The Design of Complex Weapons Systems in Scorpions: Sexual, Ontogenetic, and Interspecific Variation

Gerard A. A. Fox

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The Design of Complex Weapons Systems in Scorpions: Sexual, Ontogenetic, and Interspecific Variation

by

Gerad A. A. Fox

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biology

June 2018
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ACKNOWLEDGEMENTS

Those who know me understand that I am not much for most emotional-based responses; however this accomplishment would not have been possible without the help, support, and understanding of many people, and that should be acknowledged. It has been a long process to get to this point and without the encouragement of those around me I know that I would not have succeeded. To my parents, who sacrificed that I might make it here, I owe the most. My appreciation for the natural world was instilled from my father, Geoffrey, whom I wish had been well enough to visit me here to see some of what I was doing, or at least seen me finish, but I took too long. My mother, Geneva, has been an unending source of encouragement and aid, without whom I would never have had the will or ability to complete this journey. For the rest of my family there are too many to mention all here but I likewise thank them for their interest, encouragement and understanding.

During my time as a grad student I was taken in by Hilbert Lentz and Elsie Mclellan, a wonderful couple who treated me like family, giving me a place to go, support throughout this experience, and letting my little Miss stay there when she could not be with me in the lab. Bert’s quiet support and Elsie’s enthusiasm for everything I did (even coming with me to collect scorpions on occasion) were ever present.

I am very thankful to the EBS department as a whole for their kindness and willingness to support me during my time here, and had done so much to help me to succeed. Further, I wish to thank my committee for their support: Dr. Zia Nisani without whom I would likely not have worked with scorpions in the first place; Dr. Leonard Brand, for his insistence on considering alternate hypotheses; Dr. Stephen Dunbar, for his
interest and encouragement to continue on; and Dr. Penelope Duerksen-Hughes, for her thought-provoking questions. I especially want thank Dr. William Hayes for his indomitable enthusiasm, patience, encouragement, and friendship. He guided someone who had an active dislike for statistical models to put together a dissertation almost fully dependent upon them, so that now there is at least an uneasy alliance. I also wish to thank my lab mates for their comradery and willingness to brainstorm ideas and occasionally venture into the desert on hot, dry nights collecting fluorescent treasures.
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ABBREVIATIONS

SSD  Sexual Size Dimorphism
SBCD Sexual Body Component Dimorphism
Tot L  Total Length
Pro L  Prosoma Length
Pro W  Prosoma Width
Chela L  Chela Length
Chela W  Chela Width
Chela H  Chela Height
Met 1 L, MS1L  Metasoma Segment 1 Length
Met 1 W, MS1W  Metasoma Segment 1 Width
Met 5 L, MS5L  Metasoma Segment 5 Length
Met 5 W, MS5W  Metasoma Segment 5 Width
Tel L  Telson Length
Tel W  Telson Width
Tel H  Telson Height
Pec L  Pectine Length
PCA  Principal Component Analysis
PC1  Principal Component 1
PC2  Principal Component 2
ANCOVA  Analysis of Covariance
MANCOVA  Multiple Analysis of Covariance
DFA  Discriminant Function Analysis
<table>
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<th>SMA</th>
<th>Standard Major Axis</th>
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<td>SVL</td>
<td>Snout Vent Length</td>
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ABSTRACT OF THE DISSERTATION

The Design of Complex Weapons Systems in Scorpions:
Sexual, Ontogenetic, and Interspecific Variation

by

Gerad A. A. Fox

Doctor of Philosophy, Graduate Program in Biology
Loma Linda University, June 2018
Dr. William K. Hayes, Chairperson

Scorpions possess two integrated multifunctional weapons systems. Anteriorly, they maintain a grasping system comprised of a pair of pedipalps ending in chelae that seize and manipulate prey, ward off predators, and secure mates. Posteriorly, they wield a venom delivery system consisting of a tail-like metasoma with a stinger at the tip of the terminal segment (telson) that can be thrust into prey, predators, or mates to inject venom. Given the complexity of these systems, I hypothesized that weaponry design is subject to selective forces arising from differences in usage between the sexes, during ontogeny, and among closely-related species occupying different habitats. In the first of three studies, I examined sexual dimorphism in the North American scorpion *Hadrurus arizonensis* to develop a suitable statistical approach for disentangling sexual size dimorphism (SSD) and sexual body component dimorphism (SBCD), and to characterize the allometry of weaponry components. In the second study, after thoroughly reviewing the literature on venom yield in scorpions, I relied on the methodology developed in the first study to characterize venom availability in *H. arizonensis*. Venom yield was strongly and exponentially related to overall body size and weakly proportional to relative telson size. Venom protein concentration was weakly and negatively associated with body size,
and slightly greater in females than in males. In the third study, I examined both weapon systems of two sister Smeringurus species that co-occur with H. arizonensis but occupy distinct habitats: the psammophile S. mesaensis and the lithophile S. vachoni. Males trended toward more robust chela, especially in S. vachoni. Metasoma length averaged longer in males of both species, but the telson was larger and the venom supply greater in females. Venom availability increased exponentially during ontogeny for both species. Smeringurus vachoni possessed significantly larger venom stores than S. mesaensis. Sexual and species differences likely result from different selective regimes related to survival and reproductive demands, priority in securing mates, and possibly population density and cannibalism. These findings highlight the multiple factors that influence weapons design in scorpions, and underscore the functional importance of these complex systems that are relied upon in varying roles and contexts.
CHAPTER ONE
INTRODUCTION

Scorpions represent an ancient taxa, with fossil representatives displaying a consistent body plan dating back to the Silurian, and fossil forms from the Carboniferous differing little from modern species (Dunlop et al., 2008; Jeram, 2001; Lourenço, 2015). Scorpions inhabit diverse terrestrial habitats across the globe, ranging from shorelines to mountains, and including forests, grasslands, and desert ecosystems. Although scorpion diversity is somewhat limited, with roughly 2000 extant species (Borges and Graham, 2016; Lourenço, 2015; Soleglad and Fet, 2003), up to twelve species have been identified at a single location (Due and Polis, 1986; Jimenez-Jimenez and Palacios-Cardiel, 2010). Their density can be high, especially in semi-arid and arid ecosystems where >3,200 individuals/ha have been observed (Due and Polis, 1985; Fet et al., 1998; Polis, 1990; Williams, 1969). Scorpions appear well suited to desert environments, possessing an impermeable cuticle that limits water loss, and a low metabolic rate that minimizes energy demands (Hadley, 1990; Warburg and Polis, 1990). Scorpions tend to be generalist predators that employ a sit-and-wait strategy (Formanowicz et al., 1991; Kaltsas et al., 2008; Polis, 1990; Skutelsky, 1995), allowing their prey to come to them, thereby limiting energy output; however, some species may actively pursue prey (Formanowicz et al., 1991; Polis, 1990; Skutelsky, 1995).

Scorpions possess two integrated multifunctional weapons systems. Anteriorly, they possess a grasping system comprised of a pair of pedipalps ending in chelae that seize and manipulate prey, ward off potential predators, and secure potential mates.
Posteriorly, they wield a venom delivery system consisting of a tail-like metasoma with a
stinger at the tip of the terminal segment (telson) that can be thrust into prey items,
predators, or mates to inject venom. Upon encountering potential prey, scorpions tend to
follow a stereotypical pattern of behaviors, highly conserved across genera, which
involves their pedipalp chelae and often a venomous sting to subdue their live prey (Bub
and Bowerman, 1979; Casper, 1985; Rein, 1993; 2003; Stewart, 2006). Similar
stereotyped behaviors also occurs in defensive contexts, wherein the scorpion may flee or
defend itself using its chelae and sting (Carlson et al., 2014; Heatwole, 1967; Newlands,
1969; Nisani and Hayes, 2011; van der Meijden et al., 2013). Interplay between these
weapons makes scorpions formidable members of the communities they occupy, and aid
in their success. Given the complexity of these systems, we can hypothesize that
weaponry design should be subject to selective forces arising from differences in usage
between the sexes, during ontogeny, and among closely-related species occupying
different habitats.

The purpose of this dissertation is to characterize the weapon systems in two
representative scorpion genera, and to test hypotheses that relate to their design. To
achieve these goals, I first grappled with the issue of overall body size and sexual
dimorphism—the differences in morphology that exist between males and females. Both
of these attributes must be taken into account when comparing weapons among different
groups. Sexual dimorphism in scorpions can exist in virtually every body part. Using a
novel statistical approach, I was able to identify a relatively unbiased measure of overall
body size, which was equivalent in males in females, and used this to control for body
size in subsequent analyses. Next, I evaluated the venom delivery system of H. 
arizonensis to determine whether sexual differences and other influences on design exist, particularly for venom yield. Finally, using the morphological, venom extraction, and statistical approaches developed for H. arizonensis, I examined both weapons systems—venom delivery and the pedipalps/chelae—in two sister species of the genus Smeringurus that inhabit different environments.

Identifying Sexual Dimorphism

Most studies that document scorpion dimorphism have reported differences in one or several body components, or their ratios, usually within the context of taxonomic descriptions. Although these measures have their place in the literature, and greatly ease rapid identification of species or sex, they may lead to wrong inferences or spurious correlations (Jackson and Somers, 1991), and cannot be used to discern which particular feature or body part might be under the influence of selection. When one sex is larger overall than the other, differences in body components may simply reflect this bias. In one notable example, the conventional interpretation that sexual selection favors large male head size relative to overall length of lizards has been reinterpreted as fecundity selection favoring, instead, a larger trunk in females (Kratochvíl et al., 2003; Scharf and Meiri, 2013). Thus, more refined approaches are required to understand the selective pressures that generate or maintain dimorphism. And even with a better approach, differentiating the influences of natural and sexual selection on individual body components can be especially challenging (Pélabon et al., 2014; Shingleton and Frankino, 2012).
Multivariate statistical methods, such as regression and analysis of covariance, are ideally suited for examining dimorphism and character scaling, as they can better normalize data, control for confounding variables, and are far more sensitive for evaluating subtle characters (Packard and Boardman, 1999) that may still be under the control of natural or sexual selection. Potentially dimorphic characters or deviations from isometry, are often identified by controlling for one body component, which acts as an overall indicator of general body size, followed by evaluation of how each body component of interest responds to changes in body size. The optimal scenario is to use a reference character that correlates with size, is independent of nutritional state (van der Meijden et al., 2012), and is itself non-dimorphic (Kratochvíl et al., 2003). However, the choice of an appropriate reference character can be fraught with difficulty (Braña, 1996; Kratochvíl et al., 2003; Prenter et al., 1995; Scharf and Meiri, 2013; Suter and Stratton, 2011), and may require the measurement of numerous body components. Choice of a reference character for body size can profoundly affect the assessment of dimorphism and its interpretation.

In Chapter two, I used discriminant function analysis (DFA) to identify which among 16 covarying body components were most and least discriminating between the sexes of *H. arizonensis*. The DFA approach indicated that metasoma segment 1 width was the least biased (most appropriate) measure of overall body size. I compared this character to alternative reference characters for overall body size (prosoma length, prosoma area, total length, and principal component 1 from a principle component analysis), and showed that identification of sexual size dimorphism (SSD, differences in overall size between the sexes) and sexual body component dimorphism (SBCD,
differences in individual body components between the sexes) depended on which character was used as the reference. My findings were consistent with the conclusions of others that fecundity selection likely favors a larger prosoma in female scorpions, whereas sexual selection may favor other body parts being larger in males, especially the metasoma, pectines, and possibly the chela. Although this study underscored the need for researchers to avoid conflating SSD and SBCD, and to broaden their consideration of an appropriate reference character to overall body size, the most practical outcome was identifying a body component that could be used for comparative analyses of weapons design in my subsequent studies.

**Examining the Venom Delivery System**

Most studies relating to design of the venom delivery system have focused on venom yield and composition. Few, by comparison, have evaluated the grasping (pedipalp/cheliped) system (Simone and van der Meijden, 2017; van der Meijden et al., 2013; 2012; 2010). Scorpions comprise a good model system to evaluate the factors that influence venom availability (yield) and composition: They can often be collected in large numbers (Polis, 2001; 1990), maintained in captivity at low cost with relative ease (Brenes and Gómez, 2016; Bücherl, 1953; Candido and Lucas, 2004; Gopalakrishnakone et al., 1995; Whittemore et al., 1963), and as invertebrates require minimal institutional oversight. The venom supply is maintained within paired glands housed in the telson, which is the terminal segment of the tail-like metasoma (Hjelle, 1990). The telson terminates in a pointed tip, the aculus, which can be thrust into the soft tissues of prey or potential predators functioning as an hypodermic needle delivering venom into the target...
(Hjelle, 1990). In some species venom may even be delivered by spraying an attacker (Newlands, 1974). As in other venomous animals (Cooper et al., 2015; Hayes et al., 2002; Nelsen et al., 2014; Wigger et al., 2002), scorpions are able to control venom expenditure, metering doses relevant to the situation (Bub and Bowerman, 1979; Casper, 1985; Edmunds and Sibly, 2010; Nisani and Hayes, 2015; 2011; Rein, 1993). Because the amount of venom expended during stings and sprays is influenced, in large part, by the quantity of venom available, as well as the duration and rate at which venom is expelled (van der Meijden et al., 2015), knowledge of venom yields can be helpful in understanding the strategies used during venom deployment, selection acting on design of the system, the regimens used for sustainable venom production, and the medical risks associated with scorpionism.

