The Effect of Diet on Venom Gene Expression in *Tetragnatha versicolor*

By

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Tetragnatha versicolor is an orb weaving spider that can be found throughout North America from Alaska to Nicaragua. This along with the fact the *T. versicolor* is a habitat generalist means that the species encounters a diverse array of insect prey species throughout its range. The purpose of this study is to determine if two populations of *T. versicolor* that feed on different insect species also differentially express venom genes.

First, I determined whether two populations of *T. versicolor* were eating different prey species by measuring the $\delta^{15}N$ values of *T. versicolor* from each site. The results of this analysis were that the $\delta^{15}N$ values for the two populations of *T. versicolor* were significantly different. This suggests that the two populations are at different trophic levels which indicates that the two populations of *T. versicolor* are eating different insect species.

Next, I used the RNA isolated from the venom glands of three males and three females from each of the two populations to construct a venom gland transcriptome which I used to perform a differential expression analysis comparing the males of each population, the females of each

population, and the males and females within each population. The results of this analysis showed that when comparing the females of each population, they were differentially expressing genes that are likely to be venom related. Also, when comparing the males to females, the males of each population were differentially expressing potential venom related genes despite the fact that their isotopic signatures were not significantly different from those of the females in the same population.

The Effect of Diet on Venom Gene Expression in Tetragnatha versicolor

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Chapter 1: Literature Review of Venom Evolution and Composition

Venom can be broadly defined as a mixture of proteins, small organic molecules, and salts secreted from a specialized gland of one animal that is actively injected into another animal to disrupt one or more physiological pathways. Venom has evolved independently multiple times throughout the animal kingdom from cnidarians, to spiders, to the platypus and for good reason. Not only does venom serve as a deterrent against predators and rival conspecifics, but it also allows a venomous animal to subdue prey larger than itself while at the same time reducing the chance of being harmed by combative prey (Casewell et al., 2013).

Many venomous animals produce a diverse array of venom proteins and this diversity comes from three sources. First, duplicates of non-venom related genes are recruited into the venom gland transcriptome (Casewell et al., 2013; Fry et al., 2005). For example, the latrotoxins found in the black widow spider, *Latrodectus spp,* are homologous to *Drosophila melanogaster* dTRPA1 gene suggesting that latrotoxins are essentially weaponized calcium ion channels (Garb and Hayashi, 2013) while the geographer cone snail, *Conus geographus,* uses a weaponized insulin to stun nearby fish by inducing insulin shock (Menting et al., 2016). Second, venom genes go through high rates of gene duplication and mutation which can lead to emergence of novel functions (Casewell et al., 2013). While the genes coding for endogenous insulin in cone snails have remained conserved, the genes coding for exogenous insulins have accumulated mutations that have altered the structure of the protein to the point that they now resemble the insulin found in the cone snail's prey (Menting et al., 2016). Similarly, the venom of the black widow spider contains multiple latrotoxins with some being more effective on vertebrates while other latrotoxins are more effective on invertebrates (Garb and Hayashi, 2013; Haney et al.,

2014). Finally, venom genes can be spliced in different ways producing different isoforms (Machado et al., 2005; Faure et al., 1991).

This diversity in venom proteins along with the differential expression and the alternate splicing of venom genes allows a venomous species to explore its fitness landscape and reach local peaks faster than it would through a series of small genetic changes. This rapid capitalization of ecological niches by a venomous species is best exemplified by the adaptive radiation of the marine snail superfamily Conoidea (Olivera et al., 2012). Modern cone snails hunt a wide range of prey species including worms, fish, crustaceans, and even other snails while the common ancestor of these cone snails most likely preyed only on worms (Imperial et al., 2007; Olivera et al., 2012).

Venom Recruitment vs Restriction

Venom toxins are diverse in both function and specificity. Some venom toxins, like the cysteine inhibitor knot peptides, are short peptides that target specific ion channels in specific animal taxa while other toxins, like the phospholipases, are large proteins that indiscriminately breakdown phospholipids in the cell membrane (Fry et al., 2009). Many taxa that have evolved venom contain a multitude of unique toxins. It is estimated that the taxa Scorpiones and Conoidea contain 50,000 (Olivera and Cruz, 2001; Norton and Olivera 2006) and 100,000 (Possani et al., 1999) unique venom peptides respectively. However, these estimates are eclipsed by the 1.5 million unique venom peptides suspected to be found in the order Aranea (Tedford et al., 2004b; Escoubas et al., 2006).

Our understanding of venom gene evolution comes primarily from snake venom research. Current research suggests that venom evolved once in snakes in the colubrid taxon

around 60-80 million years ago (Fry, 2005). The prevailing theory of how venom evolved is that over time different genes were recruited into the venom gland transcriptome from other tissue trancriptomes. It is predicted that twenty-four such recruitment events have occurred in the snake venom transcriptome producing a complex venom cocktail that consists of a wide array of multigene families such as metaloproteinases, cystatins, serine inhibitors, and epithelial growth factors. These recruitments occur through gene duplication events where one duplicate continues to be expressed in non-venom related tissue while the other begins to be expressed in the venom transcriptome as a new venom protein. These gene duplication events are likely the result of segmental duplications which are more likely to occur in snakes because of the repetitive nature of the reptilian genome allows for more frequent unequal crossing over events by providing regions of psuedo-homology (Hargreaves et al., 2014). Spider genes are also likely to duplicate themselves through segmental duplication as spider genomes are also highly repetitive (Sangaard et al., 2014). Another possible mechanism of recruitment is that instead of duplication, alternative splicing produces isoforms of a gene that are expressed in non-venom related tissue while another isoform is expressed in the venom glands. Evidence of this isoform-based scenario has been found in the banded krait, *Bungarus fasciatus*, which has one coding gene for the protein acetylcholinesterase. One isoform of the protein is found in the muscle and liver of the snake while a different isoform is only produced in the venom glands (Cousin et al., 1998).

An alternative explanation to the origin of venom toxins calls into question the idea that proteins from different tissues are recruited into venom gland (Hargreaves et al., 2014). The main concern is that recruitment requires the evolution of new transcription factor binding sites adjacent to the venom gene precursor so that it can be transcribed in the venom gland. In the

restriction model of venom evolution, ancestral venom genes are expressed throughout the organism including the venom gland meaning that the gene is adjacent to multiple transcription factors allowing for expression in multiple tissues. After a duplication event, one copy begins to accumulate mutations, which accomplishes two things. First, the mutations increase the toxicity of protein and second, the mutations remove specific transcription binding sites which restricts the transcription of the gene duplicate to only the venom gland. This not only increases the lethality of the new toxin but it also reduces the chance of the organism being harmed by the toxins they produce. Hargreaves et al. (2014) suggested the events leading to a series of mutations that remove transcription binding factors to restrict transcription from multiple tissues to only the venom gland is more likely than the events that leading to a series of mutations that result in the evolution of novel transcription factor binding sites resulting in a gene duplicate being transcribed in the venom gland when it was not before.

The Role of Gene Duplication and Mutation in the Evolution of Novel Venom Toxins

Regardless of how a gene duplicate is recruited into a venom transcriptome, once in the transcriptome, these genes evolve into large multigene families through the birth-and-death model of genome evolution. In this model genes are "born" through duplication and "die" when a gene duplicate is no longer transcribed and becomes a pseudogene (a.k.a "non-random loss) (Hurles, 2004). One explanation as to why venom gene duplication might be selected for is that there is a positive correlation between the number of venom gene copies and the level of venom

protein production. We see a similar trend in the amylase gene in humans. Populations that have adopted diets heavy in carbohydrates have more copies of the amylase gene which has a positive correlation with the amount of amylase being produced (Perry et al., 2007).

Over time, venom gene duplicates that remain in the venom gland transcriptome begin to diverge in function as they accumulate mutation which venom genes have higher rates of compared to non-venom genes (Olivera et al., 2012; Pineda et al., 2014). These mutations normally occur in regions that encode for the mature toxin while portions of the gene that are code for cysteine, which are involved in molecular scaffolding, and sequences that encode for the propeptide regions involved in the recruitment of the correct modifiers on the other hand remain conserved across the genes (Pineda et al., 2014).

There are two possible outcomes to gene duplicates that remain in the transcriptome. The first outcome is that the functionality of the ancestral gene is distributed between the duplicates, which is known as subfunctionalization. Subfuctionalization may be beneficial for an organism if the ancestral gene coded for a protein that had more than one function. In this situation, a gene duplication event would allow the copies of the ancestral gene to code for proteins that are more efficient in performing specific functions that the ancestral protein had. The second outcome is that one of the duplicates retains the function of the ancestral gene while the other evolves to have a novel function (Casewell et al., 2013), a process known as neofunctionalization.

Venom Composition

Venom components can be broadly categorized by their effect on the target organism as either neurotoxic and cytotoxic. Neurotoxins can immobilize an animal by targeting the nervous and muscular systems. Many neurotoxins, like the cysteine inhibitor knot peptides found in spiders and other invertebrates, induce paralysis in animals by interacting with the ion channels found on neurons resulting in either a hyperpolarization or depolarization of the cell membrane potential. Other toxins achieve a similar effect by targeting the cell membranes of neurons. An example of this are the phospholipase A2 toxins found in the venom of snakes and other invertebrates which are enzymes that attack the cell membrane by cleaving fatty acid chains from the glycerol of phospholipids (Donato et al., 1996). Toxins that lead to the destruction of cells are referred to as being cytotoxic. The medically significant component of *Loxosceles* venom, sphingomyelinase D, causes general cell death through the conversion of sphingomyelin to ceramide 1-phosphate which results in the formation of dermonecrotic lesions (Chaves-Moreora et al., 2017).

Structurally, venom toxins can be divided into three categories: polyamines, polypeptides, and proteins (Escoubas et al., 2000; Rash et al., 2002). In my study I focused on the latter two because they are directly coded for by an organism's venom genes

Polypeptides

Polypeptides are venom components that are between 3 to 8 kDa. One group of polypeptides contain multiple disulfide bonds that form a structural motif called a cysteine inhibitor knot which incorporates a cysteine knot and a triple-stranded, antiparallel ß-sheet (Norton et al., 1998). Most venom polypeptides target various membrane ion channels in their prey and their effect depends on which ion channel the toxin targets, which receptor sites on the

channel the toxin binds to, and the exact structure of the toxin. For example, toxins that bind to receptor site 4 of insect Na_v channel can result in a flaccid or spastic paralysis depending on the exact structure of the toxin (King et al., 2008).

The second group of venom polypeptides form alpha helices with hydrophobic regions. These venom polypeptides are cytotoxic, attack the cell membrane, and are believed to form protein pores that are capable of lysing prokaryotic cells and dissipating electrochemical gradients in nerve and muscle cells. Therefore, in addition to immobilizing prey, they may also help protect a venomous organism from any pathogens its prey may have (Yan et al., 1998).

Proteins

Proteins are the largest venom components. Many of these proteins are enzymes that facilitate digestion and aid in the diffusion of other venom components by breaking down proteins and lipids in the extracellular matrix (Escoubas et al., 2000). For example, many venomous animals have the enzyme hyaluronidase in their venom (Fry et al., 2009). It has been suggested that the function of this enzyme is to facilitate the diffusion of venom toxin through the body by breaking down the hyaluronan which is a major component of the extracellular matrix (Girish and Kemparaju, 2006). Other proteins like metalloproteinases, have a general effect of destroying cells and tissue. Not only does this kill a prey species but it also aids in pre-digestion.

