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# Preliminary Investigation of the Effects of Sex and Electrical Venom Extraction on Venom Composition in the Southern Devil Scorpion, *Vaejovis carolinianus*

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## Introduction

Venom is of particular interest, not only for its medical impact in humans, but also for its therapeutic potential. Many publications have detailed the usage of venoms in treatments of various conditions, such as cancers and inflammation (Gomes, et al., 2010; Vyas, et al., 2013) as well as in current pharmaceutical research (Nunes et al., 2013). However, before venins can be analyzed for their therapeutic potentials, they must be extracted from their host. The choice of extraction technique is typically guided by animal size and ease of access to the venom glands (Besson et al., 2016; Hayes, et al., 2020). However, an issue emerges for small species, where it is difficult to extract large enough quantities of venom and/or where the extraction technique itself may influence venom composition or harm the animal. Therefore, investigative studies into venom extraction, specifically in a manner that maximizes both efficiency and animal safety, are of great importance (Tobassum et al., 2018).

Venom can be obtained by different methods such as manual extraction, venom gland maceration, and electrical stimulation (Hayes, et al., 2020; Oukkache, et al., 2013). However, for smaller animals, such as venomous arthropods, electrical stimulation has emerged as the preferred extraction method, as it typically results in a greater venom collection and a lower

percentage of impurities (Besson et al., 2016; Hayes, et al., 2020; Oukkache, et al., 2013; Tobassum et al., 2018).

Some of the most studied arthropods venoms are those from the class Arachnida, specifically scorpions, ticks, and spiders, due to their medical relevance (Corderio, et al., 2015). However, most studies to date do not adequately describe their milking methods (reporting voltage or current but never both) or report any effects on the health of their specimens. Thus, there is a significant gap in our understanding of how to successfully milk venom from some arthropods while maintaining the health and wellbeing of the animals.

In addition to how venom extraction affects arachnids, their venoms are known to vary in composition, and thus this is also vital to understand if we want to fully utilize these complex chemical cocktails as therapeutics or in the efficient treatment of envenomations. Differences in venom composition, and thus the abundance of certain compounds, is known to vary between sexes (Binford et al., 2016; Herzig et al., 2002), throughout ontogenesis (Barlow et al., 2009; Herzig, 2010), and across populations (De Sousa et al., 2010; Sentenská et al., 2017). Thus, disentangling how the venom extraction technique itself influences venom composition is vital to understand if we want to study how venoms vary naturally and in order to make antivenin that accurately and consistently reflect the natural composition of wild populations.

In choosing a model specimen to study electrical venom extraction, we believe arachnids, and more specifically scorpions, are an ideal candidate. Scorpions are a diverse group of around 2,200 species that have a worldwide distribution and are usually abundant (Lourenco, 2018). Given their unique and memorable morphology, their venom has been relatively well studied (Tobassum et al., 2018). The venom contains many low molecular weight compounds of interest for the medical relevance and industrial therapeutic potential: including enzymes, peptides,

phospholipases, mucoproteins, biogenic amines, and other substances capable of producing pathophysiological effects in victims (Bringans et al., 2008; Santibáñez-López, 2015; Southard, 2016; Tobassum et al., 2018). The Southern Devil Scorpion, *Vaejovis carolinianus*, is a member of the family Vaejovidae and is abundant in its home range of the lower Appalachian Mountain region of the United States, being particularly concentrated in Kentucky, Tennessee, Alabama, and Georgia (Kang and Brooks, 2017; Shelley and Sissom, 1995). We chose to use *V. carolinianus* due to our ability to collect a large sample size of both sexes, and its ease of care.

In this study we investigated the effects of electrical milking on venom expression, body condition, and venom composition, as well as intersexual venom variation in *V. carolinianus*.