In Chapter three, I reviewed our understanding of venom yield in scorpions. I began by describing the various methods of venom milking or extraction, and then summarized what we know about the many factors that potentially influence venom synthesis and yield. These factors include a host of internal (e.g., genetics, age, sex, body size, health, reproductive state, recent usage, regeneration rate, production costs) and external (e.g., season, temperature, humidity, prey availability, prey size, prey susceptibility to venom) influences. Although a large body of research exists on scorpion venom, with most work looking at the biochemistry and mode of action, those studies that provide details on venom yield are almost entirely descriptive, without any examination of the factors that influence venom availability. Thus, few generalizations can be made, which underscores the need for renewed attention to venom yield.
In Chapter four, I examined how body size and other variables affect volume yield and protein concentration of electrically extracted venom in *H. arizonensis*. Venom yield was strongly and exponentially related to overall body size and weakly proportional to relative telson size, but was similar for the two sexes, independent of relative mass (body condition), and similar for the two milking groups (season and/or duration in captivity). Compared to venom yield, venom protein concentration was much less dependent on overall body size, though there was a weak negative relationship. Protein concentration varied most among the milking groups (declining with duration in captivity and/or shift from fall to winter), and to a lesser extent between the sexes (greater in females than in males), with relative telson size and body condition having no measurable influence. When individual scorpions were subjected to repeated venom extractions at 21-day intervals, each extraction resulted in consistent volume yields, but reductions in protein concentrations were evident over time. These findings offer meaningful insights regarding the constraints on venom deployment and weapons design by scorpions, appropriate milking regimens for sustainable venom production, and the medical risks and symptoms associated with scorpionism.

**Examining Weapons Design in Two Sister Scorpion Species**

To build upon the knowledge acquired from detailed study of *H. arizonensis*, I turned my attention toward additional species, and expanded the level of analysis to include, within a single study, both weapons systems, sexual differences, and ontogenetic influences. I therefore searched for a group of scorpions in which I could test my hypothesis that weaponry design is potentially subject to selective forces arising from
differences in usage during ontogeny, between the sexes, and among closely-related species occupying different habitats. If selection is important, statistical differences with reasonably large effect sizes should exist.

In Chapter five, I compared the design of both the venom delivery and the pedipalp/chelae weapons systems of two sister species in the genus *Smeringurus*. These two taxa occupy very different environments: *S. mesaensis* is a psammophile (sand dweller), whereas *S. vachoni* is largely a lithophile (rock-associated dweller). I showed that SBCD existed in physical weaponry, and was most exaggerated for adults of each species. Males trended toward more robust chela, especially in *S. vachoni*. Metasoma length averaged longer in males, with *S. mesaensis* demonstrating greatest divergence. The telson housing the chemical weapon stores was larger in females of both species, as was the venom volume. Venom availability increased exponentially during ontogeny for both species. Although both species were of similar adult size, *S. vachoni* possessed significantly larger venom stores. Differences in weapon design likely result from differential allocation of resources and different selective regimes both within and among these species. Female-biased venom supply is associated with survival and increased reproductive demands, whereas male investment in the chela and metasoma could represent greater priority in securing mates. In the dense populations of *S. mesaensis*, adult males seldom live beyond a single breeding season, and the exaggerated metasoma length may help ward off cannibalistic females. The robust and modified chela of male *S. vachoni* may aid in securing mating opportunities where fewer opportunities exist at lower population density.
**Novel Insights**

My studies have advanced our understanding of numerous aspects of scorpion biology. First, as a matter of necessity arising from the complex body design of scorpions, I developed a statistical approach that can be used to disentangle the properties of SSD and SBCD. Such measurements previously were often conflated, and many wrong conclusions have been reached—for many different taxonomic groups—regarding the presumed influences of natural selection and sexual selection on individual body parts. This insight in particular extends well beyond our understanding of scorpions. Second, I have provided the most detailed characterization of venom yield and the factors that influence it in scorpions. Third, whereas virtually all prior studies have focused on just a single weapon system in scorpions, my research takes the most integrated approach to date to evaluate both weapons systems simultaneously using a modern comparative approach. Collectively, these findings highlight the multiple factors that influence weapons design in scorpions, and underscore the functional importance of these complex systems that are relied upon in varying roles and contexts.


CHAPTER TWO
THE DILEMMA OF CHOOSING A REFERENCE CHARACTER FOR
MEASURING SEXUAL SIZE DIMORPHISM, SEXUAL BODY COMPONENT
DIMORPHISM, AND CHARACTER SCALING: CRYPTIC DIMORPHISM AND
ALLOMETRY IN THE SCORPION HADRURUS ARIZONENSIS

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This chapter has been published under the following citation:
Abstract

Sexual differences in morphology, ranging from subtle to extravagant, occur commonly in many animal species. These differences can encompass overall body size (sexual size dimorphism, SSD) or the size and/or shape of specific body parts (sexual body component dimorphism, SBCD). Interacting forces of natural and sexual selection shape much of the expression of dimorphism we see, though non-adaptive processes may be involved. Differential scaling of individual features can result when selection favors either exaggerated (positive allometry) or reduced (negative allometry) size during growth. Studies of sexual dimorphism and character scaling rely on multivariate models that ideally use an unbiased reference character as an overall measure of body size. We explored several candidate reference characters in a cryptically dimorphic taxon, *Hadrurus arizonensis*. In this scorpion, essentially every body component among the 16 we examined could be interpreted as dimorphic, but identification of SSD and SBCD depended on which character was used as the reference (prosoma length, prosoma area, total length, principal component 1, or metasoma segment 1 width). Of these characters, discriminant function analysis suggested that metasoma segment 1 width was the most appropriate. The pattern of dimorphism in *H. arizonensis* mirrored that seen in other more obviously dimorphic scorpions, with static allometry trending towards isometry in most characters. Our findings are consistent with the conclusions of others that fecundity selection likely favors a larger prosoma in female scorpions, whereas sexual selection may favor other body parts being larger in males, especially the metasoma, pectines, and possibly the chela. For this scorpion and probably most other organisms, the choice of reference character profoundly affects interpretations of SSD, SBCD, and allometry. Thus, researchers need to broaden their consideration of an appropriate reference, and
exercise caution in interpreting findings. We highly recommend use of discriminant function analysis to identify the least-biased reference character.

**Introduction**

The morphology of animals can be shaped by both natural selection and sexual selection (Darwin, 1871; 1859). Natural selection favors morphologies that enhance growth, reproduction, and survival, resulting in increased fitness for a given environment. Sexual selection favors morphologies that facilitate mating success via intrasexual competition, intersexual mate choice, and post-copulatory success (Andersson, 1994; Eberhard, 1996; Simmons, 2001). Sexual dimorphism—the different appearances of females and males of the same species—can arise from either of these adaptive processes, but it may also result from non-adaptive processes such as body-size scaling, genetic correlations between female and male body size, and phylogenetic constraints or inertia (Cox et al., 2003; Fairbairn, 1990; Gosnell et al., 2009; Stillwell and Fox, 2007). Sexual dimorphism can encompass an overall increase in size of one sex over the other (sexual size dimorphism, SSD), or it can be restricted to certain body parts, affecting their size, shape, or both (sexual body component dimorphism, SBCD). To distinguish between effects on overall size and effects on the size or shape of individual components (≈characters) that may or may not result in overall size differences, we introduce the latter term.

Dimorphism can also be considered from the perspective of allometry, as both often exist at the interface of natural and sexual selection. Allometry describes how body characters interact over the size range of an organism. Differential scaling of individual
features results as selection favors either exaggerated (positive allometry) or reduced (negative allometry) size of some body components as body size increases, whereas others may remain proportional (isometry). Differences in scaling result from several interacting forces, including the physics of the structural shape in relation to the physical properties of the materials (McMahon, 1975; Ravosa et al., 2000), and biological considerations of optimal use under natural selection with sometimes confounding effects of sexual selective pressures (Bonduriansky and Day, 2003; Eberhard et al., 1998; Green, 2000). Whereas most characters follow negative allometry or isometry (Bertalanffy and Pirozynski, 1952; Bonduriansky, 2007; Eberhard, 2002), characters shaped by sexual selection often exhibit strongly positive allometries (Emlen and Nijhout, 2000; Green, 1992; Petrie, 1992; Tomkins and Simmons, 1996). However, the preponderance of sexual characters with positive allometries in the literature may be biased by extensive examination of exaggerated or extreme examples (Bonduriansky, 2007). Indeed, a recent literature review demonstrated that many sexual signals, weapons, and other sexual traits exhibit isometry or even negative allometry. Thus, because positive allometry may actually occur in a minority of sexual traits, sexual selection alone may be insufficient to produce a positive allometric trend, and the presence of positive allometry may not be indicative of sexual selection (Bonduriansky, 2007; Cuervo and Moller, 2009; Outomuro et al., 2014; Schulte-Hostedde et al., 2011).

A growing body of literature documents sexual differences in overall size and/or body component proportions of numerous animal species. This has certainly been the case for scorpions, although few authors have established a single measure or set of measures of overall body size, on average female scorpions show larger body sizes in
terms of area or mass (Polis and Sissom, 1990); however, total length is often skewed
toward males due to their often more elongate metasoma segments (van der Meijden et
al., 2010). The exaggerated size of the pectines in males represents the most consistently
dimorphic body component, resulting from an increase in both the number and size of the
pectinal teeth (Polis and Sissom, 1990). Pectines comprise sensory organs that detect both
physical (Kladt et al., 2007) and chemical cues from the substrate (Gaffin and Brownell,
1997; Steinmetz et al., 2004; Taylor et al., 2012). The enhanced pectines of males are
associated with mating, as they can follow the pheromonal trails laid down by females
(Gaffin and Brownell, 1992; Melville et al., 2003; Miller and Formanowicz, 2010) and
assess appropriate substrates for spermatophore deposition (Abushama, 1968; Alexander,
1957; Jiao and Zhu, 2009a; Melville, 2000; Tallarovic, 2000).

Several other body parts are frequently dimorphic in scorpions. The variably
modified chelae structure of males (Benton, 1991; Booncham et al., 2007; Kovařík et al.,
2010; 2011) presumably aids in holding the female during the mating dance (promenade
aux deux) (Benton, 1992; Peretti et al., 2001). The more elongate metasoma of males
(Francke and Jones, 1982; Graham et al., 2012; Koch, 1977; Sánchez-Quirós et al., 2012)
potentially facilitates a sexual sting, fencing (le arbre droit), clubbing, and maybe even
sexual identification while maintaining distance from a potentially aggressive female
(Carlson et al., 2014; Polis and Sissom, 1990). Sexual differences in prosoma and
mesosoma size and shape may relate to the female’s role of producing and carrying
offspring (Brown, 2004; Formanowicz and Shaffer, 1993; Francke, 1981; Lourenço et al.,
1996; Outeda-Jorge et al., 2009). The functions of other occasionally dimorphic traits
remain less clear, including differences in the telson and aculus shape (Booncham et al.,
2007; Lourenço and Duhem, 2010), and in the presence of male accessory glands (e.g., subacicular glands in several scorpion species Peretti, 1997; Williams, 1970) and the acular bulb in mature male Anuroctonus (Soleglad and Fet, 2004; Williams, 1966)).

Most studies that document scorpion dimorphism have reported differences in one or several body components, usually within the context of taxonomic descriptions. Often, the differences have been expressed by comparing the range of values for females and males, or the ratios for a single body part (e.g., length-to-width) to one or more other components (e.g., prosoma length, metasoma segment 5 length; Stahnke, 1970). Although these measures have their place in the literature, and greatly ease rapid identification of species or sex, they may lead to wrong inferences or spurious correlations (Jackson and Somers, 1991), and cannot be used to discern which particular feature or body part might be under the influence of selection. When one sex is larger overall than the other, for example, differences in body components may simply reflect this SSD. And in the classic case for ratios, the conventional interpretation that sexual selection favors large male head size relative to overall length of lizards has been reinterpreted as fecundity selection favoring, instead, a larger trunk in females (Kratochvíl et al., 2003; Scharf and Meiri, 2013). Thus, more refined approaches are required to understand the selective pressures that generate or maintain dimorphism, and even then differentiating the influences of natural and sexual selection on individual body components can be especially challenging (Pélabon et al., 2014; Shingleton and Frankino, 2012).

Statistical methods such as analysis of covariance and regression are ideally suited for examining dimorphism and character scaling, as they can better normalize data,
control for confounding variables, and are far more sensitive for evaluating subtle characters (Packard and Boardman, 1999) that may still be under the control of natural or sexual selection. Potentially dimorphic characters or deviations from isometry are often identified by controlling for one body component, which acts as an overall indicator of general body size, followed by evaluation of how each body component of interest responds to changes in body size. The optimal scenario is to use a reference character that correlates with size, is independent of nutritional state (van der Meijden et al., 2012), and is itself non-dimorphic (Kratochvíl et al., 2003). However, the choice of an appropriate reference character can be fraught with difficulty (Braña, 1996; Kratochvíl et al., 2003; Prenter et al., 1995; Scharf and Meiri, 2013; Suter and Stratton, 2011), and may require the measurement of numerous body components. Choice of a reference character for body size can profoundly affect the assessment of dimorphism and its interpretation.

Here, we address the difficulties associated with measuring sexual dimorphism and character scaling through rigorous analyses of morphological variation in the desert hairy scorpion, *Hadrurus arizonensis*. Specifically, we used several alternative reference characters to evaluate SSD and SBCD for 16 morphological characters. We also assessed sexual differences in the static allometry of multiple body components to better understand their relationships to sexually dimorphic traits and the potential selective forces that shape them.

The desert hairy scorpion has long been viewed as non-dimorphic in characters other than the pectines (Stahnke, 1969; 1945). Although Williams (1970) mentioned that adult males have a longer metasoma than females, Stahnke (1971) questioned the finding, and called for a more robust analysis beyond the raw data, including the use of ratios and
statistical tests for comparison. Tallarovic (2000) indicated there was no exaggerated
dimorphism. While collecting specimens for other studies, one of us (GAF) became
convincing that cryptic dimorphism existed in the species. The methodology presented
here not only confirmed this suspicion, but should be useful for assessing sexual
dimorphism and allometry in other scorpions. As our findings indicate for this scorpion,
and probably for most other organisms, the choice of reference character can profoundly
affect interpretations of SSD, SBCD, and the ways in which selection might act on these
traits.