Venom proteins can also target the nervous system and cause neurotoxic effects. Some proteins like phospholipase break down the phospholipid bilayer at the axon terminal. Other neurotoxic proteins, like latrotoxins, form protein pores on the surface of neurons resulting in the efflux or influx of different ions (Nicholls et al., 1982).

Many snake venoms also contain high amounts of nucleases though it is unclear as to what the function of these proteins is. A possible explanation is that by digesting nucleic acids, venom nucleases release free adenosine and guanosine. Free adenosine can lead to prey immobilization by binding to neuronal adenosine A1 receptors which prevents the release of acetylcholine from motor neurons while free guanine can lead to hypotension by inducing vasodilation. Therefore, it is plausible the nucleases work in junction with venom toxins that free nucleic acids by lysing cells to subdue prey (Dhananjaya et al., 2009).

Conclusion

There is still much to be learned when in it comes to venom. Even though a diverse array of animal taxa use venom, only a handful of the taxa of been well studied. Of the 40,000 described species of spiders, only a handful of medically significant species have been well studied and it is possible, that within the venoms of these understudied spiders could be toxins as unique as the latrotoxins found in the *Latrodectus* genus. The mechanisms involved in venom evolution still remain unclear especially when it comes to how genes are recruited into the venom gland transcriptome. While most studies on venom evolution have focused on venomous snakes, it may be beneficial to move our focus to spiders because unlike snakes, there are currently three sequenced spider genomes: *Stegodyphus mimosarum (*Sangaard et al., 2014), *Parasteatoda tepidariorum* (Gendreau et al., 2017), and *Nephila clavipes* (Babb et al., 2017). Having a genome to work with may help shed light onto what role transcriptome factor binding sites play in determining what genes are transcribed in the venom gland and determine to what extent gene duplication plays in the evolution of novel venom toxins.

Chapter 2: Diet and Transcriptome Analysis of Two Populations of *Tetragnatha Versicolor*

Variation in Venom Composition and its Relationship to Variation in Diet

Local adaptation and phenotypic plasticity through differential expression, alternative splicing, and differences in post-translational modifications allow for significant differences in the venom composition amongst individuals of the same species. A proteomic analysis of the venoms of vermivourus cone snail, *Conus vexillum,* by Abdel-Rahman et al. (2001) showed that there was a significant difference in the peptide composition of individual venoms which corresponded to a difference in how lethal the venom was. Similarly, a proteomic analysis of the venom of the bark scorpion *Centruroides edwardsii* by Estrada-Gomez et al. (2014) also demonstrated intraspecific variation that also corresponded with a difference in venom toxicity. While both papers presented differences in prey species as a possible explanation for these geographic variations, there is still not enough evidence for a direct correlation between the differences in diet and the differences in venom composition in the two species.

The best evidence of a relationship between variation in venom composition and a variation in diet has been found in venomous snakes. For example, the vipers of the genus *Echis* feed on a variety of prey species including both vertebrates and invertebrates. A study of *Echis* venom by Barlow et al. (2009) showed that species that feed more on invertebrates had venom that was more toxic to the Israeli gold scorpion *Scorpio maurus,* a natural prey species, than the *Echis* species that rarely feed on invertebrates.

A similar study on *Sistrurus* rattlesnakes demonstrated interspecific variation in the venom between closely related species and a possible link to differences in the diets of the species. The genus *Sistrurus* includes multiple species of small rattlesnakes that are found in different regions of North America. The diets of these species vary to the degree to which they

specialize on endothermic or exothermic prey. For example, the subspecies *Sistrurus catenatus tergeminus* are adapted to mesic environments and feed primarily on endothermic prey while the subspecies *Sistrurus catenatus ewdwardsii* are adapted to xeric environments and feed primarily on ectothermic prey. Proteomic analysis of four species of *Sistrurus* (*S. c. edwardsii, S. c. tergeminus, Sistrurus catenatus catenatus, and Sistrurus miliarius barbouri*) by Sanz et al. (2006) showed that although these species are closely related, their venom composition varied significantly. A study by Gibbs et al. (2009) looking at the functional differences between the venoms of different species of *Sistrurus* showed that there was a significant variation in toxicity toward mammals, where those species of *Sistrurus* that feed primarily on rodents had venom that was more lethal to mammals.

Evidence of intraspecific variation in venom can be found in the Malayan pit viper *Calloselasma rhodostoma. C. rhodostoma* is found throughout Southeast Asia and feeds on a diverse array of prey species including arthropods, fish, amphibians, reptiles, birds, and mammals. However, this dietary range narrows when looking at specific areas. For example, adult *C. rhodostoma* found in North Thailand feed primarily on reptiles while adult *C. rhodostoma* found in Indochina feed primarily on mammals and birds (Daltry et al., 1998). A study by Daltry et al. (1997) demonstrated that there is a significant correlation between the geographic variation in the venom composition and the geographic variation in the diet of this species. The study also showed that this variation is inherited and suggested that this variation is the result of natural selection where venom components that are toxic to the most abundant prey species in the area are selected over venom components that target prey species that the snake rarely encounters (Daltry et al., 1998).

Daltry et al. (1998) also demonstrated that there was ontogenetic variation in the venom composition in *C. rhodostema*. This makes sense since smaller juveniles would not be able to capture the same kind of prey larger adults are able to capture. It may even be that the geographic variation found in *C. rhodostema* is the result of paedomorphosis in certain geographical areas.

Unfortunately, there is little evidence of a relationship between variation in venom compositions and a variation in diet when it comes to other taxa of venomous animals including the order Aranea.

Spider Venom

The order Araneae has one of the largest venom toxin repositories of any other venom producing taxon. Using MALDI–TOF mass spectrometry coupled with chromatographic separation and the analysis of venom-gland cDNA libraries, Escoubas et al. (2004) demonstrated that Australian funnel spiders *Atrax robustus* and *Hadronyche versuta* had approximately 600 and 1000 unique venom peptides respectively. Even if these values are unique to funnel web spiders, it is estimated that the order Araneae could hold an estimated 1.5-1.9 million unique venom peptides assuming that each species of spider has an average of 200 unique peptides and that there are around 80,000 species of spiders. These estimates far outnumber the 100,000 (Possani et al., 1999) and 50,000 (Olivera and Cruz, 2001; Norton and Olivera 2006) unique peptides predicted to be in the venoms of the cone snails and scorpions respectively (Tedford et al., 2004b; Escoubas et al., 2006).

Only a few medically significant spiders have been thoroughly studied for their venom along with a handful of large mygalomorph species which were chosen for studies because of their high venom output. We know little to nothing of the venoms of the 5800 described jumping

spiders (Salticidae) (World Spider Catalog 2017) or of the 11,000 described orb weaving spiders (Hormiga and Griswold, 2014). What we do know is that, like the venom genes in snakes, spider venom genes are first recruited into the venom transcriptome through a gene duplication event and then undergo further gene duplication events and hypermutation in sequences encoding the mature venom toxins (Pineda et al., 2014). This, like in other venomous animals, has allowed spiders to rapidly evolve to take advantage of novel niches which has enabled some taxa of spider to undergo adaptive radiations. For example, Dr. Greta Binford (2001) has observed evidence of such an adaptive radiation event occurring in the long-jawed orb weavers (Araneae: Tetragnathidae: *Tetragnatha*) of Hawaii. Hawaiian *Tetragnatha* share a common North American ancestor that underwent an adaptive radiation after colonizing Kauai (the oldest high island in the archipelago) five millions ago, resulting in two clades that differ in hunting strategies. One lineage employs species-specific web architecture while the other lineage no longer produce webs and has adopted cursorial hunting strategies with concomitant species-specific ecomorphologies (e.g., cryptic coloration corresponding to habitat substrate). As expected, an analysis of the venoms of the orb weaving and the non-orb weaving Hawaiian *Tetragnatha* indicated differences in venom composition between the two clades (Binford, 2001). Remarkably, the cursorial *Tertagnatha* had lost many low molecular weight proteins of their venom along with their ability to spin webs.

Although there is little evidence of a relationship between a variation in venom compositions and a variation in diet in spiders, there is sufficient evidence of ontogenetic variation in the spider *Phoneutria nigriventer* where larger older spiders produce venom that is more lethal to mammals compared to the venom produced by smaller younger spiders (Herzig et al., 2004). This makes functional sense for the species because smaller instars would not be able

subdue mammalian prey. If this holds true for other species of spiders then it could mean that further analysis into order's venom will show many examples of intraspecific variation in venom composition. This is because if a venom gland's transcriptome changes over time, then it would require the presence of different transcription factor proteins (Gibbs et al., 2009) and possibly miRNA (Durban et al., 2013). Therefore, any mutation affecting either transcription factor or miRNA could result in a significantly different venom transcriptome in the adult.

Study Organisms

There are over 46,000 described species of spiders comprising 114 different families (World Spider Catalog, 2017). Spiders can be a found in almost every terrestrial biome on the planet ranging from the balmy tropics to arid deserts and utilize a wide range of hunting techniques to catch a variety of prey species. Around 25% of spiders belong to the Orbiculariae clade with an important synapomorphy - the use of orb webs to ensnare their prey (Hormiga and Griswold, 2014). The evolution of orb web building facilitated better access to flying insects for this lineage of spiders and likely enabled certain species to undergo adaptive radiation.

Currently, 347 species of orb weavers belong to the genus *Tetragnatha* (World Spider Catalog, 2017), which are easily recognizable by their large chelicerae, elongated abdomen, and long first, second, and fourth legs (Levi, 1981), hence their common names "long-jawed orb weaver" and "stretch spider". *Tetragnatha* are found on every continent except Antarctica and are present in arctic, temperate, and tropical environments. Most species can be found near water (with a few exceptions) and build loose orb webs that tend to have an opening in the center (Levi, 1981). Almost all *Tetragnatha* are generalist predators with the one exception of a Hawaiian *Tetragnatha* that has been observed to exclusively feed on terrestrial amphipods

(Binford, 2001). Male *Tetragnatha* can be distinguished from females by their modified pedipalps, relatively larger chelicerae, and projections on the distal ends of their chelicerae that are used during courtship (Levi, 1981; Dondale et al., 2003).

T. versicolor is found throughout North and South America from Alaska to Nicaragua (Levi, 1981; Dondale et al., 2003). They are generally found near the water where they build rectangular orb webs used to ensnare emerging flying insects, such as mosquitos and midges, though they have been found in drier habitats (Aiken et al., 2000; Dondale et al.2003). *T. versicolor* is also a habitat generalist. Both Levi (1981) and Aiken et al. (2000) have collected *T. versicolor* from a wide range of microhabitats including mixed oak forests, floodplain forests, grasslands, wetlands, and marshes. Because of this and its extensive range, it is likely that *T. versicolor* encounter a wide array of prey species. If this is the case, then it is also likely that the venom composition of *T. versicolor* varies from one location to another coinciding with the presence of different insect prey species.

General Overview of Isotope Analysis

Previous studies looking at the diets of predators have used methods such as field observations and gut content analysis. Observing predation in the wild can be time consuming and impractical for species that are cryptic or hunt infrequently like many species of non-web building spiders. Performing field observations to analyze the diet of web building spiders is more practical but it would still be time consuming and would only provide a snapshot of the spider's diet. Although gut content analysis has been used to study the diet of many animals, it has limited use in spiders because spiders liquefy their prey before consuming them.