## Methods

### *Scorpion Collection and Care*

We collected scorpions from the forest surrounding Southern Adventist University (35°02'57" N, 85°03'21W) from late August through early October ($N$ = 104). Scorpions were housed individually in 16 oz (DeliPRO) containers with a mulch substrate (Miracle-Gro, All Purpose Garden Soil). We watered scorpions *ad libitum* and fed them one ⅜" house crickets (*Acheta domestica*) once every 2 weeks.

### *Electrical Extraction of Venom*

Prior to venom extraction, scorpions were individually moved to 250 mL Narrow Mouth Erlenmeyer Flasks (Pyrex), in which $CO_2$ was introduced before sealing the flask. We kept the scorpion in the container for approximately 10 minutes. We then restrained the scorpion between two foam pieces (dimensions of 2 x 2 in) secured by a rubber band (see Fig. 1). The method used for extraction of venom was electrical stimulation of the telson, following the method described

by Yaqoob et al. (2008) with some modifications. Using featherweight forceps, immersed in a hypersaline solution, we electrically extracted venom by applying a 0.1 amp and 9-volt pulse (power = 0.9 watts) to the base of the telson until venom expulsion ceased. Expressed venom was collected in 5 uL capillary tubes with an internal diameter of 0.44 mm (Drummond, #1-000-005). After each successful collection, the capillary tubes were placed within a 15 ml Falcon tube and continuously stored in a freezer (-20 °C). We labeled and used a unique falcon tube for each sex and extraction attempt (a total of six tubes). Each scorpion was milked a total of three times, with a 14-day interval between each milking to allow for venom regeneration. One group was permitted a 21-day regeneration time to examine the effects of a longer period for regeneration; the condition was later omitted as we could not distinguish between 14- and 21-day regeneration periods in our analysis (data not reported).

**Figure 1.** Restrained Metasoma and Electrified Forceps.

*Protein Quantification*

We pooled venom samples to ensure that the protein concentration in each sample was within the detectable limits of our Agilent 1260 infinity series high-performance liquid chromatography (HPLC) instrument ne and to minimize background noise. Each sample analyzed contained venom from three individuals pooled into a 1.5 mL microcentrifuge tube (Eppendorf). As mentioned above, we attempted to collect venom from each scorpion three times with a 14-day regeneration period between venom extraction. In total, we analyzed six pooled samples from the first milking (e.g. female and male week 1A, 1B, 1C respectively) and six from the second milking (female and male week 2A, 2B, 2C respectively); however, due to the small number of males that released venom during the third milking attempt we only had enough for two pooled samples (female week 3A, 3B, 3C and males 3A and 3B). To create the pooled samples, we removed the capillary tubes containing the venom from the freezer and permitted them to thaw. We then used a microcapillary syringe (Drummond, model 3-000-752) to add the venom to 40 μL of HPLC running buffer A (described below). We centrifuged the microcentrifuge tubes at 13,200 rpm at 4° C for 60 seconds.

We quantified protein concentration using a Thermo Fisher Scientific NanoDrop One Spectrometer measured at 280 nm following manufacturer's guidelines (Thermo Fisher Scientific, 2017). A drop of pure $H_2O$ was used as a blank between each protein quantification run. Once the protein concentration of each sample was determined, we diluted all pooled venom samples to the weakest sample's concentration, so that each pooled sample had a known concentration of 2.380 mg/ml. The microcentrifuge tubes were then placed into a refrigerator (4° C) until they were run on HPLC.

*Chromatography*

We analyzed each sample with reverse-phase HPLC. Each chromatographic run consisted of a 15 μL sample solution loaded onto a ZORBAX 300SB-C8 3.5μm, (Agilent 3.0 x 150 mm), Buffer A was 0.1% trifluoroacetic acid (TFA) in $H_2O$ and Buffer B was 0.1% TFA in acetonitrile. We used a flow rate of 1.5 mL/min at 40 °C. We washed hydrophilic components off the column by running an isocratic 0% B for two minutes, then we ran a shallow linear gradient from 0% to 55% B over 45 minutes, followed by a steeper gradient from 55% to 100% B over 15 minutes, and finally washed our column by holding at 100% B for an additional 10 minutes. The total run time was 75 minutes.