**Materials and Methods**

*Ethics Statement*

All methods in this study complied with the requirements of the Institutional
Animal Care and Use Committee of Loma Linda University, which regulates animal
research at this institution. At the time of the study, no protocol reviews or permits were
required for any studies of invertebrates. However, the research met the ethical and
academic integrity policies set forth by the Office of Research Affairs, and was reviewed
and approved by the Faculty of Graduate Studies. This study also complied with federal
and state laws, as *H. arizonensis* is not an endangered or protected species, and
collections were made from public lands, where no permits or permissions were required
for the activities performed.
Scorpions

We collected adult specimens of *H. arizonensis* from the western Sonoran Desert between Cabazon and Whitewater, Riverside County, California, USA (33.898354, -116.682936; 33.910966, -116.651685). We captured them at night during the months of July to October using ultraviolet light sources (Stahnke, 1972). We acquired a sample of 184 adult scorpions consisting of 90 males and 94 females (81.2–111.7 mm overall body length).

Morphological Measurements

Using electronic calipers, we measured to the nearest 0.1 mm the following characters (Fig. 1): total length (Tot L, edge of prosoma to end of metasoma); prosoma length (Pro L) and width (Pro W, at median eye); chela length (Chela L), width (Chela W), and height (Chela H); metasoma segments 1 and 5 length (Met 1 L, Met 5 L) and width (Met 1 W, Met 5 W); total metasoma length (Met L); length (Tel L), width (Tel W) and height (Tel H) of the telson; and pectine length (Pec L) (Stahnke, 1970). We visually determined sex by relative length and arrangement of the pectines. We could have measured numerous additional characters reported in other studies (e.g., femur, patella, and other chela dimensions), but focused on what we believed were the most frequently reported dimorphic characters in scorpions. A secondary consideration was that the chosen measures could easily and reliably be done in the field for future comparisons. Although we measured mass and mesosoma size, we chose not to analyze these characters because both vary substantially with nutrition (Brown, 2001; van der Meijden et al., 2012; 2013). Taking measurements caused no apparent injury to the animals.
Figure 1. Morphology of representative Desert Hairy scorpion (*Hadrurus arizonensis*). Body components measured in this study are labeled.

Statistics

Prior to all statistical tests, we screened the data to verify compliance with parametric assumptions. We removed a small number of statistical outliers (studentized residuals >1.96) for specific body components while retaining other measurements of those individuals. Unless specified otherwise, statistical tests were conducted using SPSS.
20.0 for Macintosh (Statistical Package for the Social Sciences, Inc., Chicago, 2011), with $\alpha = 0.05$. Following Nakagawa (Nakagawa, 2004), we chose not to adjust $\alpha$ for multiple tests. As an intuitive indicator for the magnitude of sex differences, we computed the percent difference for all characters analyzed (c.f. (Lovich and Gibbons, 1992; Smith, 1999)) using the mean of each sex (i.e. [male – female] divided by 0.5 [male + female]).

We subjected the morphological measurements to five sets of analyses involving parametric tests (Mertler and Vannatta, 2009; Field, 2009). Although pectine length and arrangement were used to determine sex, we elected to include Pec L in some analyses for comparative purposes, but omitted it from several analyses, as specified below.

First, we directly compared all body size components of females and males using independent-samples $t$-tests. We computed Cohen's $d$ as a measure of effect size, with values of ~0.2, ~0.5, and ≥0.8 loosely corresponding to small, medium, and large effects, respectively (Cohen, 1988). Second, we employed discriminant function analysis (DFA) to determine which characters in multivariate space best discriminated between the sexes and those that were most neutral. We used an omnibus model including 14 variables (Pro L, Pro W, Chela L, Chela W, Chela H, Met 1 L, Met 1 W, Met 5 L, Met 5 W, Met L, Tel L, Tel W, Tel H, Tot L); the model excluded Pro A, a derived character which violated multicollinearity (tolerance = 0.00), and Pec L, which we used to determine sex. The DFA model was constructed with equal probability for group assignment and leave-one-out cross-validation. To determine the discriminating power of prosoma area, a second DFA was run which substituted Pro A for the components Pro L and Pro W. Following DFA, contrasts were conducted using ANCOVAs to determine which characters reliably
separated the sexes after adjustment for the other characters or predictors (Tabachnick and Fidell, 2013). In each ANCOVA, the variable of interest was declared the DV, sex was treated as a between-subjects factor, and the remaining characters were entered as covariates. Third, we conducted a principal component analysis (PCA) with Varimax rotation to evaluate covariance among the body size components and to create more general and uncorrelated measures of body size and shape. We excluded Pec L from the PCA model.

Fourth, we examined sexual dimorphism using five candidate reference characters via multiple analysis of covariance (MANCOVA) and analysis of covariance (ANCOVA) models. These models included sex as a between-subjects factor and one of the five covariates (reference characters) to control for overall body size. The covariates, tested in separate models, included Pro L, Pro A, and Tot L, as each has been used previously as an estimator of scorpion size and to evaluate sexual dimorphism (Brown, 2001; Carrera et al., 2009; Polis and Sissom, 1990; Sánchez-Quirós et al., 2012). We used principal component 1 (PC1) as the fourth covariate, which comprised a more general measure of body size based on multiple characters and has been recommended as a useful reference character for scaling (Bookstein, 1989; Zelditch et al., 2004). Our fifth covariate, Met 1 W, was chosen because it contributed least to the discrimination between sexes in the DFA model. To our knowledge, no study has demonstrated dimorphism of this character in any scorpion. For MANCOVA and ANCOVA models, we always tested the assumption of homogeneity of regression slopes by including an interaction term, and then removed the term from the final model if the interaction was non-significant.
Finally, we used standard major axis (SMA) regression (Falster et al., 2006; Smith, 2009; Warton et al., 2006) to assess static allometry in females and males separately. Static allometry deals with comparisons among individuals in a population which are all at the same developmental stage, and can be distinguished from ontogenetic or developmental allometry, which makes comparisons across developmental stages either within the individual or at the population level (Pélabon et al., 2013). We conducted bivariate analyses using the program SMATR (Falster et al., 2006), with $\alpha = 0.05$, iterations (used for testing for common slope, Likelihood ratio test) = 10000, and $H_0$ slope = 1 ($F$-test). We log$_{10}$-transformed all variables including the square root of the prosoma area (Sánchez-Quirós et al., 2012). We compared the results from using four different reference characters to control for body size: Pro L, Pro A, Tot L, and Met 1 W. If male and female slopes were found to be the same, we conducted follow-up Wald tests to evaluate differences in elevation and shifts along the slope (Falster et al., 2006; Warton et al., 2006).

**Results**

When morphological characters were considered individually via $t$-tests, adult female and male *H. arizonensis* exhibited sexual dimorphism in some but not all body components (Table 1). Females had significantly larger prosomas, averaging 2.06%, 1.52%, and 3.37% larger in length, width, and area, respectively. However, males had significantly larger Chela L (2.17%), Met 1 L (5.33%), Met 5 L (6.57%) and Met 5 W (1.74%), Met L (7.81%), Tot L (2.89%), and Pec L (17.09%). The remaining characters were not significantly different between the sexes (<1% difference).
The initial DFA model, which included 14 characters measured from 137 scorpions, confirmed that morphological differences between the sexes were highly significant (Wilks’ $\Lambda = 0.12, \chi^2 = 266.87, \text{df} = 14, P < 0.001$, canonical correlation = 0.936), with means for the discriminant function scores of -2.22 (range = -0.38 – -4.33).
and 3.12 (range = 1.25 – 6.01) for females and males, respectively. Every scorpion (100%) was correctly assigned for both original and cross-validated classification. The three best discriminating characters were Met L, Met 5 L, and Pro L (standardized coefficients of 1.52, 1.10, and -1.03, respectively; all other characters ≤ |0.56|; Table 2). Squared structure coefficients indicated that the function accounted for 12%, 7% and 1% of the variance in these characters, respectively. Signs for the function coefficients indicated that the difference between the sexes could largely be explained by the difference between metasoma length (represented by Met L and Met 5 L) and prosoma length, with males characterized by a longer metasoma relative to the prosoma. Contrasts using ANCOVA revealed that, after adjustment for all other predictors, only five characters provided significant discrimination between the sexes (listed in order of effect size): Met L ($P < 0.001$, partial $\eta^2 = 0.28$; adjusted marginal means for females and males, 48.5 ± 0.2 and 51.0 ± 0.2 mm, respectively); Met 5 L ($P < 0.001$, partial $\eta^2 = 0.20$; adjusted marginal means for females and males, 12.9 ± 0.1 and 13.6 ± 0.1 mm, respectively); Pro L ($P < 0.001$, partial $\eta^2 = 0.11$; adjusted marginal means for females and males, 13.2 ± 0.04 and 12.8 ± 0.1 mm, respectively); Pro W ($P = 0.005$, partial $\eta^2 = 0.06$; adjusted marginal means for females and males, 10.5 ± 0.05 and 10.2 ± 0.1 mm, respectively); and Tel L ($P = 0.036$, partial $\eta^2 = 0.035$; adjusted marginal means for females and males, 12.7 ± 0.1 and 13.0 ± 0.1 mm, respectively).
Table 2: Standardized canonical coefficients of morphological characters of *Hadrurus arizonensis* from two separate discriminant function analyses (DFAs).

<table>
<thead>
<tr>
<th>Character</th>
<th>DF 1</th>
<th>DF 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met L</td>
<td>1.518</td>
<td>1.620</td>
</tr>
<tr>
<td>Pro A</td>
<td>-1.392</td>
<td></td>
</tr>
<tr>
<td>Met 5 L</td>
<td>1.099</td>
<td>1.091</td>
</tr>
<tr>
<td>Pro L</td>
<td>-1.025</td>
<td></td>
</tr>
<tr>
<td>Pro W</td>
<td>-0.563</td>
<td></td>
</tr>
<tr>
<td>Tot L</td>
<td>-0.354</td>
<td>-0.505</td>
</tr>
<tr>
<td>Tel L</td>
<td>-0.430</td>
<td>-0.399</td>
</tr>
<tr>
<td>Tel W</td>
<td>-0.323</td>
<td>-0.373</td>
</tr>
<tr>
<td>Met 5 W</td>
<td>0.308</td>
<td>0.369</td>
</tr>
<tr>
<td>Met 1 L</td>
<td>0.231</td>
<td>0.203</td>
</tr>
<tr>
<td>Chela W</td>
<td>-0.200</td>
<td>-0.219</td>
</tr>
<tr>
<td>Chela H</td>
<td>-0.196</td>
<td>-0.171</td>
</tr>
<tr>
<td>Chela L</td>
<td>0.147</td>
<td>0.078</td>
</tr>
<tr>
<td>Tel H</td>
<td>-0.125</td>
<td>-0.073</td>
</tr>
<tr>
<td>Met 1 W</td>
<td>0.020</td>
<td>-0.068</td>
</tr>
</tbody>
</table>

DF1: Discriminant function for DFA that excluded the character prosoma area due to multicollinearity

DF2: Discriminant function for DFA that excluded the characters prosoma length and width to test the influence of prosoma area

The second DFA model testing the influence of Pro A included 13 characters and was similarly significant (Wilks’ $\Lambda = 0.13$, $\chi^2 = 265.75$, df = 13, $P < 0.001$, canonical correlation = 0.935), with female and male discriminant function means of -2.20 (range = -4.36 – 0.15) and 3.09 (range = 1.09 – 5.92) respectively. Every scorpion (100%) was correctly assigned for both original and cross-validated classification. The three best discriminating characters were Met L, Pro A, and Met 5 L (standardized coefficients of
1.62, -1.39, and 1.09, respectively; all other characters ≤ |0.51|; Table 2). Squared structure coefficients indicated that the function accounted for 13%, 1%, and 7% of the variance in these characters, respectively. As in the first model, signs on the discriminant function coefficients indicated that the difference between the sexes could largely be explained by the difference between metasoma length (represented by Met L and Met 5 L) and size of the prosoma (Pro A). Contrasts performed using ANCOVA revealed that, after adjustment for all other predictors, only Met L (\( P < 0.001 \), partial \( \eta^2 = 0.33 \); adjusted marginal means for females and males, 48.3 ± 0.2 and 51.2 ± 0.2 mm, respectively), Pro A (\( P < 0.001 \), partial \( \eta^2 = 0.24 \); adjusted marginal means for females and males, 140.5 ± 0.8 and 129.4 ± 1.1 mm\(^2\), respectively), Met 5 L (\( P < 0.001 \), partial \( \eta^2 = 0.20 \); adjusted marginal means for females and males, 12.9 ± 0.1 and 13.6 ± 0.1 mm, respectively), and Tel L (\( P = 0.049 \), partial \( \eta^2 = 0.031 \); adjusted marginal means for females and males, 12.7 ± 0.1 and 13.0 ± 0.1 mm, respectively) reliably separated the sexes.

In both DFA models, Met 1W was a poorly discriminating character (Table 2), and ANCOVA contrasts supported this conclusion (contrast following DFA model 1, \( P = 0.92 \), partial \( \eta^2 = 0.001 \); contrast following DFA model 2, \( P = 0.74 \), partial \( \eta^2 = 0.001 \)). Thus, we considered Met 1W to be the most suitable (i.e., most neutral) reference character, and added it to the remaining analyses.

The two principal components extracted from the PCA captured 77.4% of the variance (Table 3). The first (PC1), explaining 45.8% of the variance, was comprised largely of prosoma and telson size and shape, width of the two metasoma segments, and chela shape (width and height). The second (PC2), explaining 31.6% of the variance,
included primarily overall metasoma length, length of the two metasoma segments, total length, and chela length. Females averaged significantly larger for PC1 \( (t_{135} = 5.36, P < 0.001, \text{Cohen's } d = 0.93) \), and significantly smaller for PC2 \( (t_{135} = 15.17, P < 0.001, \text{Cohen's } d = 2.65) \).

### Table 3. Factor loadings for the two principal components (PC1, PC2) extracted from the principal component analysis of *Hadrurus arizonensis* morphological characters.