A new method has been proposed to analyze the insect DNA found in the guts of spiders by using the fact that the nucleases in the spider's gut quickly degrade nucleic acids to isolate

prey DNA using size exclusion which should isolate degraded prey DNA from spider DNA (Krehenwinkel et al., 2017). However, this would still only give a snapshot of the spider's diet because the mitochondrial COI DNA needed to identify insect prey are degraded beyond use after a few hours of digestion (Kuusk et al., 2008).

The importance of a particular food source to a consumer's diet can be determined by comparing the isotope ratio of the consumers and their food sources. Isotope ratios are expressed as a delta notation in parts per thousand. It is calculated using this formula;

$$\delta X = ((R_{sample}/R_{standard})-1)*1000$$

R is the ratio of heavy to light isotopes for a certain element. R_{sample} is the isotope ratio of the sample while $R_{standard}$ is the reference standard ratio for each element. The formula for a consumer's isotope value can be expressed as:

$$\delta X_{consumer} = \delta X_{food} + \Delta\delta X$$

ΔδX is the value of the isotope fractionation or the trophic shift in the isotope ratio. The enrichment of the carbon stable isotope ratio between consumer and diet is assumed to be between 0.5‰ and 1‰ based on several other previous isotope studies (DeNiro and Epstein, 1978; Mackoet al., 1982; Lajtha and Michener, 1994) including one on the spider *Pardosa lugubris* by Oelbermann et al. (2001). Therefore, $\delta^{13}C$ values can be used to determine the contribution of different food sources to an organism's diet.

Unfortunately, this is complicated by the fact that *T. versicolor*, like most orb weavers, are generalist predators that will feed on a large variety of flying insects. Another problem is that because *T. versicolor* build their webs over bodies of water, it is likely that mosquitoes are a major contributor to their diets. This is a problem because there may be a significant difference

between the isotope ratios of a mosquito that has fed and one that has not fed (Gomez-Diaz & Figuerola, 2010).

Instead of using $\delta^{13}C$ values, I used the $\delta^{15}N$ values of individual *T. versicolor* to examine their trophic level. Trophic levels can be estimated from the $\delta^{15}N$ values of an organism because, in general, this ratio is enriched by 3‰ to 4‰ compared to the organism's diet (Perkins et al., 2014). This enrichment of $\delta^{15}N$ values is a result of a preferential removal of amine groups containing ^{14}N by enzymes involved in deamination and transamination (Macko et al. 1986). Therefore, ^{14}N is preferentially excreted from the organism, while ^{15}N is retained in the organism's protein. The simplest formula to calculate the trophic level is

$$Trophic\ level = (\delta^{15}N_{consumer} - \delta^{15}N_{baseline})/\Delta_n + \lambda$$

(Gannes et al., 1997) where λ is the trophic level of the organisms used to measure $\delta^{15}N_{baseline}$, Δ_n is the isotope fractionation or trophic shift in $\delta^{15}N$ values per trophic level, and $\delta^{15}N_{baseline}$ and $\delta^{15}N_{consumer}$ are the isotope signatures of the baseline and the consumer respectively. Since plants were used to measure $\delta^{15}N_{baseline}$, λ=1. Using this formula, I estimated the trophic levels of each population of *T. versicolor*. To determine $\delta^{15}N_{baseline}$, I collected different plant species from each site and took the average of their $\delta^{15}N$ values. I assumed an enrichment per trophic level of 3‰.

Geographic Variation of $\delta^{15}N$ and $\delta^{13}C$ Values

Although a difference in $\delta^{13}C$ values between the two populations of *T. versicolor* may suggest that they are eating different prey species, $\delta^{13}C$ values are difficult to interpret if the organisms being compared are from two different geographical areas because the $\delta^{13}C$ values can vary depending on factors such as different types of soil, the amount of precipitation an area receives, and the different types of plants in the area (Gannes et al., 1997). For example, C_4, crassulacean acid metabolism (CAM), and C_3 plants will have different $\delta^{13}C$ values from the way

these plants separate carbon isotopes to make glucose (O'Leary, 1988). In general, this leads to C_3 plants having more negative $\delta^{13}C$ values ranging from -25‰ to -37‰ while C_4 and CAM plants tend to have less negative $\delta^{13}C$ values ranging from -10‰ and -20‰ (O'Leary, 1988). However, C_4 and CAM plants are normally associated with arid and semiarid environments (Yamori et al., 2014); CAM plants in particular possess an important adaptation for environments that experience frequent droughts (Herrara, 2009). Therefore, it is safe to assume that both sites used in this study are dominated by C_3 plants. Even so, there is still a significant amount of variation between the different C_3 plant species. Also, prey species can acquire carbon from other sources such as detritus and algae which have different $\delta^{13}C$ values (Benner et al., 1987; DeNiro et al., 1978). In conclusion, although a difference in $\delta^{13}C$ values between the two populations may be evidence supporting a difference in diet, it could just mean that there are different sources of carbon in both areas.

Nitrogen can also vary from one area to another. Environments that are hot and arid, are more prone to losing ^{14}N resulting in higher $\delta^{15}N$ values in both plants and animals compared to organisms found in cooler moist environments (Gannes et al., 1997). Nutrient availability in an environment can also affect $\delta^{15}N$ values. Environments high in nitrogen preferentially lose more ^{14}N resulting in higher $\delta^{15}N$ values. However, environments low in nitrogen, such as environments that experience high levels of precipitation resulting in dissolved organic nitrogen being leached from the soil and deposited into nearby streams and rivers, are more likely to have plants that rely on mycorrhizal fungi for nitrogen (Neff et al., 2003). This is important because nitrogen obtained from mycorrhizal fungi is inorganic and tends to be ^{15}N depleted (Neff et al., 2003).

Experimental Design, Hypotheses, and Predicted Results

Although venom is useful for spiders, it is also energetically costly to make (Nisani et al., 2012) and populations of spiders might not express venom proteins that do not aid in survival. Therefore, one would expect to observe geographic variation in the venom composition of a species spider if these areas contain different prey species and that much of this variation would be the direct result of the differential expression of venom related genes.

In my study, I first determined if two populations *T. versicolor* fed on different insect prey species. I used the $\delta^{15}N$ values of *T. versicolor* from Highlands Biological Station in Highlands, North Carolina (35°03’17.8” N, 83°11’19.2” W) and the Phil Carol Nature Reserve in Greenville, North Carolina (35°37'29.7"N, 77°24'30.4"W) along with the $\delta^{15}N$ values of nearby plants to determine the trophic levels of the spiders.

To make sure any difference in $\delta^{15}N$ values between the two *T. versicolor* populations is the result of a difference in trophic levels and not a result of geographic variation in $\delta^{15}N$ values, I also collected plants and insects from both sites. In a scenario where one site has higher $\delta^{15}N$ values for plants, spiders, and insects overall, it would be safe to assume that the difference is likely the result of one site having higher levels of ^{15}N than the other. However, if $\delta^{15}N$ values of the two *T. versicolor* populations are different but the $\delta^{15}N$ values of the plants and insects are similar, then it is safe to assume that the difference is the result of *T. versicolor* populations being at different trophic levels.

A difference in trophic levels provides enough evidence that the two populations of spiders are eating different prey species. Once I determined this I sequenced the venom gland transcriptome of three female and three male *T. versicolor* spiders from each of the two populations and performed differential expression analyses to determine if the two populations

were transcribing venom genes at different levels. I compared the venom gland transcriptomes the males of each population, the females of each population, and the males and females within each population. The reasoning for this was that previous observations of *T. versicolor* and *Tetragnatha* species suggested that male *T. versicolor,* once reaching maturity, rarely eat and instead devout most of their time to finding a mate (Levi, 1981). However, the males were still included in this study because of the unique sexual dimorphism of this species' venom.

H0: There is no relationship between venom composition and diet. Therefore, I will find no differentially expressed genes between the venom transcriptomes of the two *T. versicolor* populations regardless of whether or not they prey on different insect species.

H1: There is a relationship between venom composition and diet. Therefore, I will find differentially expressed genes between the venom transcriptomes of the two *T. versicolor* populations if there is a difference diet.

Taxon Sampling and Animal Care

T. versicolor were collected from two sites, the Phil Carol Nature Reserve (35°37'29.7"N, 77°24'30.4"W) near the city of Greenville, NC located in Pitt county, and the Highlands Biological Station (35°03'17.8" N, 83°11'19.2" W) near Highlands, NC located in Jackson county. The spiders were collected near the water at night when they are most active. Bioquip soft touch forceps were used to pluck the spiders from their webs. Once collected, the *T. versicolor* were kept individually in 50 mL conical tubes until they were brought back to the lab and were then transferred into medium sized deli containers. Because the spiders were only alive for five days and because prey in the spider's gut can affect isotope ratio measurements, the spiders were starved. However, their environmental humidity was kept constant to prevent the

spiders from desiccating by placing a moistened paper towel in the bottom of their containers. This paper towel was replaced daily to prevent mold growth.

Methods

Sample Preparation for Isotope Analysis

Once collected, the *T. versicolor* were starved for four to five days to make sure any prey in the guts had been expelled. Afterwards, the spiders were sacrificed and stored in a -80° Celsius freezer. Insects were collected using a malaise trap and were immediately placed in a -80° Celsius freezer along with the collected plant samples. To prepare the samples for isotope analysis, the spiders, plants, and insects were desiccated in a 60° Celsius oven for two days and then were crushed into a powder. The powdered samples were then placed in individual tin capsules (one milligram of powdered sample for invertebrates and five milligrams of powdered sample for plants) and were then sent to the University of North Carolina at Wilmington Isotope Ratio Mass Spectrometry Facility.

Venom Extraction

To collect venom from the spiders, I induced envenomation via electrical stimulation following a modified version of Dr. Jessica Garb's venom extraction protocol (2014). A GRASS SD9 Stimulator was used to provide the spiders a non-lethal amount of electricity. Seven and fifteen volts of electricity were used for small and large spiders respectively. The remaining settings on the electro-stimulator were: Frequency= 1 pulse per second, delay= 0 milliseconds, duration= 200 milliseconds, twin pulses switch= regular, and mode switch= off. Alligator clips were connected to the electro-stimulator with the negative clip attached to the bottom prong of a pair of Bioquip soft touch forceps and the positive clip was attached to a blunt hypodermic

needle that was connected to a vacuum. The bottom prong of the forceps was wrapped in cotton thread and soaked in saline to increase conductivity.

The spiders were first anesthetized via exposure to carbon dioxide until they could no longer right themselves dorsoventrally. Once the spiders had been anesthetized, they wereplaced between the prongs of the soft touch forceps. The forceps were attached to a clamp that was placed underneath a stereo microscope.

The chelicerae of the spiders were examined and cleaned with water if necessary and excess water was removed using the vacuum probe. The vacuum probe was then placed on the rostrum of each of the spiders and an electrical pulse was administered. This caused the spiders to release the contents of its venom glands and, in some cases, vomit. To prevent contamination, regurgitant was quickly vacuumed up by the probe. Venom droplets were collected using stretched microcapillary tubes. After collection, the microcapillary tubes were placed in 0.5 mL microcentrifuge tubes containing 100 microliters of molecular grade water to prevent the venom from crystallizing. The contents of the microcapillary tube were dispensed into the microcentrifuge tube and stored at -80° Celsius (Garb, 2014). Two days after venom extraction, the spiders were sacrificed, and their venom glands were removed and stored at -80° Celsius.

