using the samples collected as outlined above, two different experiments were completed. Experiment one was conducted to test the effects on latrotoxin expression when fed a vertebrate (n=5) compared to being starved (n=5), while experiment two compared being fed an invertebrate (n=8) relative to being starved (n=8). The spiders in the invertebrate-fed condition were fed mealworms, and those in the vertebrate-fed condition were fed baby house geckos (*Hemidactylus turicicus*). Regardless of condition, all female spiders were starved between 16 and 23 days. When spiders in the fed condition latched on to the prey, they were removed and placed into the freezer immediately. In the starved condition, the web of the spiders were stimulated with the feeding forceps in the same way that the fed spiders were, the only difference was that they did not receive food. The samples were kept in the freezer for around five minutes, and each spider was then dissected longitudinally. One half of each spider was used in the extraction as described above, and the other half was stored in the freezer.

(d) RNA Extraction and cDNA Synthesis

For each of the experiments, the RNA extraction procedure outlined in the ThermoFischer Scientific Ambion PureLink RNA Mini Kit was followed. Each sample was homogenized in a microcentrifuge tube with 600 μL of lysis buffer containing 1% 2-mercaptoethanol. A DNase treatment was used to rid the sample of contaminating DNA. Between Wash Buffer I and the first wash with Wash Buffer II, 80 μL of Appendix On-column PureLink DNase was prepared with 8 μL of 10X DNase I Reaction Buffer, 10 μL of Resuspended DNase, and 62 μL of RNase Free Water. The treatment was incubated on the sample for 15 minutes and the rest of the protocol was completed. 100 μL of RNase-free water was run through the column at the end to collect RNA for each female spider. For the males, only 50 μL of water was run through and collected to increase the RNA output due to the smaller sample size. At the completion of the protocol, the RNA product was analyzed to assess the quality and quantity of RNA using a Nanodrop Spectrophotometer. The purified RNA was used to prepare complementary deoxyribonucleic acid (cDNA). Extracted RNA was converted to cDNA using the Applied Biosystems 2X Real Time Buffer Mix, 20X Real Time Enzyme Mix, and Nuclease-free water for a total volume of 20 μL . The cDNA protocol was adjusted for each sample to include 250 ng of RNA per reaction using the data from the Nanodrop Spectrophotometer. The cDNA protocol was run on Thermocycler at 37 °C for 60 minutes, 95 °C for 5 minutes, and held at 4 °C until preparation of the qPCR reaction.

(e) qPCR

Three sets of primers were designed for the genes of interest: a housekeeping gene (Histone 3A), the α -latrotoxin gene, and the α -latroinsectotoxin gene. I designed primers from sequence data collected over previous years (Table 1). A 3.5 μ M solution of each primer was used in each reaction. Three technical replicates for each primer and sample were made using 5 μ L SYBR Green, 2 μ L deionized water, primers, and 2 μ L cDNA, totaling a volume of 10 μ L. The samples were run in the qPCR starting with an initial 95 °C holding stage. The samples were then run through 40 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds. Following these cycles, the samples went through a melt stage.

Table 1. Forward and reverse primer sequences for the qPCR reaction.

	Forward Primer Sequence	Reverse Primer Sequence	Efficiency
Housekeeping	5'- AGGGAAGTT TGCGGATGAG-3'	5'- CACCAAAGCT GCACGTAAAAG-3'	97.65
α -Latrotoxin	5'-CCTGGCTAAC CACAATTACGA-3'	5'-GAACCCACAA GGGACGATTTA-3'	99.27
α -Latroinsectotoxin	5'-GCTCAAGGAA GTGCAGAAAC-3'	5'-CGTGTACCGTA TTACCGAAATTG-3'	100

(f) Statistical Analysis

Relative expression was calculated using the Pfaffl method with expression of latrotoxins standardized to expression of the histone 3A gene. Three technical replicates

were run for each primer and sample, and C_T values of the technical replicates were averaged. For the sex-biased expression study, the females were used as the control sample. In the fed versus starved condition, starved females were used as the baseline for comparison. The efficiency of each primer was estimated using C_T values of serial dilutions for each primer. All data was log transformed before statistical analysis was completed.