<table>
<thead>
<tr>
<th>Character</th>
<th>Factor Loadings</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro A</td>
<td>0.931</td>
<td>0.168</td>
<td></td>
</tr>
<tr>
<td>Pro L</td>
<td>0.894</td>
<td>0.175</td>
<td></td>
</tr>
<tr>
<td>Pro W</td>
<td>0.886</td>
<td>0.158</td>
<td></td>
</tr>
<tr>
<td>Tel W</td>
<td>0.824</td>
<td>0.201</td>
<td></td>
</tr>
<tr>
<td>Tel H</td>
<td>0.812</td>
<td>0.301</td>
<td></td>
</tr>
<tr>
<td>Tel L</td>
<td>0.792</td>
<td>0.378</td>
<td></td>
</tr>
<tr>
<td>Met 1 W</td>
<td>0.787</td>
<td>0.329</td>
<td></td>
</tr>
<tr>
<td>Met 5 W</td>
<td>0.768</td>
<td>0.376</td>
<td></td>
</tr>
<tr>
<td>Chela H</td>
<td>0.732</td>
<td>0.258</td>
<td></td>
</tr>
<tr>
<td>Chela W</td>
<td>0.675</td>
<td>0.339</td>
<td></td>
</tr>
<tr>
<td>Tot L</td>
<td>0.619</td>
<td>0.688</td>
<td></td>
</tr>
<tr>
<td>Chela L</td>
<td>0.604</td>
<td>0.671</td>
<td></td>
</tr>
<tr>
<td>Met 5 L</td>
<td>0.426</td>
<td>0.833</td>
<td></td>
</tr>
<tr>
<td>Met 1 L</td>
<td>0.373</td>
<td>0.736</td>
<td></td>
</tr>
<tr>
<td>Met L</td>
<td>0.295</td>
<td>0.906</td>
<td></td>
</tr>
<tr>
<td>Pec L</td>
<td>-0.066</td>
<td>0.924</td>
<td></td>
</tr>
</tbody>
</table>

The variance explained was 47.8% for PC1 and 28.9% for PC2.

The five characters selected for use as the reference or covariate for overall size in the MANCOVA and ANCOVA models (Pro L, Pro A, Tot L, PC1, and Met 1 W) provided incongruent results (Fig. 2, Appendix 1a-e Tables). Use of Pro L, Pro A, and PC1 yielded largely identical interpretations (Pro L and Pro A both showing 12 of 15 characters dimorphic), with PC1 showing the greatest number of differences (14 of 16 characters dimorphic, and the other two characters displaying an interaction between sex...
and PC1). Most measures for the chela, metasoma, telson, pectine, and total length were substantially larger in males. Use of either Tot L (10 of 15 characters dimorphic) or Met 1 W (11 of 15 characters dimorphic) as the covariate indicated that females had significantly greater size for all prosoma measures. Remarkably, the dimorphism of some body components was reversed depending on which reference character was used. Prosoma characters were male-biased when PC1 was the reference and female-biased when Tot L and Met 1 W was the reference. Chela W was female-biased with Tot L as the reference, and male-biased with Pro L, Pro A, and PC1 as the reference. Telson W was female-biased with Tot L and Met 1 W as the covariate, and male-biased with Pro L and Pro A as the reference. Telson W was female-biased with Tot L as the reference, and male-biased with Pro L, Pro A, and PC1 as the reference. When multiple characters were combined in MANCOVA models, the results generally conformed with the ANCOVA models for individual characters.
**Figure 2.** Sexual body component dimorphism (SBCD) in *Hadrurus arizonensis*, comparing the results of alternative reference characters. Analysis of covariance (ANCOVA) results are expressed as percent difference in marginal means between the sexes (y-axis) for each body component (x-axis groupings) when using different reference characters (covariates; indicated by bar pattern). Alternative reference characters included prosoma length (Pro L), prosoma area (Pro A), total length (Tot L), principal component 1 (PC1), and metasoma segment 1 width (Met 1 W). Percent difference was calculated as \(\frac{(\text{male marginal mean} - \text{female marginal mean})}{2}\) x 100. Thus, bars above zero indicate body components showing male-biased SBCD, and bars below zero indicate female-biased SBCD. Bars with an asterisk (*) indicate a significant difference between sexes. Missing bars (indicated by arrows) occur where a significant interaction between sex and the covariate (heterogeneous regression slopes) existed, precluding ANCOVA and obfuscating male-female differences. Note the incongruent interpretations of SBCD depending on which reference character is used in the ANCOVA. Additional details are provided Appendix 1a-e Table.
A small number of interactions existed between sex and the covariate in the MANCOVA and ANCOVA models (14 of 99 models; 14.1%). In these models, the direction of sexual dimorphism could not be inferred because of a violation of the assumption of homogenous regression slopes. Detailed explanation of each interaction goes beyond our purposes.

Based on SMA regression and SMATR output, we categorized allometric relationships (slope relative to 1.0) among the 16 body components and four reference characters as either positive, isometric, or negative. Allometric relationships were most often identical between the sexes, with only 28.3% of the models (17 out of 60) demonstrating a contrasting allometry (Fig. 3; Appendix 2a-d Table). Three body components (Pro A, Met 1 L, and Met 5 W) displayed the same allometry pattern across all four reference characters, whereas 13 body components showed contrasting allometries among the four reference characters. Prosoma L and Pro A as reference characters were similar to each other, showing congruent allometries for 9 of 14 body components. Total L and Met 1 W as reference characters were also similar to each other, yielding congruent allometries for 12 of 14 body components. However, allometric relationships derived from the two pairs of reference characters differed substantially from each other. Use of Pro L and Pro A as reference characters showed primarily positive allometry and isometry for both females (Pro L: 12 positive, 3 isometric; Pro A: 9 positive, 5 isometric, 1 negative) and males (Pro L: 7 positive, 8 isometric; Pro A: 8 positive, 7 isometric). In contrast, use of Tot L and Met 1 W as reference characters yielded comparatively more isometry and/or negative allometry for females (Tot L: 3 positive, 10 isometric, 2 negative; Met 1 W: 2 positive, 11 isometric, 2 negative) and
males (Tot L: 4 positive, 11 isometric; Met 1 W: 3 positive, 12 isometric). Negative allometry was rare and only present for body components Pro L and Chela L in females. Although differences existed between sexes in designation of allometry as positive, isometric, or negative, only one body component differed statistically between the sexes in slope, and that was Tel W (Fig. 4D, Appendix 1a-d Table).

Representative comparisons in allometry between males and females for Met 1 W (the least biased) as the reference character are illustrated in Fig. 4 and in Appendix 2a-d Table. Three body components (Pro L, Pro W and, Pro A) exhibited only a shift in elevation (y-intercept) between females and males. Seven body components (Chela L, Met 1 L, Met 5 L, Met 5 W, Met L, Tot L and, Pec L) showed a shift in both elevation and along a common slope. One body component (Tel W) showed a difference between slopes. Four body components were identical for the two sexes, showing no shifts in elevation or common slope (Chela W, Chela H, Tel L and, Tel H)
Figure 3. Effects of reference character on allometric trends of body components. Allometric slopes (± 95% CI) determined from four alternative reference characters are paired against each of 16 y-axis characters for females (N = 84–90) and males (N = 65–83). The reference characters included A: prosoma length (Pro L); B: prosoma area (Pro A); C: total length (Tot L); and D: metasoma segment 1 width (Met 1 W). Bars identified with an asterisk (*) indicate a significant difference between the slope and null hypothesis of 1.0 by F-test of standard major axis regression. Significant slopes above 1.0 indicate positive allometry; significant slopes below 1.0 indicate negative allometry; and non-significant slopes indicate isometry. Additional details are supplied in Appendix 2 Table.
Figure 4. Select allometric relationships of female (open circles, dashed line) and male (closed circles, solid line) *Hadrurus arizonensis*. A–F depict static allometric scaling relationships of select body characters with metasoma segment 1 width (Met 1 W) as the reference character. A. Prosoma length (Pro L) plot illustrates a difference in y-intercept between the sexes. B. Chela height (Chela H) illustrates no difference between the sexes. C. Telson width (Tel W) illustrates a difference in slopes between the sexes. D–F Illustrate differences in both y-intercept and in shifts along the slope for metasoma length (Met L), total length (Tot L), and pectine length (Pec L). Scales are logarithmic. N = 84–90 females and 65–83 males. Additional details are supplied in Appendix 2a-d Table.
Discussion

Although most scorpion species exhibit dimorphism in overall size (SSD) or individual body components (SBCD), the methods generally relied on to detect these (ranges in character measurements, ratios, and ANCOVA using a dimorphic reference character as a covariate) usually cannot identify which body parts are subject to selection. Here, we explored several candidate reference characters for overall body size to better understand sexual dimorphism and character scaling in a cryptically dimorphic taxon, *H. arizonensis*. We begin our discussion with general patterns of dimorphism, and then describe the dilemma of choosing an appropriate reference character for assessing dimorphism and allometry. We then consider sexual dimorphism and allometry of individual body components, and the selection forces that have potentially shaped them.

**General Pattern of Dimorphism**

The most obvious conclusion from our analyses is that *H. arizonensis* could be interpreted as dimorphic in essentially every character. Simple *t*-tests demonstrated statistically significant dimorphism in multiple characters (10 of 16 measured; Table 1). Some characters had relatively small effect sizes (e.g., those of the telson), whereas others showed moderate (e.g., those of the Pro L and Chela L) or even large effect sizes (e.g., metasoma lengths and Pec L). However, univariate comparisons like these need to be viewed cautiously; if one sex is larger overall than the other sex, then a reference character for overall size needs to be controlled for. When controlling for overall size using ANCOVA, the identification of dimorphic body components varied depending on which character was used as the reference. With interactions included, 13 of 15
characters were dimorphic when each of Pro L, Pro A, Tot L, or Met 1 W was used as the covariate, and all 16 characters were dimorphic when employing PC1 as the covariate. Collectively, the ANCOVA models could suggest that every body component we measured is sexually dimorphic, even if most differences are quite small (<5%), i.e., cryptic. The fact that dimorphism exists at all in *H. arizonensis* has been largely overlooked by previous investigators (Stahnke, 1971; Tallarovic, 2000; Williams, 1970).

*Choice of Reference Character and its Implications*

The choice of reference character or covariate for analysis of body component dimorphism varies widely among studies, and can substantially influence an assessment of dimorphism (Prenter et al., 1995). Most investigations rely on some measure of overall size as the covariate, or a proxy, such as carapace width (Aisenberg et al., 2010; Hagstrum, 1971) or length (Cothran and Jeyasingh, 2010), prothorax width (Painting and Holwell, 2013; Walker et al., 2008), mass (Okada and Miyatake, 2009), total length (Bidau et al., 2013; Voje and Hansen, 2013), or snout-vent length (Cox and Calsbeek, 2010; Hayek and Heyer, 2005), usually without offering justification. In each case, one can ask which is the target of selection: the reference character itself, the body component under consideration, or both? This problem was brought to the forefront recently by those studying lizards (Braña, 1996; Kratochvíl et al., 2003; Scharf and Meiri, 2013). Previously, male-biased head size dimorphism was universally analyzed and interpreted using snout-vent length (SVL) as the reference character, and head size was considered the target of selection. Then the question arose as to whether selection was targeting the female's trunk (resulting in longer trunk via fecundity selection) or the
male's head (resulting in larger size via sexual selection). As trunk length and head length are constituents of SVL, selection on either or both of these components could affect SVL, rendering SVL an inappropriate reference character. In spiders the common reference character is carapace width (Hagstrum, 1971). However, to study the comparative allometry of fang size in three spider species (Scytodes thoracica, Loxosceles reclusa, and, Varacosa avara), Suter and Stratton (Suter and Stratton, 2011) opted to use sternum width as a proxy for size. The authors contended that use of carapace width was inappropriate, as it has been targeted by selection to a greater extent in Scytodes (indirectly due to venom gland hypertrophy (Foelix, 1996) than in other species. These examples illustrate the difficulties in choosing an appropriate reference character, the need to understand the organism of interest, and the potential for misinterpretation if these considerations are inadequately addressed.

Heretofore, scorpion sexual dimorphism and static allometry investigations have used several reference characters, including total length (Bothriurus bonariensis: Peretti et al., 2001), prosoma + mesosoma (Centruroides vittatus: Carlson et al., 2014), and prosoma area (Centruroides margariatatus: Sánchez-Quirós et al., 2012). Studies of scorpion ontogenetic allometry and life history have utilized several prosoma measures (Benton, 1991; Brown, 2001; Francke and Sissom, 1984; Polis and Farley, 1979a). We were initially interested in the use of a prosoma measure as a reference character in H. arizonensis due to precedent (Hagstrum, 1971; Santos et al., 2013), its heavy loading on PC1 (c.f. Colgoni and Vamosi, 2006), and its avoidance of both the nutritional effects of the mesosoma and the frequent dimorphism present in the metasoma, each of which can influence total length. However, based on other scorpion species (Booncham et al., 2007;
Francke and Jones, 1982; Quiroga et al., 2004) and the findings of this study, the prosoma may itself be dimorphic, and therefore less than ideal (Kratochvíl et al., 2003; Scharf and Meiri, 2013). Our DFA models support this conclusion, as prosoma variables had large unique contributions to each of the discriminant functions. We therefore considered body components that were poorly discriminating in the DFA models and demonstrated no dimorphism via t-test. Of the body components meeting these criteria (Chela W, Chela H, Met 1 W, Tel W, and Tel H), we propose Met 1 W as the best candidate reference character because it was the most neutral of all characters in the DFA models, and in contrast to other body components (Booncham et al., 2007; Fet et al., 2013a; Kovařík et al., 2011; Kovařík and Ahmed, 2013; Soleglad and Fet, 2004; Tropea et al., 2013) has a high likelihood of neutrality in other scorpion taxa.