RNA Isolation and Sequencing

RNA was extracted from the venom glands of female and male *T. versicolor* from two different populations, one from the Phil Carroll Nature Preserve (35°37'29.7"N, 77°24'30.4"W) and one from the Highlands Biological Station (35°03’17.8” N, 83°11’19.2” W). Total RNA was isolated from homogenized venom glands using a hybrid Trizol and RNeasy Kit protocol. The glands of each spider were placed in 500 μL of Trizol and were homogenized using a micro-pistil. Afterwards, an additional 500 μL of Trizol was used to dislodge any tissue stuck on the

micro-pistil. The Trizol solution was then placed in a phase lock tube along with 200 μL of chloroform. This solution was then shaken and incubated a 4° C for twenty minutes. The solution was shaken every five minutes during the incubation. After the incubation, the phase lock tubes were spun in a centrifuge at 12,000Xg for 20 minutes at 4° C. The aqueous phase above the waxy layer was then aspirated into a 1.5 mL microcentrifuge tube along with 500 μL of ethanol. The process was done carefully so that the waxy layer was not disturbed. 700 μL of the solution was then placed into an RNeasy column seated in a collection tube along with 700 μL of RW1 buffer solution. The column and the tube were then centrifuged for 30 seconds at 8,000Xg. The flow-through was then discarded and the step was then repeated for the remaining solution. Then 500 μL of RWE buffer solution was placed into the column which was then centrifuged for 2 minutes at 8,000Xg. The flow-through was then discarded and the column was centrifuged again for 1 minute at 8,000Xg to remove any remaining buffer solution. The column was then placed in a 1.5 mL microcentrifuge tube, 25 μL of RNase free water was added to the column, and it was then incubated for 1-2 minutes. After the incubation, the column was centrifuged for one minute at 8,000xg. This and the previous step were then repeated. The RNA was then stored at -80° Celsius.

To determine if the RNA was successfully isolated, the RNA concentration was measured using a NanoDrop 2000 UV-Vis Spectrophotometer. The quality of the RNA was estimated using the 260/230 absorption ratio where higher ratios indicate a higher RNA quality. From the two populations, I selected three female and three male individuals. The chosen samples were then sent to HudsonAlpha Institute of Biotechnology for cDNA library preparation and sequencing. The cDNA was paired-end sequenced using the Illumina HiSeq 4000. An

average of 40 million reads was generated for each pair of venom glands (20 million forward and 20 million reverse).

Transcriptome Assembly and Annotation

The quality of the reads was assessed using FastQC (Andrews, 2010). Low quality reads from both 5' and 3' ends were removed using Trimmomatic (Bolger et al., 2014). Default filter settings were used.

De novo transcriptome assembly was conducted using Trinity v2.3.2 software (Grabherr et al., 2011). Annotation of the assembled transcriptome was done using the Trinnotate annotation suite. First, TransDecoder (http://transdecoder.github.io/) searched for possible coding regions in each transcript and produced an output file of peptide sequences for each coding region. Next, the peptide sequences were queried against the Swiss-Prot Database (The UniProt Consortium, 2016) using BLASTx (Altschul et al., 1992). Then, protein domains were annotated for each predicted peptide sequence using the tool hmmscan (Finn et al., 2011). Finally, signalP (Peterson et al., 2011), tmHMM (Krogh et al., 2001), and RNAMMER (Lagesen et al., 2007) was used to predict signal peptides, transmembrane peptides, and identify rRNA genes respectively.

Differential Expression Analysis

The abundance of each transcribed gene from each sample was quantified using Kallisto (Bray et al., 2016). Unlike other programs that measure transcript abundance, Kallisto does not require reads to be mapped to a transcriptome. Instead, Kallisto utilizes pseudo-alignment where instead of aligning reads to the assembled transcripts, Kallisto tries to determine which transcripts a read is compatible with using the de Brujin graphs generated by the Chrysalis module of Trinity. This allows abundance measurements to be performed significantly faster

than other programs like RSEM (Li and Dewey, 2011) and eXpress (Forster et al., 2013). Then, using the Trinity script, I used the estimated transcript abundances for each sample to create matrices of counts which could then be used by the program DESeq2 (Love et al., 2014) which performs differential expression analyses using negative binomial distribution. Differential expression analyses, using DESeq2, were utilized to compare the venom gland transcriptomes of the males of the two populations, the females of the two populations, and of the males and females within each population. These steps were implemented through Trinity.

Gene Ontology Enrichment

The differentially expressed genes were analyzed using GOseq (Young et al., 2010) through the Trinity script. The annotation reports produced through Trinotate were used in place of a reference genome. GOseq categorizes overexpressed transcripts from a differential expression analysis into specific gene ontology (GO) categories. This information can then be used to determine if specific GO categories are overrepresented for a certain set of up-regulated genes. I used the cutoff of 0.05 for the false discovery rate (FDR) corrected p-value to determine if a GO category is overrepresented. GOseq can also be used to remove biases caused gene length. This bias results from the fact that longer transcripts are more likely to have reads align to it than shorter transcripts even if their expression levels are similar (Young et al., 2010).

Results

Isotope Analysis

Table 1. Isotopic signatures of the T. versicolor, plants, and insects from both the Greenville and Highlands sites including standard deviations. Standard deviations where not included for the Diptera and Lepidoptera samples because multiple individuals were pooled to attain enough dry sample for isotope measurements.

Sample	Average $\delta^{13}C$	Standard deviation of $\delta^{13}C$	Average $\delta^{15}N$	Standard deviation of $\delta^{15}N$
Greenville *T. versicolor*	-25.46	1.377	4.73	0.31
Highlands *T. versicolor*	-28.62	0.77	9.31	3.06
Greenville Plants	-29.58	2.33	-1.13	1.38
Highlands Plants	-30	1.47	-1.31	0.48
Greenville Lepidoptera	-29.16	NA	2.52	NA
Highlands Lepidoptera	-24.80	NA	1.58	NA
Greenville Diptera	-27.48	NA	3.76	NA
Highlands Diptera	-28.71	NA	0.38	NA

Table 2. $\delta^{13}C$ and $\delta^{15}N$ values of the plant species used in isotope analysis

Plant Species	Location	$\delta^{13}C$	$\delta^{15}N$
Monarda fistulosa	**Highlands**	-29.48	-0.75
Porteranthus trifoliatus		-28.84	-1.52
Peltandra virginica		-31.64	-1.64
Smilax hispida	**Greenville**	-31.96	1.17
Acer rubrum		-29.02	0.29
Microstegium vimineum		-29.83	-2.67
Smilax rotundifolia		-32.11	-0.40
Privet ligustrum		-28.79	-1.50
Lespedeza sp		-29.13	-2.71
Quercus rubra		-30.96	-1.62
Carex vulpinoidea		-24.80	-1.58

The student t test of the $\delta^{15}N$ values of *T. versicolor* showed that there is a significant difference between the two populations (t= 6.0449, p-value < 0.001) with the *T. versicolor* from the Highlands site having an average $\delta^{15}N$ of 9.31‰ (n=8) while the *T. versicolor* from the Greenville had an average $\delta^{15}N$ of 4.73‰ (n=16) (Figure 1, Figure 3). I decided not to compare the sexes separately because there was no significant difference between the sexes in the two populations and because it would have made the sample sizes very small. A student t test of the $\delta^{15}N$ values of the male and female *T. versicolor* from the Highlands site gave a t-value .466 and p-value of .328 while a student t test of the $\delta^{15}N$ values of the male and female *T. versicolor* from

the Greenville site gave a t-value .622 and p-value of .544. Using the averages of the $\delta^{15}N$ values for each population and assuming a trophic shift of 3‰, I calculated the trophic level Highlands *T. versicolor* to be 4.54 and the trophic level of Greenville *T. versicolor* to 3.96 (Figure 2).

Along with the differences in trophic levels, there were also significant differences in the $\delta^{13}C$ values between the two populations (t= 5.998, p-value < 0.001) with the *T. versicolor* from the Highlands site having an average $\delta^{13}C$ value of -28.62‰ (n=8) while the *T. versicolor* from the Greenville had an average $\delta^{13}C$ value of -25.46‰ (n=16) (Table 1, Figure 2).

Eight different plant species were collected from the Greenville site (Table 2) and were used in the isotope study. The average $\delta^{13}C$ value of the plants was -29.58‰ with a standard deviation of 2.33‰. The average $\delta^{15}N$ value of the plants was -1.13‰ with a standard deviation of 1.38‰. From the Highlands site, three different plant species (Table 2) were collected from and used in the isotope study. The average $\delta^{13}C$ value of the plants was -30‰ with a standard deviation of 1.46‰. The average $\delta^{15}N$ value of the plants was -1.31‰ with a standard deviation of 0.48‰ (Figure 1, Figure 3). A student t test of the $\delta^{15}N$ values of the plant from the two sites gave a t-value of 0.21 and p-value of .838. A student t test of the $\delta^{13}C$ values of the plant from the two sites gave a t-value of 0.28 and p-value of .785.

The insect samples were pooled into the either Diptera or Lepidoptera to ensure that there was at least 1 mg of powdered sample material for isotope measurement. The $\delta^{13}C$ and $\delta^{15}N$ values for the dipterans from the Greenville were -27.47‰ and 3.76‰ respectively. The $\delta^{13}C$ and $\delta^{15}N$ values for the lepidopterans from the Greenville site were -29.15‰ and 2.52‰ respectively. The $\delta^{13}C$ and $\delta^{15}N$ values for the Diptera from the Highlands sit were -28.71‰ and

0.38‰ respectively. The $\delta^{13}C$ and $\delta^{15}N$ values for the Lepidoptera from the Highlands were -24.81‰ and 1.58‰ respectively (Table 1, Figure 2).