*Statistical Analysis*

To compare the difference in venom expression following successive milkings, we ran a Generalized Mixed Model Omnibus test. Each week, we noted the presence/absence of damage of the aculeus and telson and the viability of each scorpion. Using this data, we ran a Log-rank test to determine if there was a difference in survival between sexes. Separation of the soluble venom components yielded many distinct peaks. We analyzed the chromatographic profiles and manually identified the most consistent peaks. We then used this peak list to identify the most representative chromatogram from each milking attempt and sex as our example chromatogram for subsequent comparisons. Using Agilent ChemStation, we qualitatively analyzed the samples for obvious changes in peak height and the presence and absence of peaks. This preliminary data was used to determine the effects of electrical venom extraction and sex on venom composition.

**Results**

*Effects of Electrical Extraction on Venom Expression and Body Condition*

We noted a significant reduction in the total number of individuals that expressed venom throughout each successive milking ($\chi^2 = 36.63$, $p < 0.001$, df =2, Fig. 2). We also observed damage to, or even the loss of, the aculeus in 11.5% of the scorpions ($N = 12$). Further, 20% of the individuals developed a "charred" appearance to their telson ($N = 21$). We did not detect a significant difference in survival between sexes (z = 0.81, p = 0.416) over the course of this experiment.

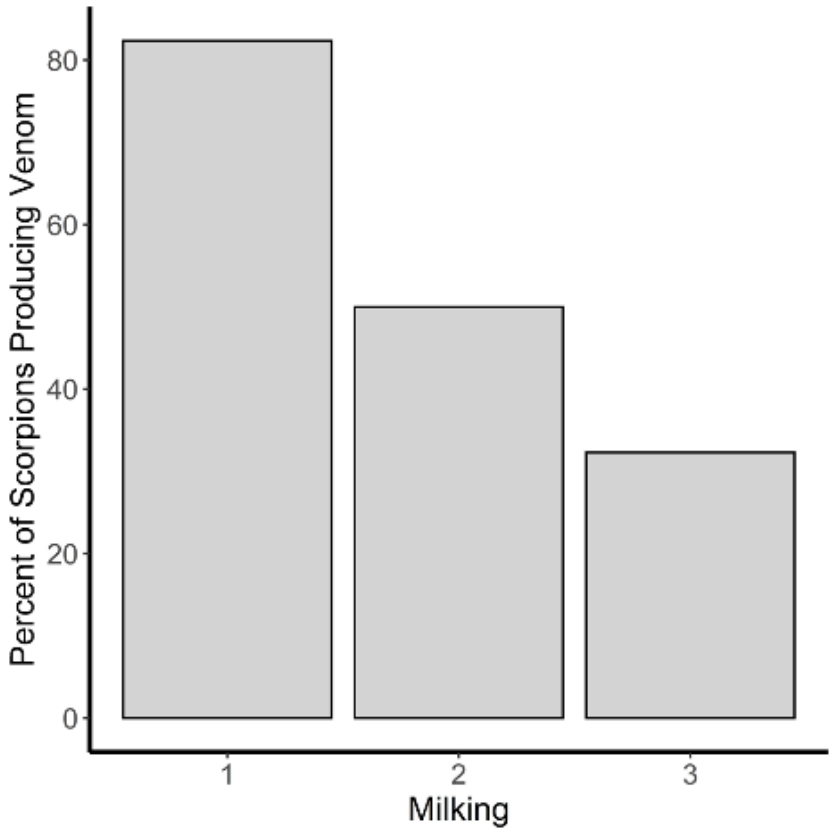


**Figure 2.** Percent of Individuals that Expressed Venom Across Each Milking Attempt.

*Effects of Electrical Extraction and Sex on Venom Composition*

We compared the effects of electrical venom extraction and sex on venom composition (Fig. 2-4). Our results indicate consecutive electrical extraction did not have any obvious effects on venom composition in either male (Fig. 3) or female (Fig. 4) *V. carolinianus*.