**Sexual Size Dimorphism (SSD)**

The question of whether overall body size dimorphism exists in *H. arizonensis* remains unclear. Some body components were larger in females, and others were larger in males. When overall body size dimorphism was evaluated by examining whether the majority of individual body components showed female-larger dimorphism (negative percent difference, with most bars of a given color below zero line in Fig. 2) or male-larger dimorphism (positive percent difference), the direction of dimorphism shifted based on the reference character (covariate) employed. Using the prosoma (Pro L or Pro A) or PC1 as the reference character, the majority of body components averaged larger in males. However, Tot L as the reference character indicated the opposite situation, with most body components larger in females, excluding those commonly larger in male
scorpions (Met 1 L, Met 5 L, Met L, and Pec L). Use of Met 1 W as the reference resulted in the most parsimonious result balancing trends of SBCD seen in other scorpions and allometric trends, though it raises the question of which characters contribute most to overall size. Body mass would be an inappropriate measure of SSD because it is subject to nutritional and reproductive status.

Principal component 1 is a commonly used measure of body size in many taxa (Bookstein, 1989; Zelditch et al., 2004), and has been used as an indicator of overall size in scorpions (Graham et al., 2012). In H. arizonensis, PC1 was positively and strongly associated with prosoma size, and averaged larger in females. However, interpretation of PC1 as a measure of overall size is complicated by the fact that it included characters representing both size (Pro L, Pro A, and Tel L) and, presumably, shape (e.g., Pro W, Tel W, Tel H, Met 1 W). Although PC1 is most commonly associated with size, it is not uncommon for both size and shape variables to load highly on a single component (Zelditch et al., 2004). For example, variables representing both size and shape loaded highly on Graham et al.’s (2012) PC1 used to differentiate scorpion species.

Considering the discordant measures of SSD, we cannot conclude which sex is larger overall. Nevertheless, we are confident that females have a larger prosoma and that males are longer overall (Tot L) due to their longer metasoma. These interpretations accord with the t-tests, ANCOVAs, and prior interpretations for scorpions in general (Polis and Sissom, 1990; van der Meijden et al., 2010).
Sexual Body Component Dimorphism (SBCD), Allometry, and Potential Selection

Our findings suggest that selection may act differently on the prosoma of *H. arizonensis* than on most of the body parts that extend from or beyond the prosoma, particularly the metasoma. The DFA models separated the sexes primarily on differences in prosoma variables (which loaded highly on PC1) and metasoma length variables (which loaded highly on PC2). In this section, we focus on inferences about individual body components. Although dimorphism (or the potential for dimorphism) is often noted in the scorpion literature (e.g., Booncham et al., 2007; Graham and Bryson, 2010; Lourenço and Duhem, 2010; Polis and Sissom, 1990; Sánchez-Quirós et al., 2012; Teruel et al., 2013), static allometry remains little studied in these taxa (Carlson et al., 2014; Peretti et al., 2001; Sánchez-Quirós et al., 2012), and the use of different methods to analyze sexual dimorphism and allometry renders comparisons among studies problematic.

Female-biased dimorphism of the prosoma is consistent with the conclusion of others that fecundity selection has favored an increase in size of the prosoma of scorpion females compared to males that, along with the mesosoma, could support larger broods, larger offspring size, or both (Brown, 2004; Formanowicz and Shaffer, 1993; Francke, 1981; Lourenço et al., 1996; Outeda-Jorge et al., 2009). We therefore suggest that the prosoma should be avoided as a reference character for assessment of dimorphism and allometry in scorpions unless detailed analysis reveals it to be neutral for a given species. Isometry was the most common allometric trend for prosoma body measures across all reference characters in *H. arizonensis*, though some discordance existed. Using Met 1 W as the reference, the presence in females of negative allometry in Pro L and isometry in
Pro W and Pro A suggests that the potential influence of fecundity selection may be constrained by other factors in this species.

Scorpions use their chela primarily to grasp items, particularly in predatory, defensive, and mating contexts, and therefore several interacting forces could influence selection on this body component. In *H. arizonensis*, choice of reference character confounded interpretation of SBCD for this particular body component, but male-biased chela length was apparent with Met 1 W as the reference character. Examples of both male-larger (Graham and Bryson, 2010; Kovařík, 2007) and female-larger chela (Booncham et al., 2007; Teruel and Roncallo, 2008) can be found among scorpions, although evaluation of dimorphism using a neutral reference character could strengthen these interpretations. Chela are of utmost importance in prey capture and defense, to the point that envenomation is rarely or never used in adults of several scorpion species (Casper, 1985; Heatwole, 1967; Jiao and Zhu, 2009b; Quinlan et al., 1995). However, because diet and predators are presumably similar for the two sexes (to our knowledge these remain unstudied), we suggest that SBCD of this character in *H. arizonensis* may have arisen largely from either intrasexual or intersexual selection (c.f. Peretti et al., 2001). Chela structure is important in mating behavior, and modifications of chela for this purpose have been suggested (Benton, 1992; 1991; Booncham et al., 2007; Kovařík et al., 2011; Peretti et al., 2001). Female Chela L was the only character other than Pro L to display negative allometry (with Pro A, Tot L, or Met 1 W as reference), whereas Chela W and Chela H showed isometry with Met 1 W as the reference character (Fig. 3). Larger females may have disproportionately shorter chela in order to maintain the ability to interact efficiently with males during the *promenade aux deux* under a “one size fits
all” model (sexual selection: Eberhard et al., 1998; Peretti et al., 2001). As *H. arizonensis* relies largely, but not exclusively, on venom to obtain prey (Bub and Bowerman, 1979; Edmunds and Sibly, 2010), natural selection may also favour increased relative Chela L for smaller females resulting in enhanced prey capture ability.

Although the metasoma is a prominent feature in scorpions, acting as the base and point of articulation for their venomous sting, the shape and structure of this tail can be variable both among species and between sexes (Polis and Sissom, 1990). Indeed, we found *H. arizonensis* males to possess a substantially longer metasoma (including segments 1 and 5) than females. Choice of reference character affected only degree of dimorphism for measures of metasoma length. Elaboration of the metasoma in the males of many species (e.g., Fet et al., 2013a; Kovařík, 2007; Kovařík et al., 2010; 2013; Polis and Sissom, 1990) argues for a sexual role for this body segment, which could include combat with other males (Carlson et al., 2014), clubbing or deflection of sting attempts by resistant females (e.g., Jiao and Zhu, 2009a; Polis and Farley, 1979b), and sexual stings toward females (e.g., Tallarovic et al., 2000; Toscano-Gadea, 2010). A male-longer metasoma could alternatively be a by-product of different selection pressures on more sedentary females compared to more vagile males (Benton, 2001; Kaltsas and Mylonas, 2010; Polis and Farley, 1979b), resulting in different foraging (Kaltsas et al., 2008) or defense/escape (Carlson et al., 2014) tactics. We suspect that sexual selection has shaped the dimorphism of this body component in *H. arizonensis*, but more study is needed. The direction of allometry in metasoma body components was largely similar across all references and isometry was the dominant trend. Positive allometry was present for Met 1 L for both sexes across all references. As the first metasoma segment is the connection
between the scorpion body and tail, a disproportional increase in the size of this segment in larger individuals may be related to mechanical constraints.

Variation in telson morphology by species and sex has been described (Fet et al., 2013b; Lourenço and Duhem, 2010; Polis and Sissom, 1990; Soleglad and Fet, 2004), but explanations invoking functional relationships seem to be absent. Interpretations of dimorphism differed depending on reference character, but subtle female-biased dimorphism (Tel W) existed with Met 1 W as the reference character. As the telson harbors the venom glands and the musculature that controls venom expulsion, SBCD in this structure could have important implications for possible sexual differences in venom availability and use. Scorpions (with a few exceptions) rely on their venom not only for predation and defense (Edmunds and Sibly, 2010; Nisani and Hayes, 2011; Sarhan et al., 2013), but males may also use their venom in a sexual sting, which has been described in *H. arizonensis* (Tallarovic et al., 2000). Stabilizing selection nevertheless may be acting on the telson to optimize venom supply for both sexes. Telson characters were generally isometric or positively allometric (Tel H for females, Tel W for males) with Met 1 W as the reference. Larger scorpions tend to possess disproportionately larger telsons, but predominantly isometrically-scaled chela suggests a consistent reliance of *H. arizonensis* on venom rather than chela for subjugation of prey. It would be interesting to compare allometry of the telson and chelae in *Pandinus imperator*, which uses venom to subdue prey when young, but relies primarily on the chelae as adults (Casper, 1985).

Variation in pectine size and structure may be the best characterized SBCD, as it is unique to scorpions and often relied upon by investigators to determine sex. Our pectine-related results align well with findings from other species: females had smaller
pectines than males, and the pectines were the most dimorphic character by $t$-test and in all ANCOVA models. Pectines function to identify physical (Kladt et al., 2007) and chemical cues (Gaffin and Brownell, 1997) on the substrate, which enable pheromonal sex discrimination (Melville et al., 2003), mate trailing (Melville et al., 2003; Steinmetz et al., 2004), and spermatophore deposition (Abushama, 1968; Alexander, 1957; Polis and Farley, 1979b), suggesting a strong influence from sexual selection. Intersexual and interspecific differences in pectine structure may reflect, for example, differing degrees of vagility in scorpions. Males typically travel more and occupy larger home ranges than females, particularly during the breeding season when males are searching for mates, and given the sensory importance of the pectines, exaggeration of this body component in males is reasonable (Allred, 1973; Kaltsas and Mylonas, 2010; Polis et al., 1985; Tourtlotte, 1974; Williams, 1966). However, the pectines may also function in prey detection (Hidalgo, 2012; Mineo and del Claro, 2006), and therefore could be under the influence of natural selection. Positive allometry with Met 1 W as the reference character (significant for males and approaching significance for females) similarly suggests selection arising from the functional roles of pectines in adults.

**Conclusions**

In our attempt to statistically characterize cryptic sexual dimorphism and character scaling in *H. arizonensis*, we encountered serious difficulties in finding a suitable reference character for overall body size. Of the reference characters we examined (Pro L, Pro A, Tot L, PC1, and Met 1 W), the prosoma-based characters and PC1 are likely poor choices in this species, as they are all dimorphic measures, and the
prosoma characters contributed unique variance within DFA models sufficient to differentiate the sexes. Although Tot L was also dimorphic, it was a poorly discriminating character in DFA models, and therefore potentially a better choice of reference for *H. arizonensis*. We selected Met 1 W as the best reference character however, as it was the most neutral of all characters examined. We suspect that Met 1 W as a reference has the greatest likelihood of utility in other scorpion taxa, as Tot L and the other body components evaluated often demonstrate greater dimorphism in other taxa than in *H. arizonensis*.

The direction of dimorphism in *H. arizonensis* for most characters mirrored that seen in other more obviously dimorphic scorpions. Our findings are consistent with the conclusions of others that fecundity selection likely favors a larger prosoma in female scorpions, whereas sexual selection may favor other body parts being larger in males, especially length measures of the metasoma, pectines, and possibly the chela. While we expected most characters to be isometric in *H. arizonensis*, we were surprised by both the negative allometry of Pro L (female) and positive allometry of Met 1 L (both sexes). As methodology for evaluating static allometry is still being established for scorpions, interspecific comparisons await future study.

For *H. arizonensis*, and probably for most other organisms, the choice of reference character can profoundly affect interpretations of SSD, SBCD, and allometry. Thus, researchers need to broaden their consideration of an appropriate reference, and exercise more caution in interpreting their findings, especially as they relate to selection. We highly recommend use of discriminant function analysis as a useful means for identifying the most appropriate (unbiased) reference character. Further studies including
more species and a wider range of morphological characters will shed further light on our understanding of sexual dimorphism and character scaling in scorpions.

Acknowledgments

We thank Eric Gren, David Nelson, and Carl Person for help in collecting scorpions. We also gratefully acknowledge the suggestions of three anonymous reviewers.
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APPENDIX 1

COMPARISON OF MARGINAL MEANS FOR MORPHOLOGICAL CHARACTERS OF ADULT FEMALE AND MALE *HADRURUS ARIZONENSIS*

FROM MANCOVA AND ANCOVA MODELS USING ALTERNATIVE REFERENCE CHARACTERS (A-E) TO ASSESS DIMORPHISM

**Appendix 1a.** Comparison of marginal means (± 1 S.E.) for morphological characters (dependent variables, DVs) of adult male (*N* = 65–83 for each character) and female (*N* = 84–90 for each character) *Hadrurus arizonensis* from MANCOVA (grey shading) and ANCOVA models using prosoma length as the reference character (covariates) to assess dimorphism. Analyses conducted using untransformed data.

<table>
<thead>
<tr>
<th>MANCOVA DVs (gray) or ANCOVA DV (white)</th>
<th>Marginal means (mean ± 1 S.E.)</th>
<th>Percent Difference (♂ to ♀)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prosoma length</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prosoma width</td>
<td>10.3±0.03</td>
<td>10.4±0.03</td>
</tr>
<tr>
<td>Prosoma area</td>
<td>135.4±0.36</td>
<td>135.5±0.41</td>
</tr>
<tr>
<td><strong>Prosoma width</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prosoma area</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MANCOVA: DVs = prosoma length x width</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chela length</td>
<td>19.6±0.04</td>
<td>20.3±0.05</td>
</tr>
<tr>
<td>Chela width</td>
<td>4.3±0.02</td>
<td>4.4±0.02</td>
</tr>
<tr>
<td>Chela height</td>
<td></td>
<td>Interaction</td>
</tr>
<tr>
<td>MANCOVA: DVs = chela length x width x height</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metasoma segment 1</td>
<td>length x width</td>
<td></td>
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<tr>
<td>-------------------</td>
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<td></td>
</tr>
<tr>
<td>Metasoma segment 1 length</td>
<td>6.8±0.03</td>
<td>7.3±0.04</td>
</tr>
<tr>
<td>Metasoma segment 1 width</td>
<td>6.7±0.02</td>
<td>6.8±0.03</td>
</tr>
</tbody>
</table>

**MANCOVA: DVs = metasoma segment 5 length x width**

| Metasoma segment 5 length | 12.7±0.04 | 13.8±0.04 | 8.31 *** |
| Metasoma segment 5 width | 5.7±0.02 | 5.9±0.02 | 2.70 *** |
| Metasoma length | 47.3±0.17 | 52.2±0.17 | 9.76 *** |

**MANCOVA: DVs = telson length x width x height**

| Telson length | 12.7±0.04 | 13.0±0.04 | 2.05 *** |
| Telson width | 5.8±0.02 | 5.9±0.03 | 1.69 ** |
| Telson height | 5.3±0.02 | 5.5±0.02 | 2.65 *** |
| Total length | 99.5±0.30 | 104.7±0.32 | 5.09 *** |
| Pectine length | 10.0±0.06 | 12.0±0.06 | 18.54 *** |

Percent difference is calculated as \(((\text{male marginal mean} – \text{female marginal mean})/((\text{male marginal mean} – \text{female marginal mean})/2)) \times 100\)

* \(P \leq 0.05\); ** \(P \leq 0.01\); *** \(P \leq 0.001\)

"Interaction" indicates a significant interaction between covariate and sex
Appendix 1b. Comparison of marginal means (± 1 S.E.) for morphological characters (dependent variables, DVs) of adult male (N = 65–83 for each character) and female (N = 84–90 for each character) *Hadrurus arizonensis* from MANCOVA (grey shading) and ANCOVA models using prosoma area as the reference character (covariates) to assess dimorphism. Analyses conducted using untransformed data.