Figure 1. Calculated trophic levels along with 95% confidence intervals. Trophic levels were calculated using $\delta^{15}N$ of *T. versicolor* and plants $\delta^{15}N$ as a baseline. An enrichment per trophic level of 3‰ was used

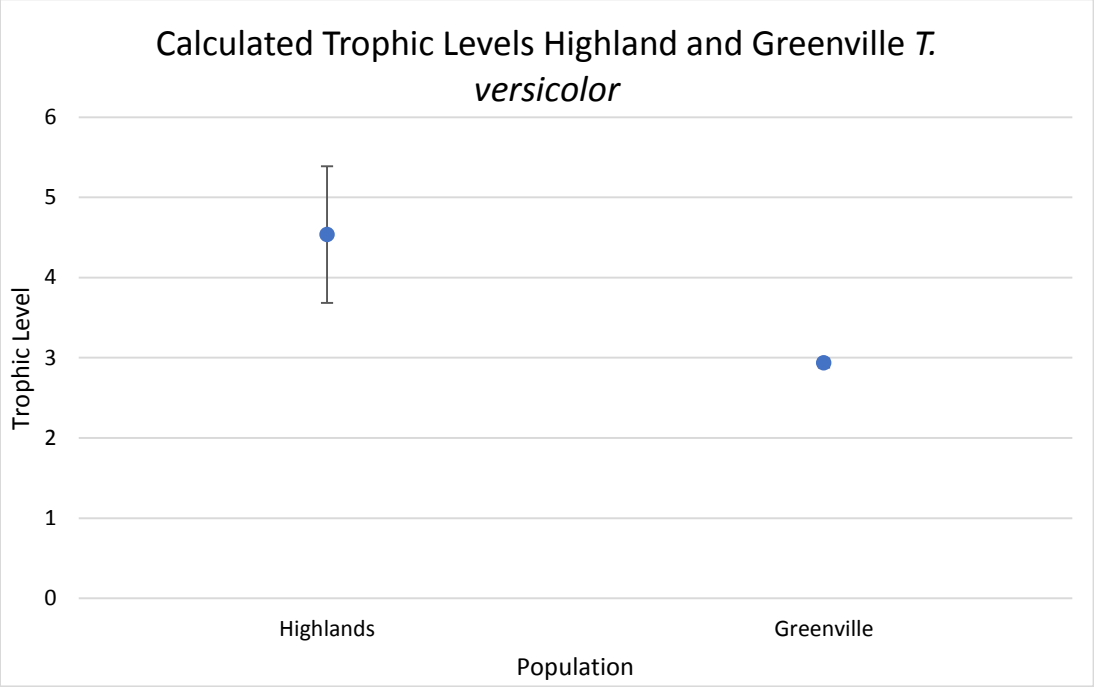
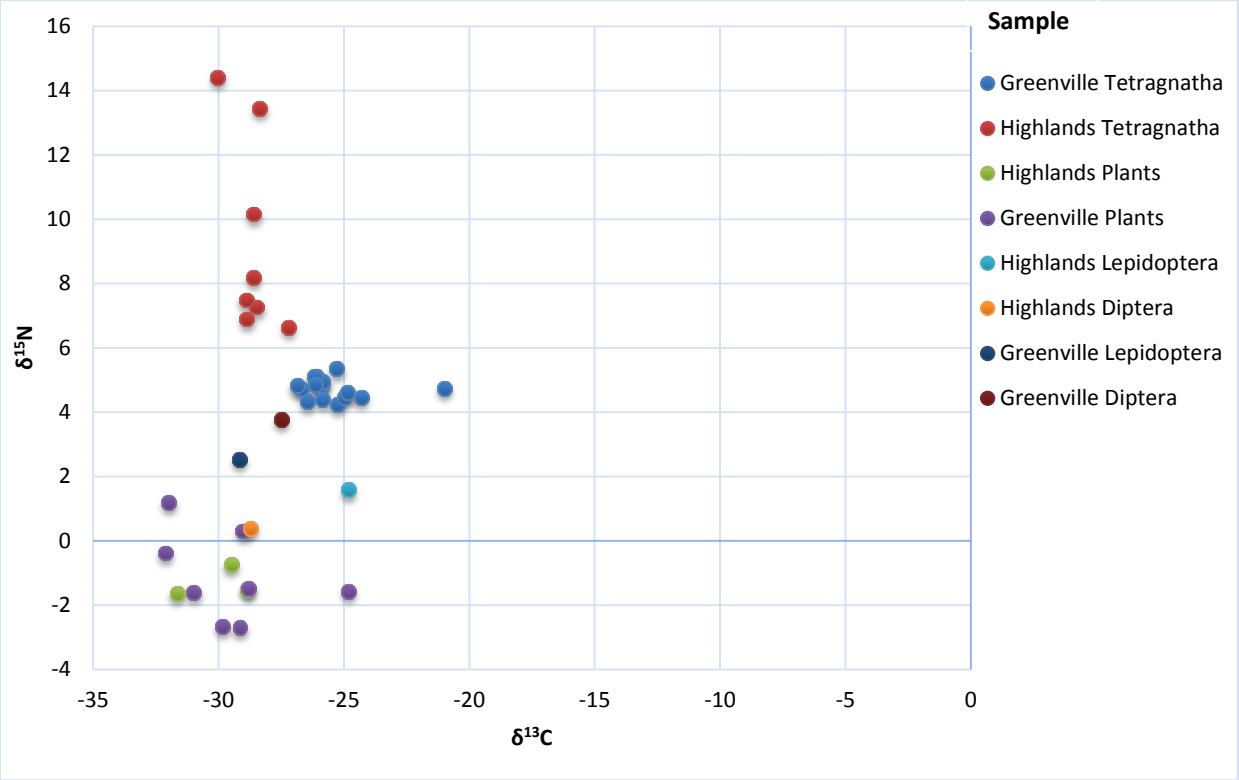


Figure 2. Isotopic signatures of Greenville and Highlands *T. versicolor* along with plants and insects from both sites.



Transcriptome Analysis

Transcriptome Assembly and Predicted Open Reading Frames

A total of 497,910 transcripts were assembled from the RNA-seq reads of the 12 *T. versicolor*. Transcript length ranges from 485 to 3,015 base pairs with the average and median lengths being 451.37 and 283 base pairs respectively. Predicted open reading frames were obtained for 55,435 genes using Transdecoder.

Highlands Females vs. Greenville Females

The DESeq2 analysis between the three females of each population showed that 42 transcripts were over-expressed in the Greenville female *T. versicolor* and 28 transcripts were over-expressed in the Highlands female *T. versicolor* (Figure 3). The GO enrichment analysis of

the differentially expressed transcripts identified six significantly overrepresented (0.05>FDR) GO categories in the Greenville *T. versicolor* females including extracellular region (GO:0005576), decidualization (GO:0046697), regulation of catalytic activity (GO:0050790), cysteine-type endopeptidase activity (GO:0004197), cysteine-type peptidase activity (GO:0008234), cellular response to thyroid hormone stimulus (GO:0097067) (Figure 4). GO enrichment analysis, however, identified no significantly overrepresented GO categories in the Highlands *T. versicolor* females.

Figure 3. Heat map of the differentially expressed transcripts of Highlands female *T. versicolor* (blue) and Greenville female *T. versicolor* (red). Purple represent down regulation while yellow represents up regulation.

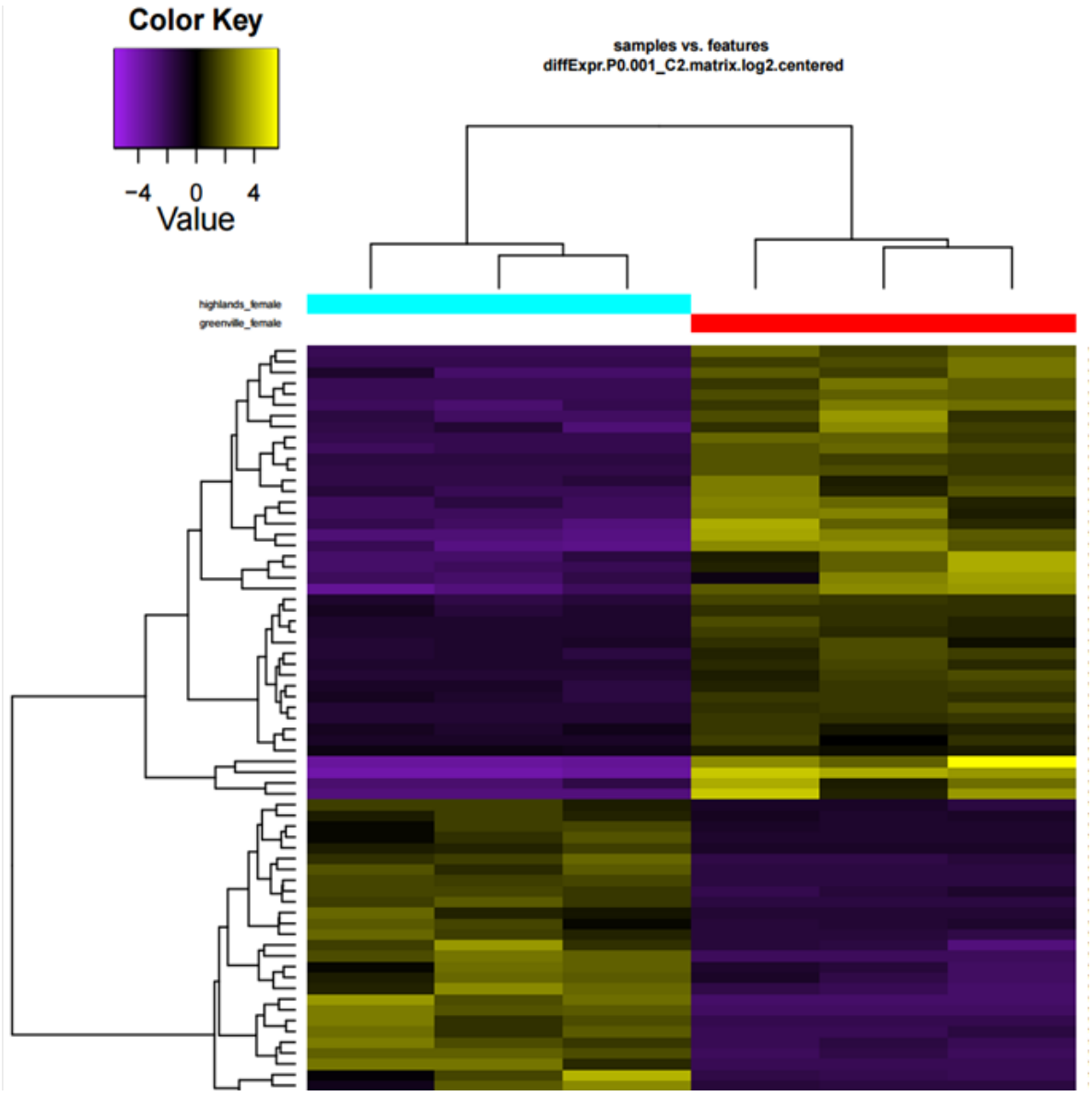
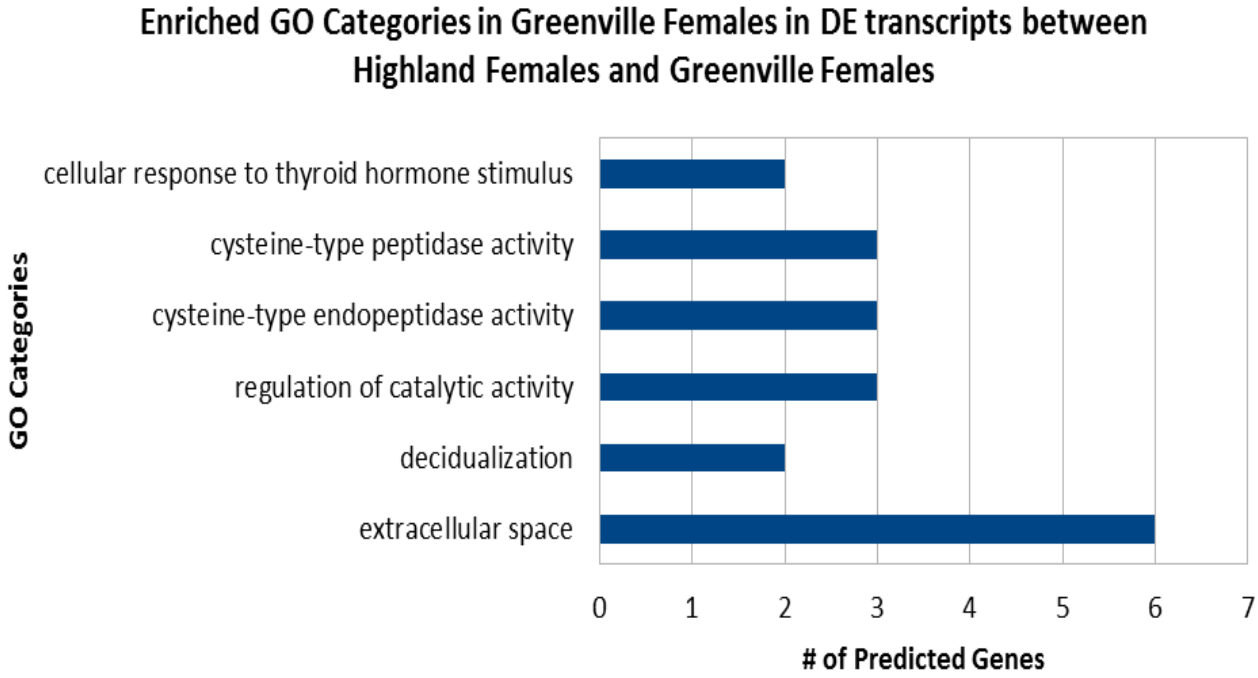