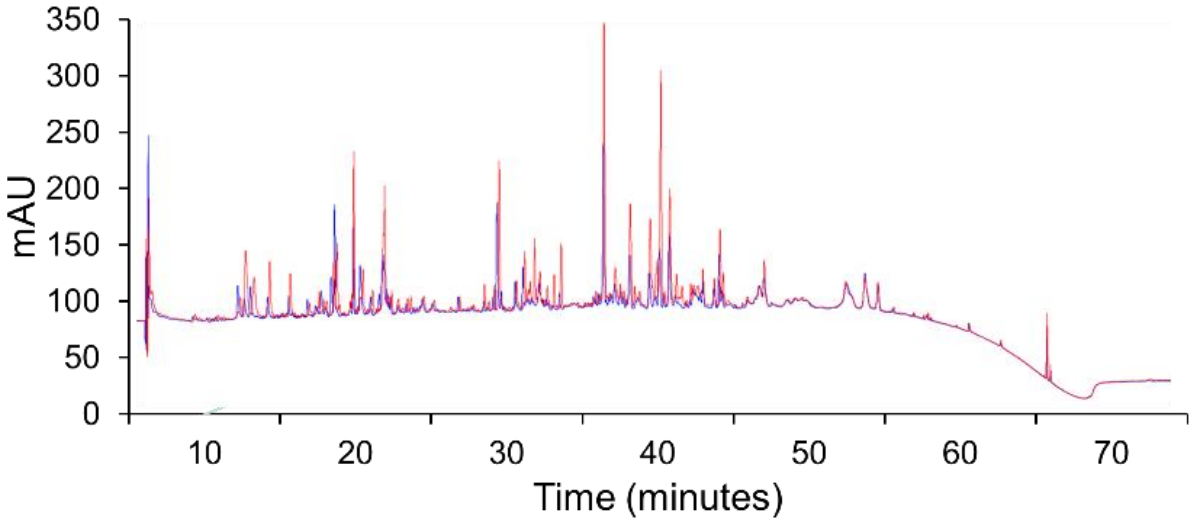


**Figure 3.** Comparison of First (Blue) vs. Second Venom Milking (Red) in Males.

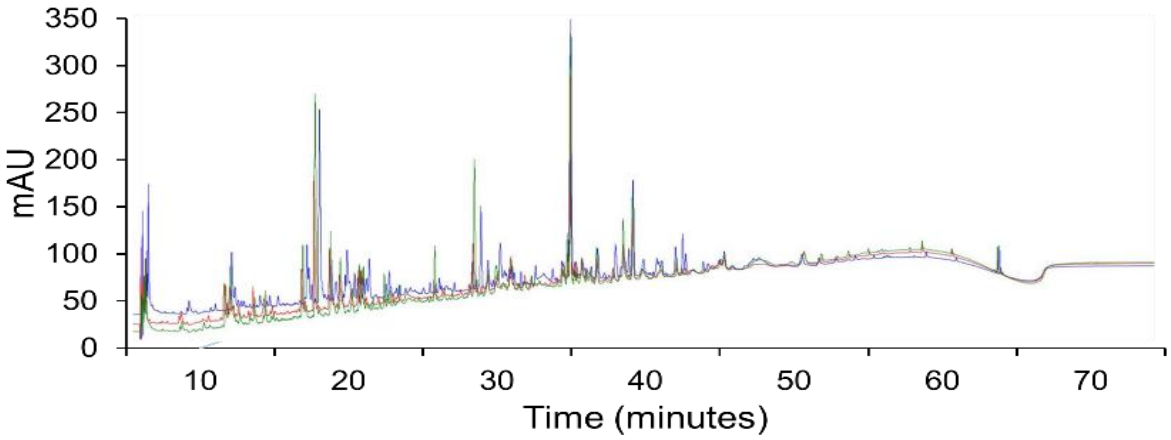


**Figure 4.** Comparison of First (Red) vs. Second (Green) vs. Third Venom Milking (Blue) in females.

However, we did observe consistent qualitative differences in retention times and peak height between sexes in several regions of the chromatograms (Fig. 5).