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<tr>
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<th>Marginal means (mean ± 1 S.E.)</th>
<th>Percent Difference (♂ to ♀)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prosoma length</strong></td>
<td>Female: 13.0±0.02  Male: 13.0±0.02</td>
<td>-0.22</td>
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<tr>
<td><strong>Prosoma width</strong></td>
<td>Female: 10.4±0.01  Male: 10.4±0.02</td>
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<tr>
<td><strong>Prosoma area</strong></td>
<td>Female: 13.0±0.02  Male: 13.0±0.02</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Chela length</strong></td>
<td>Female: 19.6±0.04  Male: 20.3±0.05</td>
<td>3.54 ***</td>
</tr>
<tr>
<td><strong>Chela width</strong></td>
<td>Female: 4.3±0.02   Male: 4.4±0.02</td>
<td>1.56 **</td>
</tr>
<tr>
<td><strong>Chela height</strong></td>
<td>Female: 6.8±0.03   Male: 7.3±0.04</td>
<td>6.90 ***</td>
</tr>
<tr>
<td><strong>Metasoma segment 1 length</strong></td>
<td>Female: 6.7±0.02   Male: 6.8±0.02</td>
<td>1.88 ***</td>
</tr>
<tr>
<td><strong>Metasoma segment 1 width</strong></td>
<td>Female: 6.7±0.02   Male: 6.8±0.02</td>
<td>1.88 ***</td>
</tr>
<tr>
<td>Metasoma segment 5</td>
<td>Length</td>
<td>Width</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>Metasoma segment 5</td>
<td>12.7±0.04</td>
<td>5.7±0.02</td>
</tr>
<tr>
<td>length</td>
<td>13.8±0.04</td>
<td>5.9±0.02</td>
</tr>
<tr>
<td>Metasoma segment 5</td>
<td>12.7±0.04</td>
<td>5.7±0.02</td>
</tr>
<tr>
<td>width</td>
<td>13.8±0.04</td>
<td>5.9±0.02</td>
</tr>
<tr>
<td>Metasoma length</td>
<td>47.3±0.15</td>
<td>5.7±0.02</td>
</tr>
<tr>
<td></td>
<td>52.3±0.17</td>
<td>5.9±0.02</td>
</tr>
</tbody>
</table>

**MANCOVA: DVs = telson length x width x height**

<table>
<thead>
<tr>
<th>Telson length</th>
<th>Telson width</th>
<th>Telson height</th>
<th>Total length</th>
<th>Pectine length</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.7±0.04</td>
<td>5.7±0.02</td>
<td>5.3±0.02</td>
<td>100.0±0.28</td>
<td>10.0±0.06</td>
</tr>
<tr>
<td>13.0±0.04</td>
<td>5.9±0.03</td>
<td>5.5±0.02</td>
<td>104.4±0.31</td>
<td>12.1±0.07</td>
</tr>
<tr>
<td></td>
<td>1.96 ***</td>
<td>1.43 **</td>
<td>2.35 ***</td>
<td>18.62 ***</td>
</tr>
<tr>
<td></td>
<td>1.96 ***</td>
<td>1.43 **</td>
<td>2.35 ***</td>
<td>18.62 ***</td>
</tr>
</tbody>
</table>

Percent difference is calculated as ((male marginal mean – female marginal mean)/((male marginal mean – female marginal mean)/2)) x 100

* P ≤ 0.05;  ** P ≤ 0.01;  *** P ≤ 0.001

"Interaction" indicates a significant interaction between covariate and sex
**Appendix 1c.** Comparison of marginal means (± 1 S.E.) for morphological characters (dependent variables, DVs) of adult male \( (N = 65–83 \text{ for each character}) \) and female \( (N = 84–90 \text{ for each character}) \) *Hadrurus arizonensis* from MANCOVA (grey shading) and ANCOVA models using total length as the reference character (covariates) to assess dimorphism. Analyses conducted using untransformed data.

<table>
<thead>
<tr>
<th>MANCOVA DVs (gray) or ANCOVA DV (white)</th>
<th>Marginal means (mean ± 1 S.E.)</th>
<th>Percent Difference (♂ to ♀)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prosoma length</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>13.3±0.03</td>
<td>12.7±0.04</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prosoma width</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10.6±0.03</td>
<td>10.1±0.04</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prosoma area</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>140.3±0.68</td>
<td>128.7±0.79</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chela length</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>19.9±0.05</td>
<td>20.0±0.06</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chela width</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4.4±0.02</td>
<td>4.3±0.02</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chela height</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6.9±0.03</td>
<td>6.7±0.03</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Metasoma segment 1 length</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6.9±0.03</td>
<td>7.2±0.04</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Metasoma segment 1 width</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>Interaction</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**MANCOVA: DVs = prosoma length x width**

**MANCOVA: DVs = chela length x width x height**

**MANCOVA: DVs = metasoma segment 1 length x width**
<table>
<thead>
<tr>
<th>Metasoma segment 5 length x width</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Metasoma segment 5 length</td>
<td>13.0±0.04</td>
</tr>
<tr>
<td>Metasoma segment 5 width</td>
<td>5.8±0.02</td>
</tr>
<tr>
<td>Metasoma length</td>
<td>Interaction</td>
</tr>
</tbody>
</table>

**MANCOVA: DVs = telson length x width x height**

<table>
<thead>
<tr>
<th>Telson length</th>
<th>Interaction</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Telson width</td>
<td>5.9±0.03</td>
<td>5.7±0.03</td>
</tr>
<tr>
<td>Telson height</td>
<td>5.4±0.02</td>
<td>5.3±0.02</td>
</tr>
<tr>
<td>Total length</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectine length</td>
<td>10.2±0.06</td>
<td>11.8±0.07</td>
</tr>
</tbody>
</table>

Percent difference is calculated as \(((\text{male marginal mean} - \text{female marginal mean})/((\text{male marginal mean} - \text{female marginal mean})/2)) \times 100 \)

* P ≤ 0.05;  ** P ≤ 0.01;  *** P ≤ 0.001

"Interaction" indicates a significant interaction between covariate and sex
**Appendix 1d.** Comparison of marginal means (± 1 S.E.) for morphological characters (dependent variables, DVs) of adult male ($N = 65–83$ for each character) and female ($N = 84–90$ for each character) *Hadrurus arizonensis* from MANCOVA (grey shading) and ANCOVA models using PC1 as the reference character (covariates) to assess dimorphism. Analyses conducted using untransformed data.

<table>
<thead>
<tr>
<th>MANCOVA DVs (gray) or ANCOVA DV (white)</th>
<th>Marginal means (mean ± 1 S.E.)</th>
<th>Percent Difference ($♂$ to $♀$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MANCOVA: DVs = prosoma length x width</strong></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Prosoma length</td>
<td>12.9±0.03</td>
<td>13.1±0.03</td>
</tr>
<tr>
<td>Prosoma width</td>
<td>10.3±0.02</td>
<td>10.5±0.03</td>
</tr>
<tr>
<td>Prosoma area</td>
<td>133.8±0.44</td>
<td>138.8±0.53</td>
</tr>
<tr>
<td><strong>MANCOVA: DVs = chela length x width x height</strong></td>
<td></td>
<td>Interaction</td>
</tr>
<tr>
<td>Chela length</td>
<td>19.5±0.05</td>
<td>20.5±0.06</td>
</tr>
<tr>
<td>Chela width</td>
<td>4.3±0.02</td>
<td>4.4±0.02</td>
</tr>
<tr>
<td>Chela height</td>
<td></td>
<td>Interaction</td>
</tr>
<tr>
<td><strong>MANCOVA: DVs = metasoma segment 1 length x width</strong></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Metasoma segment 1 length</td>
<td>6.8±0.03</td>
<td>7.4±0.04</td>
</tr>
<tr>
<td>Metasoma segment 1 width</td>
<td>6.7±0.02</td>
<td>6.9±0.02</td>
</tr>
<tr>
<td><strong>MANCOVA: DVs =</strong></td>
<td>***</td>
<td></td>
</tr>
</tbody>
</table>
metasoma segment 5
length x width

<table>
<thead>
<tr>
<th>Metasoma segment 5 length</th>
<th>12.7±0.04</th>
<th>13.9±0.05</th>
<th>9.83 ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metasoma segment 5 width</td>
<td>5.7±0.02</td>
<td>5.9±0.02</td>
<td>4.67 ***</td>
</tr>
<tr>
<td>Metasoma length</td>
<td>47.1±0.15</td>
<td>52.9±0.18</td>
<td>12.03 ***</td>
</tr>
</tbody>
</table>

MANCOVA: DVs = telson length x width x height

<table>
<thead>
<tr>
<th>Telson length</th>
<th>12.7±0.04</th>
<th>13.1±0.05</th>
<th>3.96 ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telson width</td>
<td>Interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Telson height</td>
<td>5.3±0.02</td>
<td>5.5±0.02</td>
<td>4.55 ***</td>
</tr>
<tr>
<td>Total length</td>
<td>99.1±0.27</td>
<td>105.1±0.32</td>
<td>6.43 ***</td>
</tr>
<tr>
<td>Pectine length</td>
<td>10.0±0.06</td>
<td>12.1±0.08</td>
<td>20.19 ***</td>
</tr>
</tbody>
</table>

Percent difference is calculated as (((male marginal mean – female marginal mean)/(male marginal mean – female marginal mean)/2)) x 100

* P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

"Interaction" indicates a significant interaction between covariate and sex
**Appendix 1e.** Comparison of marginal means (± 1 S.E.) for morphological characters (dependent variables, DVs) of adult male ($N = 65–83$ for each character) and female ($N = 84–90$ for each character) *Hadrurus arizonensis* from MANCOVA (grey shading) and ANCOVA models using metasoma segment 1 width as the reference character (covariates) to assess dimorphism. Analyses conducted using untransformed data.

<table>
<thead>
<tr>
<th>MANCOVA DVs (gray) or ANCOVA DV (white)</th>
<th>Marginal means (mean ± 1 S.E.)</th>
<th>Percent Difference ($♂$ to $♀$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MANCOVA: DVs = prosoma length x width</strong></td>
<td><strong>Female</strong></td>
<td><strong>Male</strong></td>
</tr>
<tr>
<td>Prosoma length</td>
<td>13.1±0.03</td>
<td>12.9±0.04</td>
</tr>
<tr>
<td>Prosoma width</td>
<td>10.5±0.03</td>
<td>10.3±0.04</td>
</tr>
<tr>
<td>Prosoma area</td>
<td>137.9±0.71</td>
<td>132.5±0.80</td>
</tr>
<tr>
<td><strong>MANCOVA: DVs = chela length x width x height</strong></td>
<td><strong>Interaction</strong></td>
<td></td>
</tr>
<tr>
<td>Chela length</td>
<td>19.8±0.06</td>
<td>20.2±0.07</td>
</tr>
<tr>
<td>Chela width</td>
<td>4.4±0.02</td>
<td>4.4±0.02</td>
</tr>
<tr>
<td>Chela height</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MANCOVA: DVs = metasoma segment 1 length x width</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metasoma segment 1 length</td>
<td>6.9±0.03</td>
<td>7.2±0.04</td>
</tr>
<tr>
<td>Metasoma segment 1 width</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metasoma segment 5 length x width</td>
<td>Metasoma segment 5 length</td>
<td>Metasoma segment 5 width</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td></td>
<td>12.9±0.05</td>
<td>13.7±0.05</td>
</tr>
<tr>
<td></td>
<td>5.8±0.02</td>
<td>5.8±0.02</td>
</tr>
<tr>
<td></td>
<td>47.8±0.17</td>
<td>51.9±0.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MANCOVA: DVs = telson length x width x height</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telson length</td>
<td>Interaction</td>
</tr>
<tr>
<td>Telson width</td>
<td>5.8±0.02</td>
</tr>
<tr>
<td>Telson height</td>
<td>5.4±0.02</td>
</tr>
<tr>
<td>Total length</td>
<td>100.4±0.33</td>
</tr>
<tr>
<td>Pectine length</td>
<td>10.0±0.06</td>
</tr>
</tbody>
</table>

Percent difference is calculated as 
\[
\frac{((\text{male marginal mean} - \text{female marginal mean})/((\text{male marginal mean} - \text{female marginal mean})/2)) \times 100
\]

* P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

"Interaction" indicates a significant interaction between covariate and sex.
APPENDIX 2

COMPARISONS OF STATIC ALLOMETRY FOR MORPHOLOGICAL CHARACTERS OF ADULT FEMALE AND MALE \textit{HADRURUS ARIZONENSIS} FROM STANDARD MAJOR AXIS REGRESSION MODELS USING FOUR ALTERNATIVE REFERENCE CHARACTERS (A-D)
**Appendix 2a.** Comparisons of static allometry for morphological characters of adult male (N=64-81) and female (N=83-91) *Hadrurops arizonensis* from standard major axis regression models using prosoma length as the reference character. Slope values of each sex were compared to the theoretical isometric value of 1.0. The male and female slopes were then compared to each other for each morphological character, testing for commonality of slopes and elevations (y-intercept) between the sexes.