Results

Sexual dimorphism plays a role in toxin production and use

Female spiders had an average relative expression (\pm SE) of 2.78 ± 1.14 and 2.53 ± 1.12 for the α -latrotoxin and α -latroinsectotoxin genes, respectively. Males had an average relative expression of 0.10 ± 0.03 for the α -latrotoxin gene and 0.07 ± 0.03 for the α -latroinsectotoxin gene. Figure 1 shows an average of 30-fold higher expression for both genes in the female widow spider. The data shows that there are sexual dimorphisms in toxin production and use.

Not all latrotoxins are constitutively produced and stored; some are induced when exposed to prey

The average relative expression of the α -latroinsectotoxin gene for spiders in the invertebrate-fed treatment was 12.75 ± 5.6 . This can be compared to the expression in the starved treatment, which was 2.1 ± 0.70 . Figure 2 shows α -latroinsectotoxin expression is upregulated from basal expression levels upon encountering insect prey. α -latroinsectotoxin expression increased 5-fold after insect feeding relative to the starved

condition. Gene specific expression changes are consistent with the insect specific function of α -latroinsectotoxin. The relative expression of the α -latroinsectotoxin gene for the vertebrate fed and starved conditions was 2.38 ± 1.22 and 2.35 ± 1.42 starved, respectively (Figure 3). Exposure to vertebrate prey did not induce significant changes in expression levels of either toxin gene.

Vertebrate specific α -latrotoxin is consistently expressed at some level

The average relative expression of the α -latrotoxin gene for the invertebrate-fed and starved conditions was 2.35 ± 1.42 and 1.81 ± 0.89 , respectively. The expression of the α -latrotoxin gene for the vertebrate-fed and starved conditions was 1.18 ± 0.36 and 1.24 ± 0.42 respectively. Figures 2 and 3 show sustained expression of the α -latrotoxin gene throughout both conditions indicating α -latrotoxin expression did not change from basal levels when exposed to any prey type.