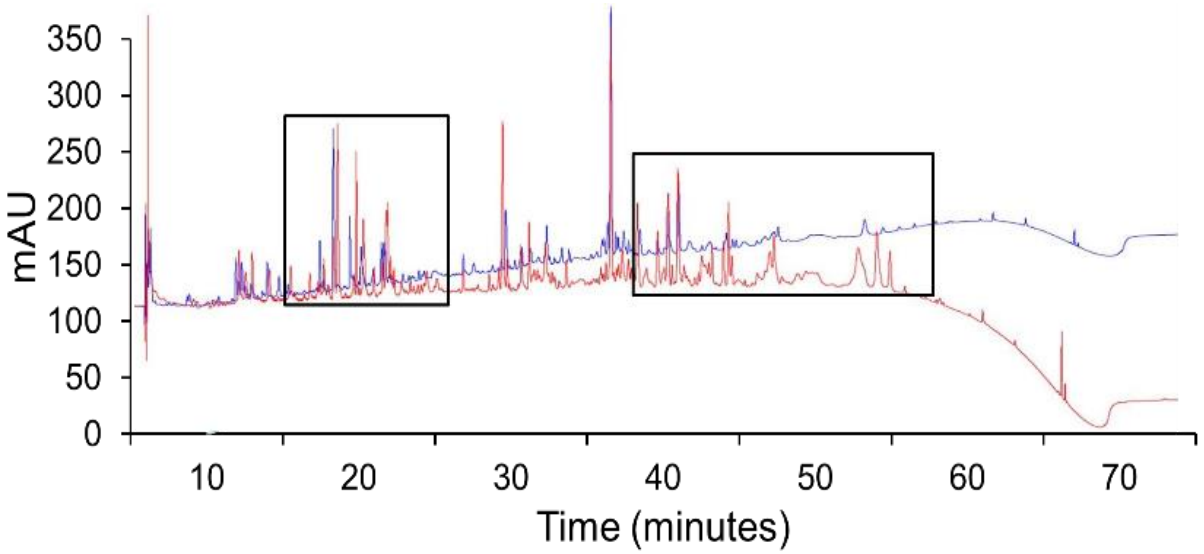


**Figure 5.** Comparison of Average Male (Red) vs. Average Female (Blue) Venom. Rectangles indicate regions where there were consistent differences in retention times and/or absorbance.

## Discussion

In this study we investigated the effects of electrical milking on venom expression, body condition, venom composition, and intersexual venom variation in *V. carolinianus*. We observed variation in venom expression and body condition across successive milkings, we also observed differences in venom composition between the sexes. However, we did not observe variation in venom composition between successive milkings.

### *Effects of Electrical Extraction on Venom Expression*

Although electrical venom extraction has been routinely used for honeybees (Owen, 1978), centipedes (Cooper et al., 2014), spiders (Besson et al., 2016), scorpions (Yaqoob et al., 2016), and snakes (McCleary & Heard, 2010), the effects of electrical venom extraction on venom expression are seldom reported; when they are, differences in how the expression is

measured can make comparisons challenging. Direct comparisons of rates of venom expulsion among different studies, and among different taxa, are also limited due to factors such as variation in testing methodologies, the species' venom repletion rate, and amount of expulsed venom. Like similar studies (Bücherl, 1971; Candido and Lucas, 2004; Yaqoob et al., 2016), we noted that previously unmilked scorpions were the most likely to express venom and that the number of individuals that expressed venom on repeated milkings decreased through each milking attempt.