<table>
<thead>
<tr>
<th>Character</th>
<th>Sex</th>
<th>R²</th>
<th>Slope (b)</th>
<th>95% CI y-Intercept</th>
<th>95% CI Allometry (H₀=1)</th>
<th>Common Slope (H₀: δ = Φ) [Shift A]</th>
<th>Common Intercept [Shift B]</th>
<th>Shift Along Common Slope [Shift C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prosoma</td>
<td>Female</td>
<td>0.74</td>
<td>1.15 1.03 1.28</td>
<td>-0.26 -0.40 -0.12</td>
<td>6.33 0.014</td>
<td>0.49 0.49</td>
<td>1.39 0.239</td>
<td>4.96 0.026</td>
</tr>
<tr>
<td>Length</td>
<td>Male</td>
<td>0.67</td>
<td>1.08 0.94 1.24</td>
<td>-0.18 -0.35 -0.02</td>
<td>1.14 0.289</td>
<td>0.85 0.36</td>
<td>0.78 0.378</td>
<td>5.60 0.018</td>
</tr>
<tr>
<td>Prosoma</td>
<td>Female</td>
<td>0.92</td>
<td>1.04 0.98 1.10</td>
<td>-0.09 -0.16 -0.02</td>
<td>1.38 0.243</td>
<td>0.02 0.90</td>
<td>145.29 &lt;0.001</td>
<td>0.48 0.489</td>
</tr>
<tr>
<td>Area</td>
<td>Male</td>
<td>0.90</td>
<td>0.99 0.92 1.07</td>
<td>-0.04 -0.12 0.05</td>
<td>0.06 0.803</td>
<td>0.71 0.40</td>
<td>13.92 &lt;0.001</td>
<td>2.03 0.154</td>
</tr>
<tr>
<td>Chela</td>
<td>Female</td>
<td>0.78</td>
<td>0.92 0.83 1.02</td>
<td>0.27 0.17 0.37</td>
<td>2.81 0.097</td>
<td>0.17 0.68</td>
<td>5.44 0.02</td>
<td>2.90 0.089</td>
</tr>
<tr>
<td>Length</td>
<td>Male</td>
<td>0.57</td>
<td>0.93 0.80 1.08</td>
<td>0.27 0.12 0.42</td>
<td>0.95 0.332</td>
<td>0.03 0.87</td>
<td>124.28 &lt;0.001</td>
<td>5.02 0.025</td>
</tr>
<tr>
<td>Chela</td>
<td>Female</td>
<td>0.54</td>
<td>1.16 1.00 1.33</td>
<td>-0.65 -0.84 -0.47</td>
<td>4.01 0.048</td>
<td>0.03 0.87</td>
<td>124.28 &lt;0.001</td>
<td>5.02 0.025</td>
</tr>
<tr>
<td>Height</td>
<td>Male</td>
<td>0.61</td>
<td>1.30 1.13 1.48</td>
<td>-0.61 -0.81 -0.42</td>
<td>15.05 &lt;0.001</td>
<td>0.27 0.61</td>
<td>27.25 &lt;0.001</td>
<td>0.31 0.576</td>
</tr>
<tr>
<td>Metasoma Segment 5 Length</td>
<td>Female</td>
<td>0.70</td>
<td>1.18</td>
<td>1.05</td>
<td>1.33</td>
<td>-0.21</td>
<td>-0.36</td>
<td>-0.06</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.68</td>
<td>1.09</td>
<td>0.95</td>
<td>1.25</td>
<td>-0.07</td>
<td>-0.24</td>
<td>0.10</td>
</tr>
<tr>
<td>Metasoma Segment 5 Width</td>
<td>Female</td>
<td>0.54</td>
<td>1.03</td>
<td>0.89</td>
<td>1.20</td>
<td>-0.39</td>
<td>-0.56</td>
<td>-0.23</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.25</td>
<td>1.15</td>
<td>0.93</td>
<td>1.42</td>
<td>-0.50</td>
<td>-0.78</td>
<td>-0.23</td>
</tr>
<tr>
<td>Metasoma Total Length</td>
<td>Female</td>
<td>0.70</td>
<td>1.24</td>
<td>1.10</td>
<td>1.39</td>
<td>0.29</td>
<td>0.13</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.58</td>
<td>1.10</td>
<td>0.94</td>
<td>1.27</td>
<td>0.50</td>
<td>0.32</td>
<td>0.68</td>
</tr>
<tr>
<td>Telson Length</td>
<td>Female</td>
<td>0.70</td>
<td>1.26</td>
<td>1.12</td>
<td>1.41</td>
<td>-0.30</td>
<td>-0.46</td>
<td>-0.13</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.49</td>
<td>1.18</td>
<td>1.01</td>
<td>1.38</td>
<td>-0.20</td>
<td>-0.41</td>
<td>0.01</td>
</tr>
<tr>
<td>Telson Width</td>
<td>Female</td>
<td>0.56</td>
<td>1.25</td>
<td>1.09</td>
<td>1.44</td>
<td>-0.64</td>
<td>-0.83</td>
<td>-0.44</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.45</td>
<td>1.56</td>
<td>1.32</td>
<td>1.84</td>
<td>-0.97</td>
<td>-1.25</td>
<td>-0.68</td>
</tr>
<tr>
<td>Telson Height</td>
<td>Female</td>
<td>0.60</td>
<td>1.32</td>
<td>1.16</td>
<td>1.51</td>
<td>-0.75</td>
<td>-0.94</td>
<td>-0.55</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.36</td>
<td>1.27</td>
<td>1.14</td>
<td>1.64</td>
<td>-0.79</td>
<td>-1.06</td>
<td>-0.51</td>
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Shift A: represents a difference in slopes between the sexes
Shift B: represents a difference in elevation (y-intercept) while having a common slope between the sexes,
Shift C: represents a shift along a common slope between the sexes
Shift D: represents both a shift in elevation (y-intercept) and along a common slope.
Note: Shift D exists only if both shifts B and C exist
Shifts are defined according to Falster et al. (2006).
**Appendix 2b.** Comparisons of static allometry for morphological characters of adult male (N=64-81) and female (N=83-91) *Hadrurus arizonensis* from standard major axis regression models using prosoma area as the reference character. Slope values of each sex were compared to the theoretical isometric value of 1.0. The male and female slopes were then compared to each other for each morphological character, testing for commonality of slopes and elevations (y-intercept) between the sexes.

<table>
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<tr>
<th>Character</th>
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<th>Slope ($b$)</th>
<th>95% CI</th>
<th>$y$-Intercept</th>
<th>95% CI</th>
<th>Allometry ($H_0=1$)</th>
<th>Common Slope ($H_0: \sigma = \Omega$)</th>
<th>Common Intercept ($H_0: \beta = \Delta$)</th>
<th>Shift Along Common Slope ($H_0: \gamma = \Gamma$)</th>
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<td>0.91</td>
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</tbody>
</table>

Shift A: represents a difference in slopes between the sexes
Shift B: represents a difference in elevation (y-intercept) while having a common slope between the sexes,
Shift C: represents a shift along a common slope between the sexes
Shift D: represents both a shift in elevation (y-intercept) and along a common slope.
Note: Shift D exists only if both shifts B and C exist
Shifts are defined according to Falster et al. (2006).
Appendix 2c. Comparisons of static allometry for morphological characters of adult male (N=64-81) and female (N=83-91) *Hadrurus arizonensis* from standard major axis regression models using total length as the reference character. Slope values of each sex were compared to the theoretical isometric value of 1.0. The male and female slopes were then compared to each other for each morphological character, testing for commonality of slopes and elevations (y-intercept) between the sexes.

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<th>Character</th>
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<th>95% CI Lower</th>
<th>Upper</th>
<th>y-Intercept</th>
<th>95% CI Lower</th>
<th>Upper</th>
<th>Allometry (H₀=1)</th>
<th>F</th>
<th>P</th>
<th>Common Slope (H₀: c = c) [Shift A]</th>
<th>Common Intercept [Shift B]</th>
<th>Shift Along Common Slope [Shift C]</th>
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**Shift A:** represents a difference in slopes between the sexes

**Shift B:** represents a difference in elevation (y-intercept) while having a common slope between the sexes,

**Shift C:** represents a shift along a common slope between the sexes

**Shift D:** represents both a shift in elevation (y-intercept) and along a common slope.

**Note:** Shift D exists only if both shifts B and C exist

Shifts are defined according to Falster et al. (2006).
Appendix 2d. Comparisons of static allometry for morphological characters of adult male (N=64-81) and female (N=83-91) *Hadrurus arizonensis* from standard major axis regression models using metasoma segment 1 width as the reference character. Slope values of each sex were compared to the theoretical isometric value of 1.0. The male and female slopes were then compared to each other for each morphological character, testing for commonality of slopes and elevations (y-intercept) between the sexes.

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<th>95% CI of y-Intercept</th>
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<th>Common Slope (H₀: ( \beta ) = ( \varnothing ))</th>
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Shift A: represents a difference in slopes between the sexes
Shift B: represents a difference in elevation (y-intercept) while having a common slope between the sexes,
Shift C: represents a shift along a common slope between the sexes
Shift D: represents both a shift in elevation (y-intercept) and along a common slope.
Note: Shift D exists only if both shifts B and C exist
Shifts are defined according to Falster et al. (2006).
CHAPTER THREE
VENOM YIELD IN SCORPIONS: A REVIEW OF METHODS, YIELDS, AND POTENTIAL SOURCES OF VARIATION

Gerad A. Fox*

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Abstract

Venom comprises a valuable trait that can increase an organism’s chances for survival. Because venom may represent a significant metabolic expense, natural selection should act to optimize its production and supply. However, venom supply (i.e. venom yield) may be subject to numerous internal (e.g., genetics, age, sex, body size, health, reproductive state, recent usage, regeneration rate, production costs) and external (e.g., season, temperature, humidity, prey availability, prey size, prey susceptibility to venom) influences. In this review, I examined the literature for references relating to venom yield in scorpions to identify any known factors that may influence the expression of venom in this group. Early research on scorpion venom relied predominantly upon extraction from whole telsons, but has transitioned to predominantly electrical extraction methods today. While improved analytical techniques and decreased costs are permitting the characterization of many more species, still only around 70 of the roughly 2,140 scorpion species have been examined, mostly within family Buthidae. The most commonly cited source of variation in venom yield is interspecific differences, but body size, both within and between species, may be equally or more important sources of variation. Other influences include extraction history, sex, geography, diet, season, and, surprisingly, circadian variation. Individual variation also has a significant influence on venom yield, even within relatively homogenous parthenogenic populations. Studies that use voluntary extraction methods must consider the circumstances of extraction, as scorpions are able to control venom expression, and thus influence measures of yield. Values representing venom yield are often referenced in research relating to venom composition, antivenom production, and medical applications of venom. In this literature, venom yield is often tangential to the primary investigation and thus lacks sufficient detail for rigorous
comparisons of yield among such studies. Future research is needed that specifically addresses venom yield and its influences to provide a better understanding of venom supply and expression in scorpions.

**Introduction**

How an organism interacts with its biotic environment is predicated on a suite of characteristics that define its activity and place in its biological community. The careful investigation of defining characters helps elucidate how an organism occupies its niche, and comparisons with other organisms occupying similar niches with alternate character suites can exemplify different strategies for survival. One such defining character is the presence of venom (Duda and Lee, 2009; Sunagar et al., 2016). The deployment of this biochemical system can aid in an organism’s survival through increased predatory efficiency (prey subjugation: Libersat, 2003; Pekár et al., 2014), tracking (Chiszar et al., 2008), digestion (Cohen, 1995; Thomas and Pough, 1979) and defensive capability (Bohlen et al., 2011; 2010; Dutertre et al., 2014; Inceoglu et al., 2003; Siemens et al., 2006). Venom may also act as antimicrobial agents (Baracchi and Tragust, 2015; Obin and Vander Meer, 1985), or as pheromonal signals (Mateus, 2011; Pasteels et al., 1989; Post and Jeanne, 1984).

The presence of venom can act via many avenues to increase an organism’s chances for survival. However, venom synthesis and storage may also represent a significant metabolic expense (McCue, 2006; Nisani et al., 2007; but see Smith et al., 2014). It seems likely that natural selection would act to modulate the supply and production of venom. Indeed, venom availability has been correlated with both an
organism’s physiology (Haight, 2012; 2008; 2002; Haight and Tschinkel, 2003; Klauber, 1997; Kuhn-Nentwig et al., 2016; Malli et al., 1993; Mirtschin et al., 2002; Nisani et al., 2012; Rocha-E-Silva et al., 2009b) and environmental factors (Cooper et al., 2015; Hayes, 2008; Hayes et al., 2002; Morgenstern and King, 2013; Wigger et al., 2002). The interplay between an organism’s internal biology and the demands of the environment predict the evolutionary success of an individual.

Scorpions make a good model system to evaluate venom availability and use. They have been reasonably well characterized in terms of venom extraction methods (Bücherl, 1971; 1953; de Roodt et al., 2012; Gopalakrishnakone et al., 1995; Meadows and Russell, 1970; Sissom et al., 1990; Yaqoob et al., 2016; Zlotkin and Shulov, 1969) and laboratory maintenance (Brenes and Gómez, 2016; Candido and Lucas, 2004; Gopalakrishnakone et al., 1995; Lucas et al., 2010; Nagaraj et al., 2015; Whittemore et al., 1963) largely due to the needs of antibody production. Still rigorous methods have not been applied to evaluate venom availability in the majority of species.