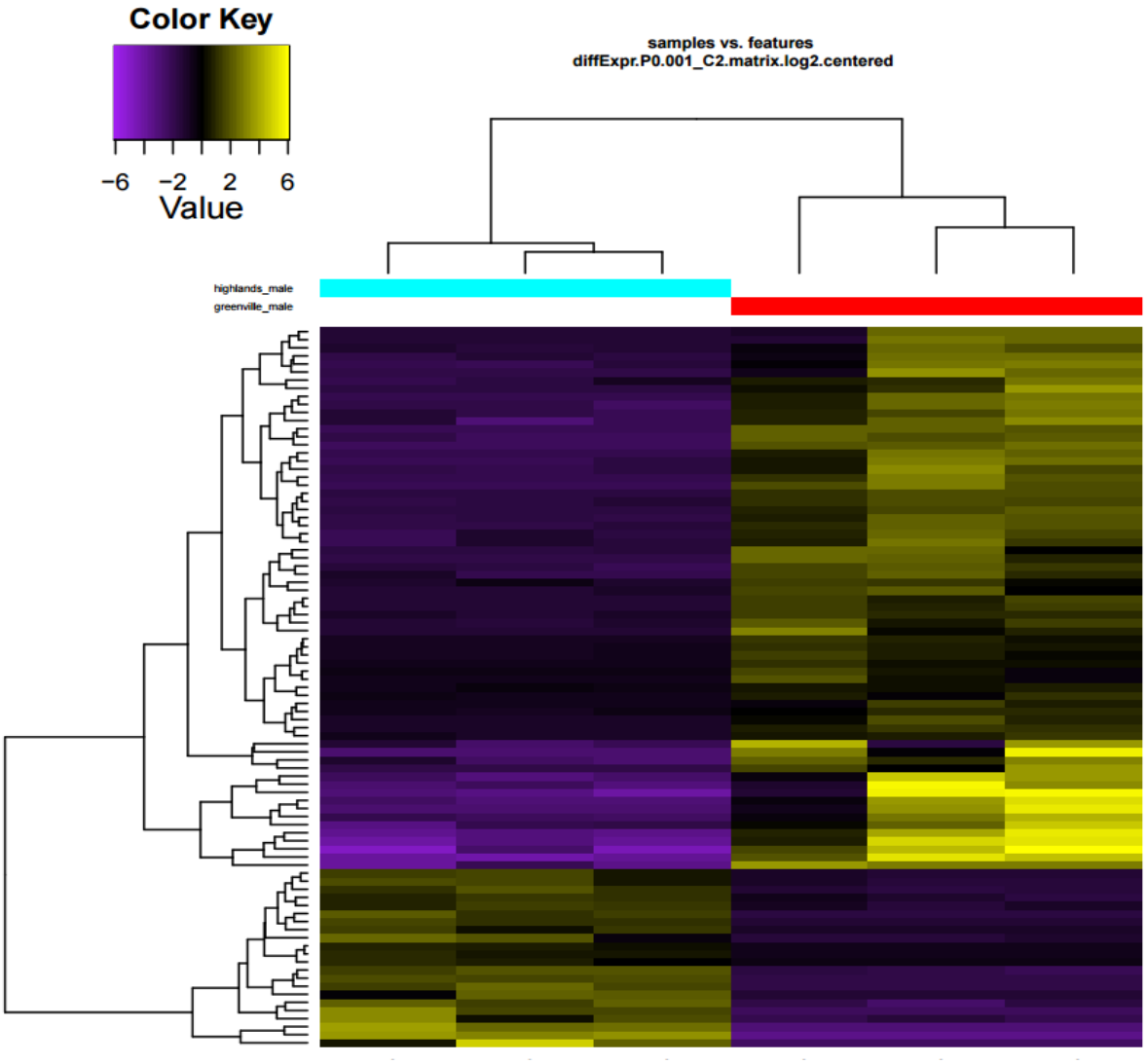
Figure 4. Bar graph of over-represented GO categories (FDR<0.05) and the number of predicted genes for each category for Greenville females in differentially expressed transcripts between Highlands females and Greenville females



Highlands Males vs Greenville Males

Between the three males of each population, 67 transcripts were over-expressed in the Greenville male *T. versicolor* and 22 transcripts were over-expressed in the Highlands male *T. versicolor* (Figure 5). The GO enrichment analysis of the differentially expressed transcripts did not identify any significantly overrepresented (0.05>FDR) GO categories for either group.

Figure 5. Heat map of the differentially expressed transcripts of Highland male *T. versicolor* (blue) and Greenville male *T. versicolor* (red). Purple represent down regulation while yellow represents up regulation.



Greenville Females vs Greenville Males

Between male and female *T. versicolor* collected from Greenville, 209 transcripts were over-expressed in the Greenville male *T. versicolor* and 52 transcripts were over-expressed in the Greenville female *T. versicolor* (Figure 6). The GO enrichment analysis of the differentially expressed transcripts identified no significantly overrepresented GO categories in the Greenville *T. versicolor* females, and fifteen significantly overrepresented GO categories in the Greenville *T. versicolor* males: oxygen transporter activity (GO:0005344), copper ion binding (GO:0005507), extracellular space (GO:0005615), chitin metabolic process (GO:0006030), chitin binding (GO:0008061), chloride ion binding (GO:0031404), structural constituent of cuticle (GO:0042302), oxidoreductase activity (GO:0016491), extracellular region (GO:0005576), steroid hormone receptor complex assembly (GO:0006463), prostate gland development (GO:0030850), negative regulation of microtubule polymerization or depolymerization (GO:0031111), male sex differentiation (GO:0046661), protein complex localization (GO:0031503), and chitin synthase activity (GO:0004100) (Figure 7).

Figure 6. Heat map of the differentially expressed transcripts of Greenville female (blue) and male T. versicolor (red). Purple represent down regulation while yellow represents up regulation.

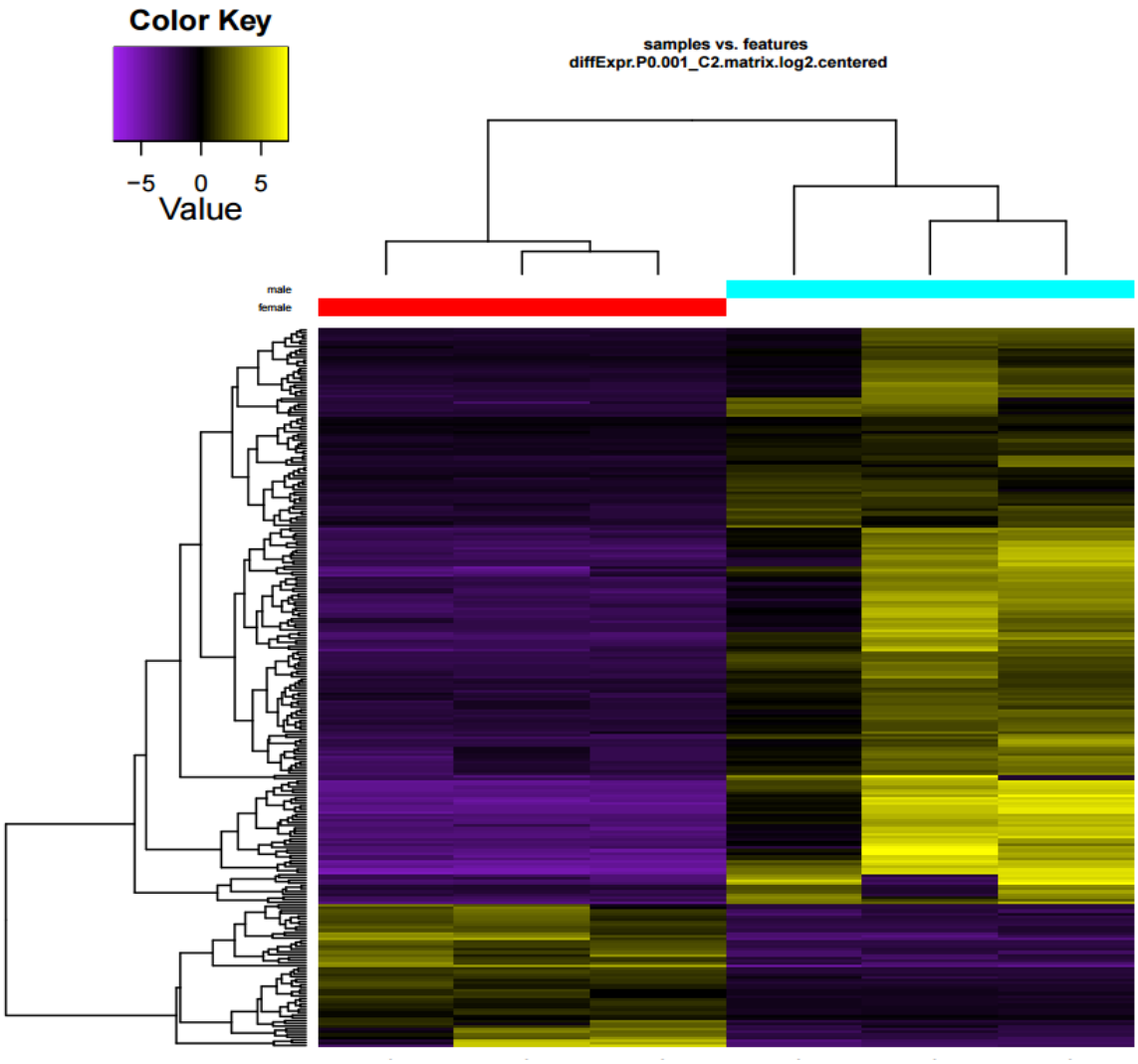
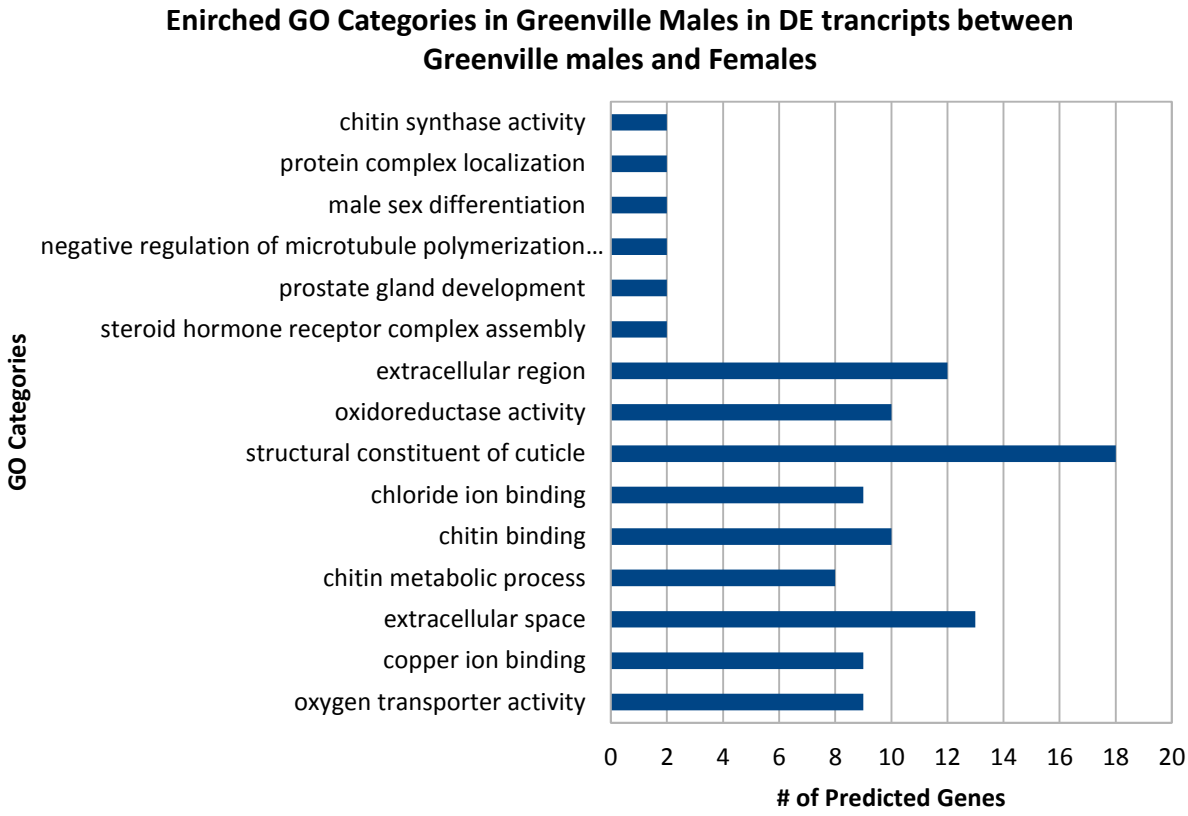