While figure 2 shows a significant difference in the proportion of scorpions producing venom when compared to each milking attempt, we are unsure as to why a decrease in venom expression is occurring. It is possible that forced electrical extraction of venom damages the venom gland and therefore the number of individuals that express venom in subsequent milkings decreases; this is discussed in the following section. Or, it may be that scorpions can resist the forced expulsion of venom and, even though they have venom to give, i.e. electrical extraction is not sufficient to compel venom expression. This is anatomically demonstrated in centipedes (Cooper, 2014), which possess a mechanism involving a sphincter and nozzle-like non-return valve, that work together to regulate venom discharge from secretory cells. Evidence of dry defensive stings of targets by *Parabuthus transvaalicus* (Nisani & Hayes, 2011) and *Latrodectus hesperus* (Nelsen et al., 2014) further support the likelihood of flexible venom expulsion. As we are unable to determine how electrical milking affected venom expulsion, our results, and those of previous studies detailing similar changes in venom expression, demand further investigations.

*Effects of Electrical Extraction on Body Condition*

Of the commonly used venom extraction methods for smaller animals, electrical stimulation has proven to be the least traumatic (Li et al., 2013) and least likely to introduce contaminants when collecting (Besson et al., 2016; Hayes, et al., 2020; Li et al., 2013; Oukkache, et al., 2013; Tobassum et al., 2018). However, some investigators have proposed that electrical milking may damage the venom glands or venom gland musculature (Cooper et al., 2014; Tobassum et al., 2018). Evidence of this has been observed among spiders, *Argiope bruennichi* quit expressing after one milking attempt (Friedel & Nentwig, 1989). Sissom et al. (1990) proposes this is also true in scorpions, whereas they can only be milked four times, on average, before gland muscles stop responding to electrical stimuli; likewise, Tobassum et al. (2018) observed this to occur after seven-eight consecutive extractions. In one instance, electrical milking proved fatal to the animal (Sahayaraj et al., 2006).

Electrical extraction of venom has also resulted in changes to body condition, as reported in the centipede *Scolopendra polymorpha*, with some receiving blunted tarsungulum while others had no apparent injuries. Like similar studies, we noted observed changes in body condition, with damage or the removal of the aculeus in the scorpions. Imperfect placement of the aculeus within the capillary tube could result in the injury or fragmentation observed. Unlike other studies, we noted roughly a quarter of scorpions developing a “charred” appearance to the location where electric shock (0.9 watts) was applied. We suspect that this “charred” appearance may be dried hemolymph, and thus the extraction technique may have resulted enough damage around the telson to result in bleeding. However, more detailed observations are necessary to substantiate this hypothesis.

In contrast to observations that link electrical extraction with changes in body condition, a few studies have reported repeated electrical milking did not produce permanent injuries

immediately or within 2-3 weeks with scorpions (Gopalakrishnakone et al., 1995) and the scorpion *Hadrurus arizonensis* (Fox et al., 2009). Additionally, studies in the spider *Coremiocnemis tropix* (Herzig, 2010) and snake *Agkistrodon piscivorus conanti* (McCleary & Heard, 2010) also did not result in reduced venom expression or obvious changes in body condition. Thus, we need more information to determine if electrical milking technique can be modified to make it safe for the animal. However, this detailed information is currently lacking in the body of literature on this topic.

Unfortunately, direct comparison of the effects of electrical stimulation on body condition between studies is limited: due to differing methodologies and as previous studies report voltage or current but never both. Additionally, other studies seldom report differences in survivorship between sexes, making our insignificant difference in survivorship challenging to compare.

Presumably, and as proposed by Cooper et al. (2014), we believe that the potential for venom gland tissue, musculature, or exoskeleton damage increases with larger power, voltage-current combinations, relative to body size and will vary depending on where the electrical stimulation is applied. Further, the time the animal is subjected to the shock may be an influencing factor. We believe investigations regarding changes in body conditions of males vs. females are also worthy of future studies; such investigations must consider the influences of their differing physiologies and size.

Although consecutive electrical milking did not appear overly detrimental to *V. carolinianus,* further studies, preferably incorporating microscopic examinations of venom gland tissue, are necessary to determine the mechanisms by which electrical milking leads to the observed damaged and decrease in venom expression we and others have reported.