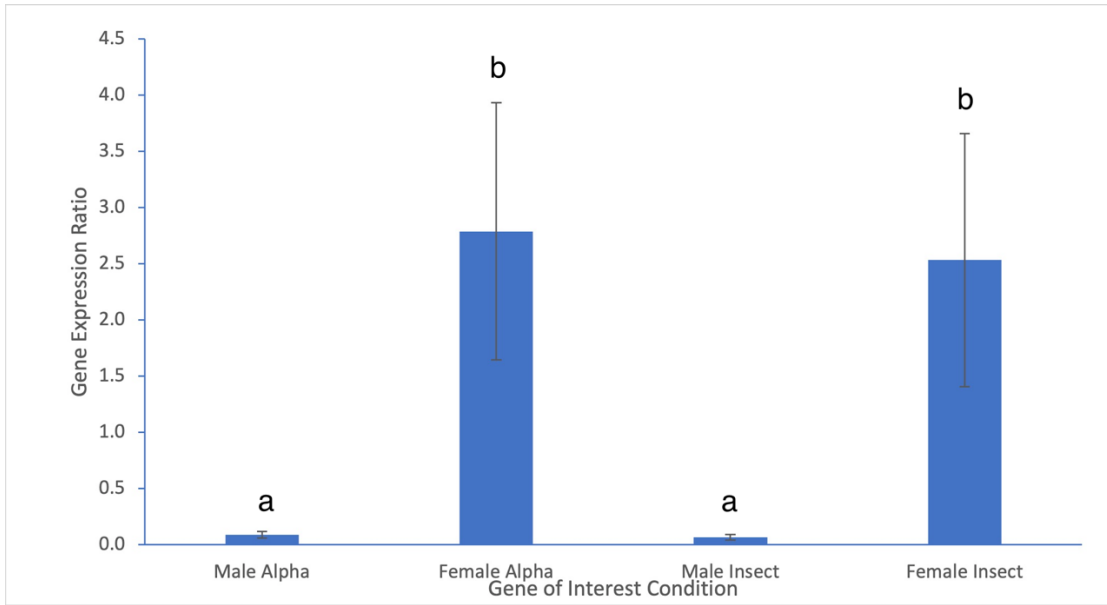


Fig 1. Average relative expression (\pm SE) of males (n=7) and females (n=8) for both α -latrotoxin and α -latroinsectotoxin genes (Males: 0.10 ± 0.03 and 0.07 ± 0.03 , Females: 2.78 ± 1.14 and 2.53 ± 1.12). Females show higher expression levels than males for both genes under standard conditions ($P \leq 0.001$).

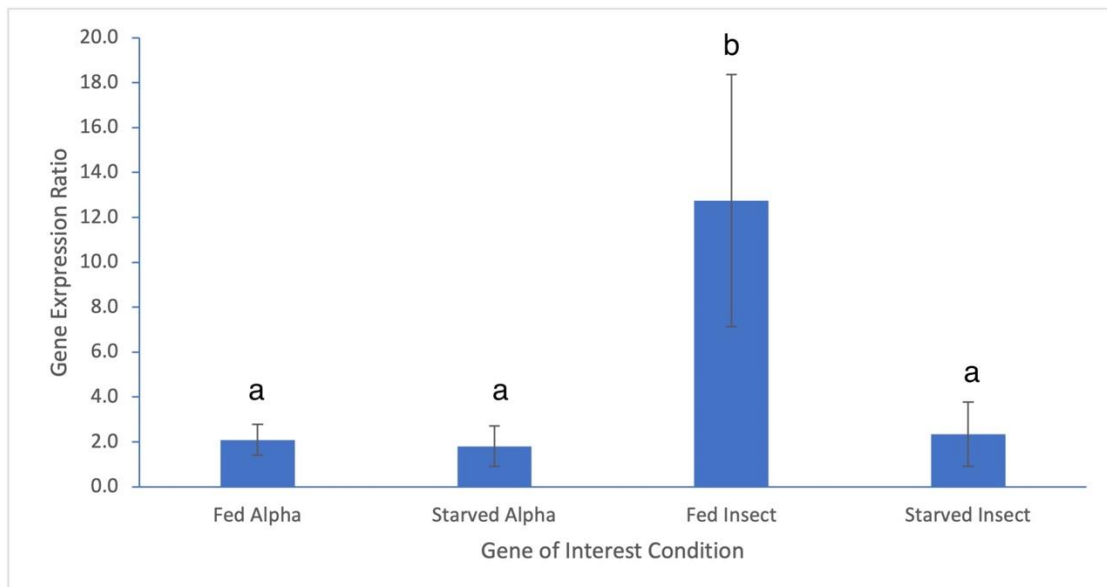


Fig 2. Average relative expression (\pm SE) of insect fed (n=8) vs. starved (n=8) spiders for α -latrotoxin and α -latroinsectotoxin genes. α -Latroinsectotoxin was expressed at higher levels after feeding relative to starved conditions (12.75 ± 5.6 vs. 2.1 ± 0.70) and relative to α -latrotoxin under both conditions ($P=0.026$). α -latrotoxin did not change between insect fed and starved conditions (2.35 ± 1.42 and 1.81 ± 0.89 respectively).