Figure 8. Bar graph of over-represented GO categories (FDR<0.05) and the number of predicted genes for each category for Greenville males in the differentially expressed transcripts between Greenville females and males.



Highlands Females vs Highlands Males

Between male and female *T. versicolor* collected from Highlands, 161 transcripts were overexpressed in the Greenville female *T. versicolor* and 374 transcripts were overexpressed in the Greenville male *T. versicolor* (Figure 8). The GO enrichment analysis of the differentially expressed transcripts identified forty significantly overrepresented GO categories in the Highlands *T. versicolor* males: endoplasmic reticulum lumen (GO:0005788), protein disulfide isomerase activity (GO:0006467), integral component of membrane (GO:0016021), cell redox homeostasis (GO:0031351), neurotransmitter catabolic process (GO:0042135), protein folding (GO:0032801), acetylcholinesterase activity (GO:0003990), germ cell repulsion (GO:0035233), maintenance of protein localization in endoplasmic reticulum (GO:0035437), transmembrane transport (GO:0055085), lipid phosphatase activity (GO:0042577), phospholipid dephosphorylation (GO:0046839), diuretic hormone activity (GO:0008613), polypeptide N-acetylgalactosaminyltransferase activity (GO:0004653), ectopic germ cell programmed cell death (GO:0035234), phosphatidate phosphatase activity (GO:0008195), hormone activity (GO:0005179), pole cell migration (GO:0007280), succinate transport (GO:0015744), extracellular region (GO:0005576), oligosaccharide biosynthetic process (GO:0009312), phospholipid-translocating ATPase activity (GO:0004012), endomembrane system (GO:0012505), peptide hormone processing (GO:0016486), ion transmembrane transport (GO:0034220), cerebellum structural organization (GO:0021589), succinate transmembrane transporter activity (GO:0015141), Melanosome (GO:0042470), citrate transmembrane transporter activity (GO:0015137), citrate transport (GO:0015746), succinate transmembrane transport (GO:0071422), carbohydrate binding (GO:0055085), unfolded protein binding (GO:0051082), phospholipid translocation (GO:0045332), plasma membrane (GO:0005886),

germ cell migration (GO:0008354), negative regulation of innate immune response (GO:0045824), phosphatase activity (GO:0016791), phospholipid metabolic process (GO:0006644), and metalloendopeptidase activity (GO:0004222) (Figure 9).

Figure 8. Heat map of the differentially expressed transcripts of Highlands female (blue) and male *T. versicolor* (red). Purple represent down regulation while yellow represents up regulation.

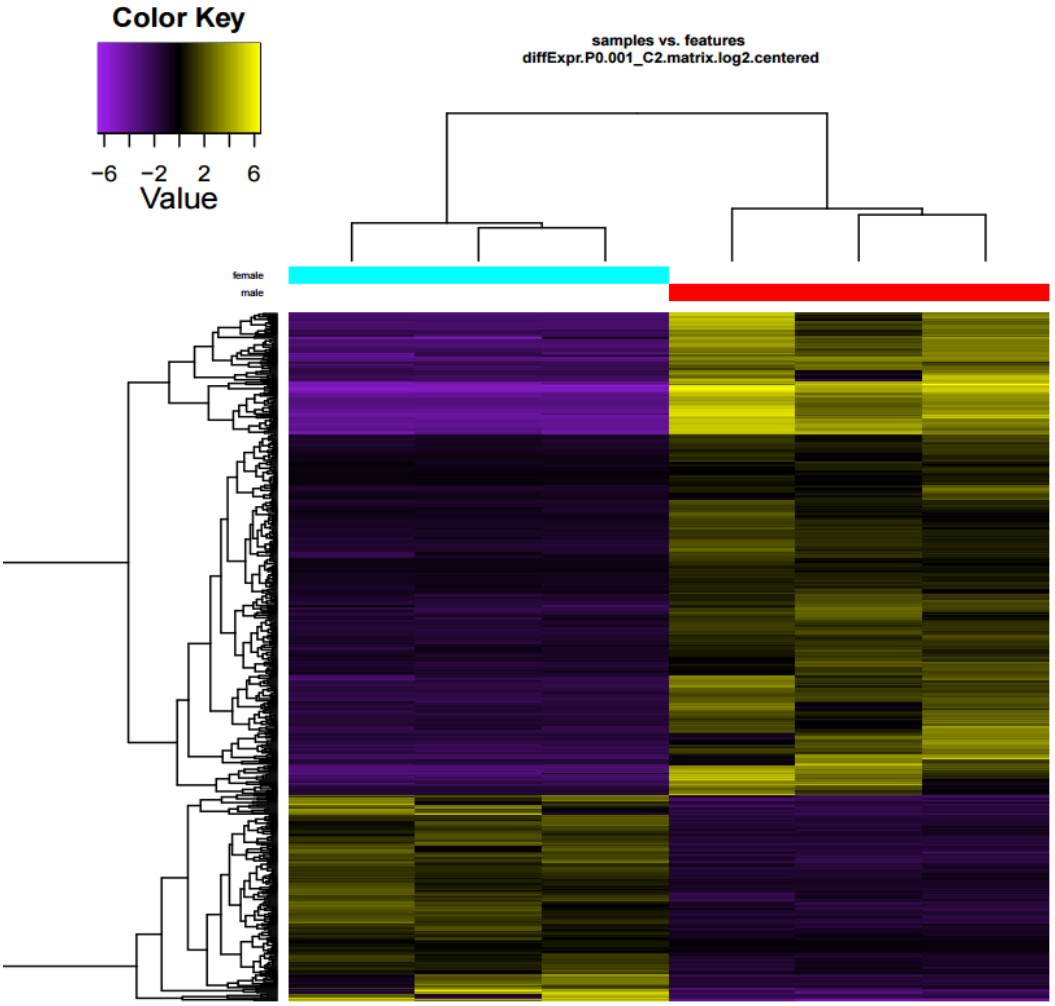
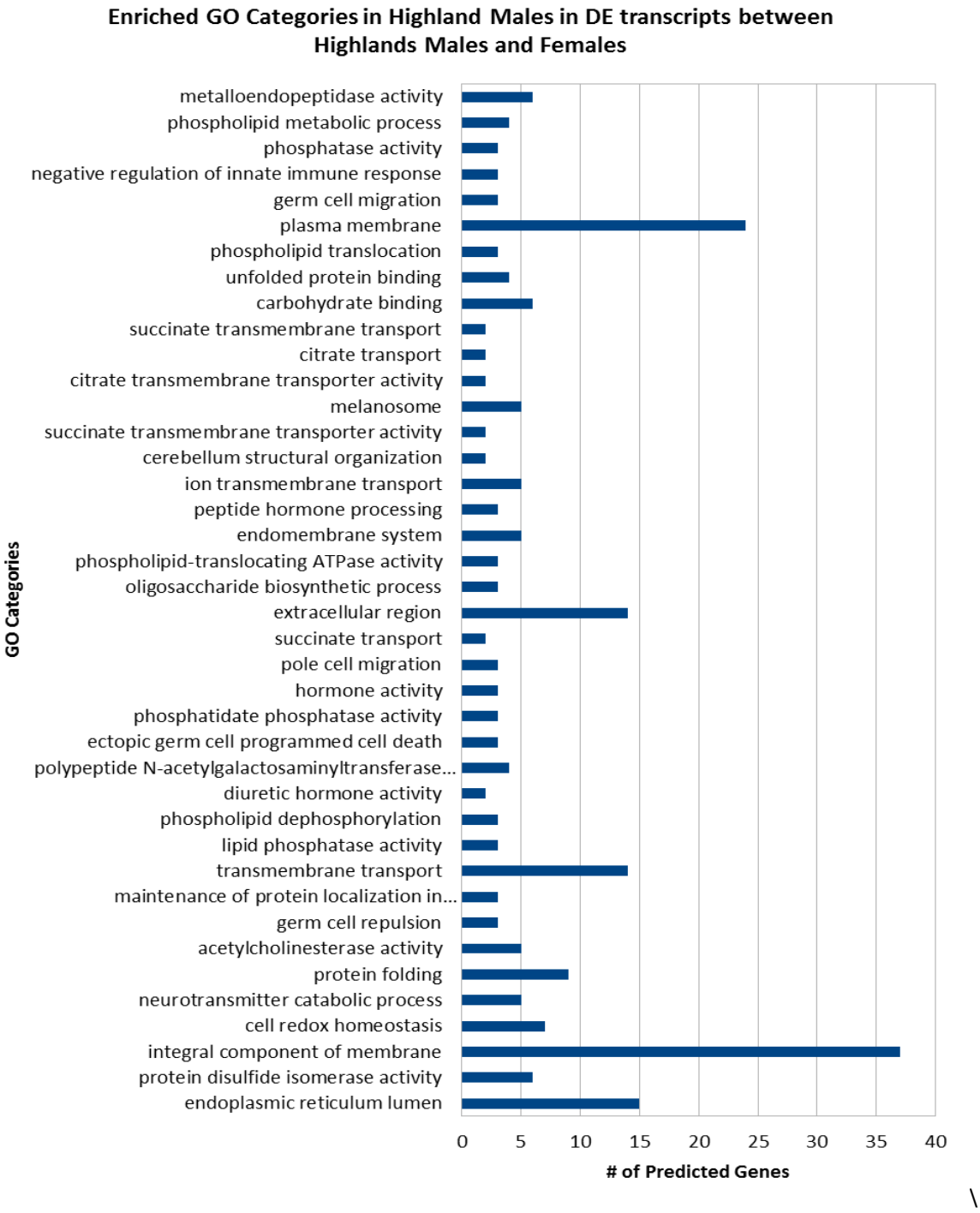