*Effect of electrical extraction on Venom Composition*

As mentioned above, electrical stimulation is a standard method for venom collection from scorpions (Candido and Lucas, 2004; Fox, 2018; Lowe and Farrell, 2011; Miller; De Sousa et al., 2010; Yaqoob et al., 2016) as well in other arachnid species (Besson et al., 2016; Garb, 2014; Kristensen, 2008; Nagdalian et al., 2018). However, some have suggested that involuntary electrical extractions may damage the venom glands of scorpions and result in variations between compositional analyses (Stahnke, 1978; Yaqoob et al., 2016). Unlike similar studies, we did not note dramatic changes in venom composition across successive electrical extractions, performed at 14-day intervals. Although figures 3 and 4 show obvious differences in peak heights, we were not sure that these differences were reflective of changes due to electrical extraction or were more reflective of variation between samples/chromatographic runs. We observed variation between pooled venom samples within a given milk attempt, even though we used the same reagents, performed tests on the same day, and diluted samples to the same final protein concentration; this was true for both sexes. Therefore, we are not able to determine if and how electrical milking affected venom composition. However, due to the results of previous studies, and the ambiguous nature of our results, we believe that this topic desperately needs further investigation.

It is also important to note that venom regeneration in scorpions has been demonstrated to be asynchronous, with volume being regenerated first followed by protein content over time (Nisani et al., 2012; Pimenta et al., 2003). Although preliminary, our data implies a period of 14-days may be sufficient for complete venom regeneration in this species.

*Effects of Sex on Venom Variation*

Sexual dimorphism in venom is observed in snakes (Furtado et al., 2006), scorpions (Sentenska et al., 2017), and spiders (Zobel-Thropp et al., 2018). Studies of arachnids have reported these variations to be differences in the abundance and of the presence/absence of specific venom components (Binford, 2001; Herzig and Hodgson, 2009). In the qualitative analysis of venom samples, we believe our results demonstrate intersexual differences in the venom composition of *V. carolinianus*; we hold this to be accurate, even given the variation observed between different samples from the same extraction attempt, as discussed above. Because of the run to run variation we observed between samples within a sex and milking attempt we limited our qualitative analysis to obvious presence/absence of peaks, and only the most consistent and obvious changes in peak height. We believe that the differences we found, as depicted in figure 5, showed greater between sex variation than within sex variation. Although we have yet to test this statistically, we are confident that further investigation will substantiate these differences and find additional differences we were too cautious to include.

As *V. carolinianus* exhibits a marked sexual dimorphism (Southard, 2016), we may suggest an ad hoc explanation for the venom variation. It is tempting to propose that a smaller and potentially weaker male would require a more effective venom in defense against potential enemies or prey capture. Research by Miller (2016) demonstrates male venom as being more irritable when injected into a mouse's paw; Miller (2016) further suggested the difference as resulting from a smaller body size necessitating greater venom efficiency. Further research into sex-related differences in venom production may be worthy of future investigations.

The results of our qualitative analysis of the sexual dimorphism of venom demand further investigations into differences in venom gland morphology and genomic expression of *V. carolinianus* that may be responsible for the differences we observed. Additionally, inter- and

intrasexual venom variation between juveniles and juveniles and adults may also be worthy of investigation. As we did not control for the age or mass of the scorpions tested, future studies can address these factors, before differences due to ontogeny occur (Barlow et al., 2009; Herzig, 2010).

*Chemical Properties of Venom and its Potentials for Therapeutics*

Scorpion venom is a complex mixture of polypeptides, lipids, biogenic amines, nucleotides, mucoproteins, and other unclassified substances (Hmed et al., 2013). Of these, peptides standout due to their structural and functional diversity, leading to an array of biological functions when acting on mammalian cells (Ammar & Albalas, 2014; Hmed et al., 2013). Scorpion venom peptides are generally classified into two primary families (Ammar & Albalas, 2014; Quintero-Hernández et al., 2015): disulfide-bridged peptides (DBPs), which typically act on membrane-bound ion channels (Ammar & Albalas, 2014), and non-disulfide-bridged peptides (NDBPs), which display multifunctional activities (Ammar & Albalas, 2014; Zeng et al., 2005).