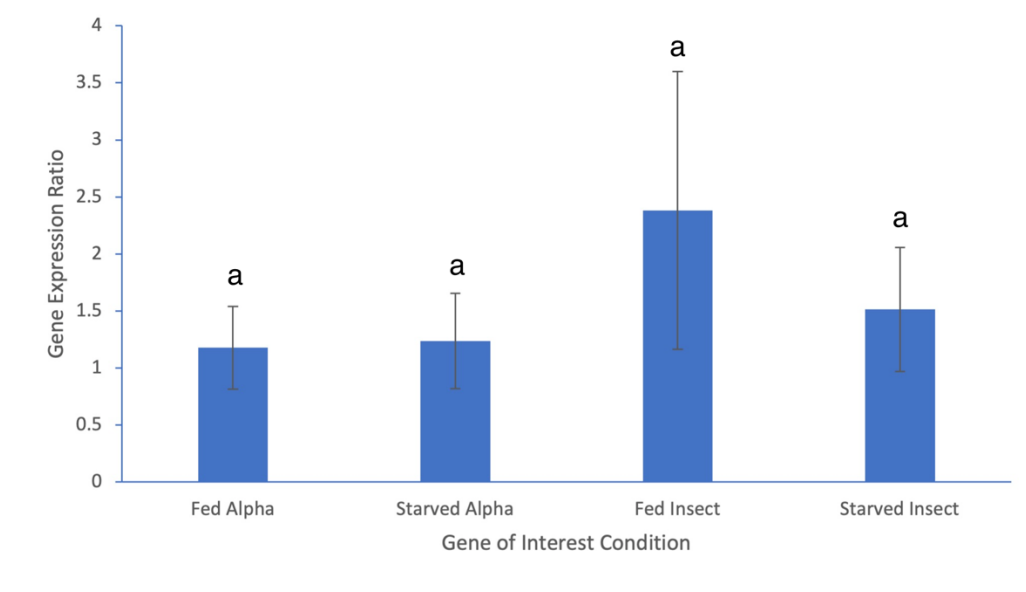


Fig 3. Relative expression ratios (\pm SE) of vertebrate fed (n=5) vs. starved (n=5) spiders for α -latrotoxin (1.18 ± 0.36 fed, 1.24 ± 0.42 starved) and α -latroinsectotoxin (2.38 ± 1.22 fed, 2.35 ± 1.42 starved). Expression levels did not differ among any conditions for either gene (P=0.912).

Discussion and Conclusions

Spider venom is a composite mixture of peptides, proteins, and other small molecules that target neuron cells (Cooper et al., 2015). In widow spider venom, latrotoxins, the main components, are specific to different taxa (Rohou et al., 2007). The known latrotoxins fall in the following categories: crustacean specific α -latrocrustatotoxins, invertebrate-specific α , β , δ , ϵ , λ -latroinsectotoxins, and vertebrate specific α -latrotoxins (Rohou et al. 2007). While widow spider toxin genes are known to be specific for various types of prey, the specific regulation of these genes when exposed to prey has not been studied. This led me to question how different latrotoxins, specifically α -latrotoxin and α -latroinsectotoxin, are regulated to give insight into the production, use, and quantity of those toxins in widow spiders. Here, I found that α -latrotoxin is maintained at a consistent level despite being starved, fed an invertebrate, or fed a vertebrate. α -Latroinsectotoxin was significantly upregulated following an invertebrate feeding when compared to being starved. This general trend was followed when the spiders were fed vertebrates, although the data was not statistically significant. This study also researched the regulation of α -latrotoxin and α -latroinsectotoxin based on the sex of the spider to determine if sexual dimorphisms are present in toxin production and use.

α -Latrotoxin is of particular importance as it targets vertebrates. For example, widow spiders are responsible for most clinically significant human envenomation's in the United States (Williams et al., 2021). Latroinsectotoxins are important as they have been widely described and identified as important in the immobilization and feeding on insect prey (Lüddecke et al., 2021). Peptides in spider venom affect acetylcholine and

glutamate receptors as well as potassium, sodium, and calcium channels (Rahmani et al., 2014). α -Latrotoxin is of medical importance to humans as it releases massive amounts of neurotransmitters from pre-synaptic neurons (Torres et al., 2021). This causes a medical condition called latrodectism, which is characterized by muscles stiffness and pain, nausea, and vomiting (Timms and Gibbons, 1986). While human deaths are uncommon from widow spider bites, cats, dogs, and other smaller vertebrates have an increased risk (Peterson 2006). To widow spiders, it is likely that vertebrates are more common as predators than prey. It would not be advantageous to adjust toxin composition when there is an ongoing predator threat to the spider. With 85% of human bites being envenomated, it also is likely that dry bites are used for predator avoidance with vertebrates (Peterson, 2006). The relatively low and constant expression of the vertebrate toxin in both the vertebrate-fed and invertebrate-fed treatment likely reflect differential responses of spiders to predator and prey.