Figure 9. List of over-represented GO categories (FDR<0.05) and the number of predicted genes for each category for Highlands males in the differentially transcripts between Highlands females and males



Discussion

Stable Isotope Analysis

The isotope analysis shows that the $\delta^{15}N$ values of the two *T. versicolor* populations are significantly different suggesting that the *T. versicolor* from the Highlands site are at a higher trophic position than the *T. versicolor* from the Greenville site. Measurements of the plants from both the Highlands and Greenville sites suggest that there is no significant difference in their $\delta^{15}N$ values. Even though *T. versicolor* from the Highlands site had higher $\delta^{15}N$ values than the *T. versicolor* from the Greenville site, I observed the opposite in the $\delta^{15}N$ values for the insects collected. The Lepidoptera and Diptera collected from the Greenville site had $\delta^{15}N$ values of 2.52‰ and 3.76‰ respectively while the Lepidoptera and Diptera collected from the Highlands site had $\delta^{15}N$ values of 1.58‰ and .38‰ respectively. Although it is unclear if these spiders are eating the insects used in this study, the $\delta^{15}N$ values of these insects, along with the $\delta^{15}N$ values of plants, provides evidence that the difference in the $\delta^{15}N$ values between the two populations of *T. versicolor* is not the result of one site having higher or lower levels of ^{15}N than the other but instead is a result of the two populations eating insects that are in different trophic levels.

An interesting result of the isotope measurements was that there was no significant difference in the $\delta^{15}N$ values between males and females which puts into question the idea that males do not eat often once they reached sexual maturity. If this were the case then we would have seen a higher $\delta^{15}N$ in the males since the spiders would have to breakdown their own fats and proteins for sustenance thus leading to elevated $\delta^{15}N$ values (Gannes et al., 1997; Hobson et al., 1993). Also of interest was the $\delta^{15}N$ values for the *T. versicolor* from the Highlands site had more variance than the *T. versicolor* from Greenville site. One possible explanation for this is that the Highlands *T. versicolor* prey on a more diverse array of insect species than the

Greenville *T. versicolor*. However, it could also be interpreted as the *T. versicolor* from both sites feed on an equally diverse amount of insect species, but the insects the Greenville *T. versicolor* feed on are all on the same trophic level.

Transcriptome Analysis

Along with a difference in the transcriptome between the two populations, the results also show that there is not only a sexual dimorphism in the venom transcriptomes but also that male *T. versicolor* are up-regulating more transcripts than the females. This supports the findings of an SDS-PAGE analysis performed by Dr. Greta Binford (2016). The SDS-PAGE showed unique more molecular weight protein bands for male *T. versicolor* than female *T. versicolor*. Unfortunately, little research has been done on sexual dimorphisms in spider venoms, and therefore it is difficult to propose a possible explanation for this. However, sexual dimorphisms in venomous snakes are normally correlated with a difference in diet and size dimorphism. This explanation is not supported by the isotope data which suggests males and females are at the same trophic level which suggests that they are eating the same prey species. However, it is possible that the males are eating different prey species and that these prey species are at the same trophic level as the prey species eaten by the females. It has been suggested by Dr. Binford (2016) that the high molecular weight proteins are involved in the mating ritual of the genus where male and female *Tetragnatha* lock chelicerae but this does not explain the presence of different low and mid molecular weight proteins many of which are likely to be toxins.

Annotation

Of the 501,038 transcripts produced by Trinity only 12% of those transcripts were annotated. One of the reasons for such a low rate of annotation for the transcriptome could be that the genes of the *T. versicolor* are very different from those currently found on GenBank and

therefore do not align well. Although there are currently three spider genomes, *Stegodyphus mimosarum (*Sangaard et al., 2014), *Parasteatoda tepidariorum* (Gendreau et al., 2017), and *Nephila clavipes* (Babb et al., 2017, both *Stegodyphus mimosarum* and *Parasteatoda tepidariorum* are not closely related to *T. versicolor*. *S. mimosarum* belongs to the family Eresidae while *P. tepidariorum* belong to the family Theridiidae. Both families diverged from *T. versicolor* and other Tetragnathida sometime in the Mesozoic era (Garrison et al., 2016). Therefore, although highly conserved genes of *T. versicolor* will likely align with the two genomes, the venom genes of *T. versicolor* may not. While *Nephila clavipes* is more closely related being an orb weaving spider, *N. clavipes* shares a closer common ancestor with the spiders of the Araneidae family than they do with the spiders of the Tetragnathidae family.

Differential Expression Analysis between Greenville female *T. versicolor* and Highlands *T. versicolor*

Numerous transcripts were differentially expressed between the Greenville females and the Highlands females. The GO enrichment analysis of these differentially expressed genes did not result in any significantly overrepresented GO categories for the transcripts overexpressed in the Highlands females but overrepresented GO categories were found for the transcripts overexpressed in the Greenville females. Of the overrepresented GO categories in the Greenville females, the category with the most predicted genes was extracellular space. This is promising because transcripts in this GO category should code for proteins that function outside the cell which suggests that many of these genes could code for venom toxins. A BLAST search of some these transcripts showed that many of these transcripts have leucine rich repeats that show a close resemblance to proteins in the RTN/Nogo gene family. Although it is unclear as to what the function of these proteins are in invertebrates, in humans Nogo (neurite outgrowth inhibitor)

proteins inhibit neuronal growth which restricts the central nervous systems ability to repair itself (Oertle et al., 2003). Another transcript matched for cathepsin B. In humans, cathepsin B is a lysosomal cysteine protease that plays an important role in intracellular protein digestion (Aggarwal and Sloane, 2014). In spiders, cathepsin B plays a similar role in digestion. Research by Fuzuta et al. (2015) suggests that spiders use astracins and trypsins to digest food extra-orally and then use various cathepsins to digest protein intracellularly although cathepsin B has been found in the digestive fluid of spiders that have been starved. However, cathepsin B has never been shown to be expressed in the venom glands. This could be a result of contamination or it could mean that the gene was recruited into the venom transcriptome of *T. versicolor*. Although cathepsin B has not been found in the venom of other spiders, it is used as a venom in the soldier cast of the eusocial aphid *Tuberaphis styraci* (Kutsukake et al., 2004).

Differential Expression Analysis between male and female *T. versicolor* in the Greenville and Highlands sites

The differential expression analysis between the males and females of the two populations not only show that that there is a significant difference in expression, but also that the males are overexpressing more transcripts than the females. This aligns with previous proteome analysis that compared the venom of female and male *T. versicolor* using SDS-PAGE.

The enrichment analysis between the female and male *T. versicolor* did not find any significantly overrepresented GO categories for the Greenville females, but the analysis did find 15 overrepresented GO categories for the Greenville males. Many of these GO categories are involved in chitin and cuticle development. It is likely that the predicted genes in these categories are not involved with the venom gland. Instead, they are likely related to development of the chelicerae and fangs. However, some of these genes could be chitinases which could potentially

be a component of the spider's venom. Sequences that closely align with known chitinases have been found in the venom gland transcriptome of the spider *Loxosceles laeta* (Fernandes-Pedrosa et al., 2008). It is also possible that chitinase found in these *T. versicolor* play a role pre-digesting prey. The other two important GO categories that were overrepresented are extracellular region and extracellular space which are likely to have potential venom genes. A BLAST search of these transcripts resulted is matches for serine protease inhibitors which are commonly found in the venoms of bees and snakes (Kirby et al., 1979; Kim et al., 2013).

A similar result was shown in the GO enrichment analysis of the differentially expressed genes between males and females of *T. versicolor* in the Highlands. While there were no overrepresented GO categories for the females, there were forty-two GO categories that were found to be overrepresented in the males. Extracellular region was one of these categories which contained predicted genes that matched for cysteine inhibitor knot toxins, serine protease inhibitors, and ribonucleases which can all potentially be venom toxins. Another GO category of interest is the metalloendopeptidase activity which could potentially contain venom metalloproteases. Another interesting set of GO categories are those that are involved in phospholipase activity. A BLAST search of these predicted genes resulted in a match with phospholipase A2 which are commonly found in the venoms of bees and certain species of snakes (Sobotka et al., 1976; Harris and Scott-Davey, 2013). The GO categories plasma membrane and integral component of membrane were also overrepresented and contained many predicted genes. Some of these predicted genes could code for cytotoxic venom proteins. Many cytotoxic venom proteins contain hydrophobic regions that allow them to interact with a cell plasma membrane and therefore may share domains with proteins normally found in the cell membrane (Gasanov et al., 2014). Finally, GO categories acetylcholinesterase and

neurotransmitter catabolic process likely contain enzymes, like acetylcholinesterase, that have neurotoxic effects.

The GOseq enrichment analysis of the differentially expressed genes between the male and female *T. versicolor* suggests that there is a difference in venom composition between the sexes. This could result from the fact that males and females have different feeding strategies and may even be eating different prey species. Another possible explanation is that while females spend most of their time at the center of their web waiting for prey, adult males travel from web to web and therefore may experience higher rates of predation. Because of this, males maybe up regulating defense related venom genes. Evidence of this has been observed in spiders in the *Atrax* genus where males produce venom that is more neurotoxic (Atkinson, 1981) and the mouse spider *Missulena bradleyi* where only the male of the species produces venom that is medically significant (Herzig et al., 2008). In both cases, the spiders share a similar life history in that mature males will leave the safety of their burrows in search of a mate (Ibster et al., 2004) and by doing so, increase their chances of predation.

Conclusion

The results of this research provide evidence supporting the existence of geographic variation in the venom composition in the spider *T. versicolor* that coincides with a difference in diet, indicating either local adaptation or phenotypic plasticity associated with dietary differences. The stable nitrogen isotope analysis of the spiders suggests that *T. versicolor* at the Highlands site are at a higher trophic level than the *T. versicolor* at the Greenville site. Also of note is that the nitrogen isotope signature of the Highlands *T. versicolor* is more varied than the nitrogen isotope signature of Greenville *T. versicolor*. This could mean that the Highlands *T. versicolor* have a more varied diet but it could just mean that the Greenville *T. versicolor* only

prey on insect species from a specific trophic level. The results also support the existence of a sexual dimorphism in the venom *T. versicolor* which is backed by previous proteomic analysis of the venom in this species and in other species in the genus *Tetrangtha* (Binford et al., 2016). The isotope analyses of these spiders do not support the existence of a difference in the diets of male and female *T. versicolor* as a possible explanation for this dimorphism despite the fact the that the results suggest that the genes that are being overexpressed in the males are venom related. If it is true that male *T. versicolor* are eating the same prey species as the females then it is possible that the venom genes that the males are overexpressing are not involved in predation but instead serve defensive purposes. This would make sense in this species because the wandering behavior of the mature male *T. versicolor* leaves it more vulnerable to predation than the mature female *T. versicolor.*

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