The NDBPs represent a smaller group, with only 40 identified peptides, that have only recently gained the interest of researchers; a large percentage of these peptide were functionally characterized within the previous decade (Ammar & Albalas, 2014; Hernández-Aponte et al., 2011; Quintero-Hernández et al., 2015). Unlike DBPs, which exhibit conserved structure-function relationships, NDBPs are structurally diverse and act on numerous targets (Ammar & Albalas, 2014). They are composed of 13-56 amino acid residues and display a broad diversity in sequences (Ammar & Albalas, 2014). As revealed either through Circular dichroism spectroscopy studies or by predictions of secondary structures from using bioinformatics, the majority of NDBPs exhibit a cationic amphipathic α-helical structure (Ammar & Albalas, 2014).

Classification into one of three groups is based on the peptide's structural conformation: presentation of a single α-helix domain and two random coiled regions at both C and N termini, two α-helix domains separated by a random coiled region, or 100% helicity. Nonetheless, a characteristic feature displayed by all NDPBs, is that all present unordered random coil conformations when placed in benign conditions, such as in aqueous solution. A transition, however, to solutions containing 50-60% aqueous trifluoroethanol and dodecylphosphocholine micelles, which mimic the membrane environment of cells, results in a rapid and dramatic conformational change to one of the cationic α-helical structures (Ammar & Albalas, 2014). It is this transitional behavior, observed in all studied NDBPs, that reflects their ability to interact with the anionic membrane of cellular targets (Ammar & Albalas, 2014). Further, the net positive charge (1-7 range) carried by the majority of NDBPs, which attracts them to the negatively charged phospholipid heads of the lipid membrane player of target cells by way of electrostatic interaction (Ammar & Albalas, 2014), heightens their ability to interact with cells.

In the mammalian body, the NDBP family of scorpion peptides display a diverse range of activity; unlike DBPs, some even show multifunctional activities without regard to a specific cellular target (Ammar & Albalas, 2014). To date, NDBPs have been found to have hemolytic, anticancer, anti-inflammatory, and immune-modulatory properties (Ammar & Albalas, 2014). The majority of scorpion NDBPs, 37 out of 40, however, are grouped as antimicrobial peptides. One of these peptides, Vejovine, has been isolated from a species within the *Vaejovis* genus (*Vaejovis mexicanus*) (Hernández-Aponte et al., 2011; Sánchez-Vásquez et al., 2013). Vejovine exhibits antimicrobial properties with high specificity for prokaryotic membranes and a unique membrane-disrupting mechanism (Hernández-Aponte et al., 2011). It has been demonstrated to inhibit the growth of several gram-negative multidrug-resistant bacteria known for nosocomial,

or hospital-acquired, infections (*Acinetobacter baumanii*, *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*) (Hernández-Aponte et al., 2011; Samy et al., 2017).

As shown, scorpion non-disulfide bridged proteins exhibit unique conformational behavior that highlights their potential to be used for therapeutics (Ammar & Albalas, 2014; Hernández-Aponte et al., 2011; Quintero-Hernández et al., 2015; Sánchez-Vásquez et al., 2013). It is our opinion that further research should explore scorpion venoms as new sources for potential antimicrobial and broad-spectrum peptides; these agents may constitute important tools in drug discovery or serve as templates for drug design and development of new therapeutic agents.

## Conclusion

Although preliminary, our study strongly suggests that electrical extraction resulted in decreased venom expression and changes in body condition in *Vaejovis carolinianus*. We could not confidently determine if/how electrical extraction affected venom composition; however, we believe that intersexual variations in venom composition is present in this species and will be supported by future investigations.

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