α and δ -Latroinsectotoxins have been the most thoroughly characterized latroinsectotoxins with respect to structure and function. Genetic sequences of β , ϵ , and λ -latroinsectotoxins have not been produced, making it harder to study those subcategories of latroinsectotoxins (Torres et al., 2021). Insects, the main prey of spiders, are abundant in lipids and proteins, making it important to have enzymes to break down these molecules. Venom of spiders is rich in protease, lipase, and carbohydrase enzymes. These enzymes are seen to play a role in the early stages of digestion before digestive enzymes are secreted (Walter et al., 2017). The upregulation of α -latroinsectotoxin that I observed when the brown widows were exposed to prey suggests that this species adjusts venom composition upon feeding. There are two described methods relating to how

venomous animals modulate their venom production, storage, and delivery. The first method is that protein composition varies throughout the bite sequence. What is first injected into the victim differs drastically from the final components injected and the difference is simply a by-product of protein storage location in the venom gland (Morgenstern et al., 2012). This pattern is what was observed in the only known study on venom gland composition change in a spider to date, the funnel web spider (*Hadronyche infensa*) (Morgenstern et al., 2012; Cooper et al., 2015). In contrast, other animals alter composition during defensive and predatory use. This type of venom modulation is used by cone snails (Cooper et al., 2015). The patterns observed in the brown widow spider in this study are consistent with the second method of modulation as it appears there were predatory and defensive venom composition changes in latrotoxin expression.

This study suggests that not all latrotoxins are constitutively produced and stored; some are induced when exposed to prey. Expression of some components are induced to change venom composition. This could mean that latrotoxin production is a response to feeding. In this study, insect specific α -latroinsectotoxin was expressed at a basal level and upregulated in a pattern consistent with its insect specific function. If the insect-specific, α -latroinsectotoxin, is upregulated in response to invertebrate feeding, it would make sense that the vertebrate-specific, α -latrotoxin, would be upregulated in response to being fed or exposure to a vertebrate.

Vertebrate specific α -latrotoxin was consistently expressed at a low level consistent with lower levels of α -latroinsectotoxin. This might suggest it might have multiple roles or be more potent than other latrotoxins. One possible explanation for the consistency of α -latrotoxin, would be that it is not truly vertebrate specific and serves as a

complementary molecule. However, this explanation does not seem to stand as one study found that the taxa-specific function of latrotoxins arises from the differences in different taxa's neurotoxin receptors in their nerve endings. The structure of the receptors in the nerve-endings differs between vertebrates and invertebrates, which explains the selectivity of the latrotoxins (Magazanik et al., 1992). It would be beneficial to further study the extent to which each taxa-specific latrotoxin plays a role in widow spider's genes. For example, the same study could be completed with the crustacean-specific latrotoxin using a roly-poly as the crustacean prey (*Armadillidium vulgare*). Understanding the production and use of these latrotoxins could be critical to understanding toxins in the arachnid family as all but two small groups of arachnids have poison glands that secrete venom (Rahmani et al., 2014).

This study showed a significant upregulation of α -latroinsectotoxin when the brown widow spiders were fed insect prey. When exposed to a small vertebrate, it did appear there was a pattern of upregulation of α -latroinsectotoxin, although the data was not significant. It is possible that initial production of taxa-specific latrotoxins might be induced when a widow spider senses prey caught in its web. When the prey type is determined, focused production of the taxa-specific latrotoxin is then activated for efficient toxin use. This would mean that spider webs play a sensory role in the toxin gene regulation process. Spider webs are made from spider silk containing proteins that come from the spidroin gene family (Correa-Garhwal et al., 2021). Widow spiders strategically build webs to ensure success in capturing prey. They have been known to have webs that have a bottom attachment that recoils when prey lands on the web. This allows the prey to be lifted towards the top of the web (Vollrath, 1992). The presence of

the captured prey is made known to the spider by vibrations or tension changes in the web (Vollrath, 1992).

The separate male and female sexes in sexually reproducing organism often have different physiology, behavior, and life history attributes. Genes that show sex-biased expression have been documented as some of the fastest evolving genes, making them be of particular importance in evolutionary genetics (Meisel 2011). Sex differences are apparent in most spider species and in many cases are extreme (Cordellier et al., 2020). In the brown widow spider, there is a considerable body size, morphology, and life history differences between the sexes. One big difference is seen in the pedipalps of adult male and female widow spiders. Upon reaching maturity, male pedipalps transition from feeding organs to reproductive organs, whereas females continue using their pedipalps as feeding and sensory organs (Cordellier et al., 2020). Before sexual maturity, there are minimal ecological, morphological, and life history differences between the sexes. Sex-biased expression of widow spider genes is not well characterized, especially upon reaching sexual maturity. This led me to question whether sex-biased expression played a role in brown widow spider toxin production following sexual maturity. For example, the transition of male pedipalps to reproductive organs upon maturity might induce males to downregulate toxin expression compared to females. Torres et al., 2021 found that immature widow spiders consistently express toxin genes. The data collected in the study presented here shows downregulation of toxin production in males after sexual maturity. Following sexual maturity, the 30-fold increase in expression for female spiders at both genes is consistent with the transition of male pedipalps to reproductive organs.

These findings strongly suggest that sexual dimorphisms are present in toxin production and use. This is likely an adaptation to different roles and morphologies of male and female spiders. With the cost of producing toxins metabolically high, there would be no need for males to produce this energy-depleting substance. Instead, they could put their energy into reproduction to help assure reproductive success. A study done on fruit flies showed that there is an exchange between survival and reproduction when it comes to sexual selection. Intense mating in the early lives of fruit flies correlated with younger deaths and vice versa following the “live fast and die” pattern seen in the life histories of many species (Travers et al., 2015). With male widow spiders living only 4 to 10 weeks, it would make sense that sexual selection would favor high reproduction rates in their short life spans. Another study showed that male brown widow spiders intentionally allow the female spiders to feed on them to increase the chances of a successful mating event (Segoli et al., 2008). It appears male widow spiders have one goal following maturity: to assure reproductive success. Female widow spiders live up to two years, resulting in a need for long-term maintenance. They can store large amounts of sperm from each mating and are known to produce a maximum of 29 egg sacs in their lifetime, which would total around 6,000 eggs (Arrington 2014). Feeding and maintenance during this time would maximize her reproductive output. This could mean that sexual selection would favor less intense reproduction frequency. More energy could then go into toxin production and use to ensure the capability to fight off predators and obtain prey for extended survival.

In conclusion, I found that sex and feeding play defining roles in latrotoxin production. There was a 30-fold increase in expression of toxin genes for female widow

spiders when compared to males. α -Latroinsectotoxin was upregulated following being fed an invertebrate. This data is consistent with the morphological, ecological, and life history differences of male and female widow spiders. This data is also consistent with the taxa-specific latrotoxins.

With the data collected and completion of this study I have identified several directions for future studies. I would like to investigate how feeding plays a role in all the taxa-specific latrotoxins. For example, is the crustacean specific latrotoxin regulated in the same manner as α -latrotoxin or α -latroinsectotoxin? I would also like to study the point in maturity at which males stop expressing latrotoxin during development. An interesting inter-species study would seek to determine if toxins are expressed and regulated differently between species to determine if this effects on the potency of different species' toxins.

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