In this review, I examine the literature for references relating to venom yield in scorpions to identify any known factors that may influence the supply and expression of venom. Initial searches were conducted predominantly with Google Scholar, which identified publications related to venom extraction techniques, toxicology, and comparative biology. Any relevant literature was examined to identify previous related literature, and Google Scholar was again used to search for relevant citing articles. Several full text keyword searches were also carried out on a personal digital library of roughly two thousand publications related to scorpion biology.
Methods of Venom Extraction

Venom availability can be evaluated by a variety of means by directly measuring some form of the venom itself, or by indirect estimates based on some feature other than the venom, such as gland size (Bettini, 1978; Bücherl, 1971; Undheim et al., 2015). While indirect methods are informative, little research has been conducted. I therefore focus on direct measures of venom yield which fall into two categories: voluntary and involuntary expression (Glenn and Straight, 1982; Meadows and Russell, 1970). The types of measures used to evaluate availability (dry mass, wet mass, volume, number of lethal doses) may be identical for both categories, although how the venom is obtained varies.

Voluntary measures are the best means to identify the amounts of venom potentially expended in predatory or defensive contexts, as the organism has full control of expulsion, and thus can exemplify "normal" usage (Yahel-Niv and Zlotkin, 1979). Voluntary methods typically induce a scorpion to express venom following sensory stimulation (Schöttler, 1954), or by allowing it to sting an object through a membrane (Zlotkin and Shulov, 1969). Voluntary collections are limited by an organism’s willingness to participate in the process, which can only demonstrate amounts normally expressed and may not equate to total capacity.

Involuntary methods of venom extraction take partial or complete control of venom expulsion away from the organism (Bettini, 1978; Glenn and Straight, 1982), and therefore attempt to demonstrate maximal availability. Expression of venom can be manipulated via electrical stimulation, glandular massage, or administration of induction chemicals, which force venom expulsion (Hill and Mackessy, 1997). More invasively, the venom can be extracted from a surgically interrupted or excised gland by either
directly capturing the glandular products or by trituration of the homogenized tissue to extract the toxic components (Bettini, 1978; Bücherl and Buckley, 1971). While involuntary methods may result in greater yields, there can be substantial drawbacks and implications depending on the nature of the study (Aili et al., 2017; Gopalakrishnakone et al., 1995; Kristensen, 2005; Möller et al., 2013; Perret, 1977; Stahnke, 1978).

**Taxonomic Coverage**

Of the roughly 2,140 extant species of scorpions categorized into 14–22 families (Borges and Graham, 2016; Lourenço, 2015; Soleglad and Fet, 2003), only a small number include any information concerning venom yield. Prior studies have examined representatives of five of the families and 76 species (Table 1). The most widespread, speciose (roughly half of all known scorpion species; Borges and Graham, 2016), and medically relevant family of scorpions is the Buthidae. The majority of species (55) with any yield data are from this family, representing 85 percent of the yield values in the literature. Of the non-Buthid scorpion families, fifteen species are represented from Scorpionidae, three from Caraboctonidae, two from Vaejovidae, and one from Iuridae. To garner a more complete picture of the relevance of venom across scorpion taxa, more non-buthids must be surveyed.

**Nature of Study**

In my search of the literature, I found 75 published accounts that mention specific venom yield values in scorpions. Yet very few of these (6.1%) have venom yield being a primary focus of investigation despite its relevance to envenomation severity (de
Rezende et al., 1996; Hafny et al., 2002; Krifi et al., 1998) and its implications to the biology and ecology of scorpions (Hayes, 2008; Morgenstern and King, 2013; Nisani et al., 2007; Nisani and Hayes, 2011; Wigger et al., 2002). Most studies (62.1%) focus on toxicity and venom characterization. Of these, most are associated with the medical aspects of scorpionism (scorpion stings and their symptomology), and all but a couple of the remaining studies are predicated on biomedical questions. Scorpionism has been an important driver for the development of methodologies to facilitate captive care and venom extraction procedures, which represent 16.7 percent of the yield related literature. General reviews on scorpion biology, venom toxicity, and venom extraction account for six of the publications (9.1%). The dearth of studies related to venom availability and use leaves many biological questions unanswered.

Factors Associated with Venom Yield

Every study which included more than one scorpion species demonstrated differences in yield between species (Table 1). While this is to be anticipated due to differences in the evolutionary history, biology, or ecology of each species, little work has been done to evaluate the correlates of these sources of variation at the family, genus, or even species level. Most of the intraspecific variation that is exhibited across studies can be attributed to methodological differences between the investigations. Differences in yield that are less likely to be methodological artifacts can be attributed to several different factors that I will examine in turn.
**Phylogenetic Variation**

Interspecific variation is the most commonly expressed factor associated with venom yield. Scorpion species that attain a larger body size, for example, should provide larger venom yields (Brenes and Gómez, 2016; Miranda et al., 1970; Nagaraj et al., 2015; Whittemore et al., 1963). Since body size can effect venom yields, it must be taken into consideration for any comparative analysis among taxa (Brown, 2001; Outeda-Jorge et al., 2009; van der Meijden et al., 2010; 2013; Warburg, 2011). However, controlling for the confounding influence of body size has not often been done. Individual scorpion species may also be subject to varying behavioral or ecological pressures that favor different (optimal) quantities of venom availability (Hayes, 2008; Morgenstern and King, 2013). To date, no comparative study of venom supply among scorpion taxa has been conducted, yet we can still identify at least one trend which suggests phylogenetic variation exists. In the comparison of six *Tityus* species, D’Suze et al. (2015b) demonstrated a significant positive linear correlation between average venom yield and mean body size. Interestingly, four of the species across the size range displayed similar toxicities, and therefore yielded increasing lethal potential with increasing species size. Nevertheless, inclusion of more species is needed to verify this relationship. I suggest that future studies which carefully examine both body size and venom yield simultaneously would be useful to identify differences among families, among different genera within individual families, and among species within individual genera. While body size and morphology should be associated with phylogeny, I predict that morphological characteristics should have a greater influence on venom yield then phylogenetic affinity.
Geographic Variation

While it is rather common to find geographic variation in venom composition in many organisms (Binford, 2001; Duda et al., 2009; Fry et al., 2003), including scorpions (Abdel-Rahman et al., 2009; Rodríguez-Ravelo et al., 2013), reports on geographic differences in venom yield are rare. I was able to find examples of variations in yield among different populations of snakes (dry mass, Mirtschin et al., 2002), centipedes (volume and protein concentration, Cooper et al., 2014), and spiders (volume, Binford, 2001). Within scorpions, only three publications provide data on geographical variation in venom yield. De Roodt et al (2009) examined two Argentinian populations of Tityus confluens at a large geographic scale (~550 km), and demonstrated an average two-fold difference in protein content of telson homogenate (with similar differences in toxicity), although no information was provided on potential biological differences between the populations. In Brazil, a scorpionid, Bothriurus bonariensis, from two localities ~300 km apart was found to have a two-fold difference in volume and a difference in protein concentration that resulted in a three-fold difference in dry mass (Santos et al., 2013). At a smaller geographic scale (~110 km), Tityus perijanensis populations were examined at the extremes of its range in Venezuela, yet exhibited no statistical difference in venom dry weight (or toxicity) obtained by voluntary extractions (Borges and Rojasrunjaic, 2007). Most extant scorpions are considered to have limited dispersal potential due to their sedentary nature and specific habitat requirements (Due, 2001; Lira et al., 2016; Lourenço, 2015; 1996; Yamashita and Fet, 2001; Yamashita and Polis, 1995). Thus, populations at greater distances apart are likely to have restricted gene flow, allowing greater divergence in traits (Yamashita and Fet, 2001; Yamashita and Polis, 1995).
Sexual Variation

Sexual dimorphism in venom composition has been demonstrated in several members of both the Buthid (D'Suze et al., 2015b; de Sousa et al., 2010; Miller et al., 2016; Ozkan et al., 2011; Rodríguez-Ravelo et al., 2015) and Scorpionid (Abdel-Rahman, 2008; Schwartz et al., 2008; Yamaji et al., 2004) families of scorpions. These variations demonstrate both qualitative and quantitative shifts in toxin inventories between the sexes, which suggests the possibility of variations in yield as well. Similarly, sexual size dimorphism and sexual body component dimorphism are relatively common in scorpions (Fox et al., 2015), which acts as further impetus to investigate variations in yield, yet this has been only minimally investigated.

While an effect of sexual variation may be mentioned as a possibility in much of the literature, only a few examples were found that provide data on the phenomenon. In Venezuela, two buthid species that both demonstrate sexual dimorphism have been tested for sexual differences in yield. Whereas *Tityus isabelceciliae* demonstrated no sexual dimorphism in yield (González-Sponga et al., 2001), *T. nororientalis* was dimorphic in both toxicity and yield, with females being more toxic, and males having greater yield (Aguilera Rodríguez et al., 2010; Chadee Burgos, 2010; de Sousa et al., 2010). Differences in yield are also present in a North American Buthid, *Centruroides vittatus*, where females demonstrated a greater dry mass of venom and delivered more venom per sting than males, which were found to have a more pain-inducing venom (Miller et al., 2016). When dimorphism was statistically controlled for, body size rather than sex was found to be the main factor influencing venom yield, with the larger size of females conferring greater venom availability.
**Body Size**

Body size is often a good predictor of venom availability in other arachnids, such as spiders (Morgan, 1969; Perret, 1974). Ontogenetic increases in venom yield of spiders has been shown to be linear with respect to body size in some species (Rocha-E-Silva et al., 2009a; Vapenik and Nentwig, 2000), and following a power function in others (Herzig et al., 2004). Similar trends have been suggested in scorpions (Brenes and Gómez, 2016; Miranda et al., 1970; Nagaraj et al., 2015; van der Meijden et al., 2015; Whittemore et al., 1963). However, apart from comparisons among different species of South American *Tityus* scorpions demonstrating a positive linear trend (D'Suze et al., 2015b), the only ontogenetic comparison in scorpions indicated an exponential increase in venom production with increasing body size (age) involved *Hadrurus arizonensis* (Fox et al., 2009). Similar relationships are likely in many scorpion species; however, some species, such as *Pandinus imperator* (Casper, 1985) and *Paruroctonus boreus* (Cushing and Matherne, 1980), exhibit an ontogenetic behavioral shift away from venom use in adults for prey subjugation or defense, relying more, instead, on their pedipal/chelae weapons. If venom is no longer a benefit to survival in the adults of these species, venom production may be limited or degenerate at this stage. Thus, species that are less reliant on venom may not follow the same trends as those that are more dependant on venom throughout their life, but further study is required to evaluate this hypothesis.

**Seasonal Variation**

Scorpion activity is tied to seasonal cycles (Araújo et al., 2010; Nime et al., 2013; Polis, 1980; Schwerdt et al., 2016; Zack and Looney, 2012), and incidences of
scorpionism tend to be related to times of high scorpion activity (Amr, 2015; Chávez-Haro and Ortiz, 2015; Chippaux and Goyffon, 2008; Chowell et al., 2005; D'Suze et al., 2015b; 2015a; Kang and Brooks, 2016; Ortiz et al., 2015; Pucca et al., 2015a). Several authors have suggested seasonal variations in venom yield are likely (Candido and Lucas, 2004; D'Suze et al., 2015b; Magalhães, 1928; Ozkan and Ciftci, 2010; Schöttler, 1954). However, the only author to provide data and statistically evaluate this hypothesis found no seasonal effect on venom yield in three scorpion species representing two families (Grasset et al., 1946). Also, no study has evaluated seasonal differences in envenomation severity. This is not surprising, as scorpionism tends to be underreported worldwide, with reports biased toward more severe cases (Chippaux and Goyffon, 2008; Kang and Brooks, 2016; LoVecchio and McBride, 2003; Pucca et al., 2015b). If seasonal variation can be tied to venom yield, toxicity, or both, envenomation events that produce a less severe symptomology are likely to be ignored, or treated without medical intervention.

Circadian Variation

As in most organisms, scorpions have circadian rhythms tied to both activity (Warburg, 2013a) and physiological function (Warburg, 2013b). Most scorpions tend to be crepuscular or nocturnal in nature (Stockmann, 2015), and the majority of human envenomations occur during these times (Amr, 2015; Chávez-Haro and Ortiz, 2015; Chippaux and Goyffon, 2008; Guerrero-Vargas et al., 2015; Kang and Brooks, 2016). To associate venom yield with envenomation events, Tare et al. (1992) compared extraction efficiency (time to full venom expression, and venom dry mass) at different times of the day in the scorpionid *Heterometrus indus*. Using electric stimulation, venom was
expelled more readily, and in greater quantities, at night than during the day. This study implies a circadian variation in tissue responsiveness to venom expression even under involuntary extraction techniques. If this is the case, scorpions may be less able to utilize their venom stores at some times of the day, resulting in a higher frequency of dry stings. Considering possible temporal variation in venom expression could also be useful in comparing inter-individual yield variations if extractions are carried out at different times, although more research into this possibility needs to be carried out.

**Dietary Influence**

Dietary shifts are likely an important factor in the evolution and maintenance of many venom systems (Barlow et al., 2009; Casewell et al., 2013; Li et al., 2005; Phuong et al., 2016). Different prey species have been implicated in both venom composition (Barlow et al., 2009; Gibbs et al., 2011; Li et al., 2005; Phuong et al., 2016), and the strategic decisions related to venom deployment (as reviewed by, Cooper et al., 2015; Hayes, 2008; Morgenstern and King, 2013). Whereas diet has been suggested to affect scorpion venom composition (Abdel-Rahman et al., 2009), only one study has tested the influence of diet on venom yield. Pucca et al. (2014) found shifts in both composition and yield (dry mass) between groups of *Tityus serrulatus* scorpions fed size-equivalent meals of either crickets or cockroaches after an initial venom extraction event. On the second extraction, the total protein extracted from the cockroach-fed group was twice that of the cricket-fed group, whereas the cricket-fed group displayed greater hyaluronidase activity. Although preliminary, these data imply a plasticity in venom production related
to prey type and nutritional status. Whether the differences are due to changes in protein expression, nutritional limitation, or some other factors remains unknown.

**Single vs Repeated Extractions**

The first milking is almost always the most productive extraction in scorpions (Bravo Salazar, 2010; Bücherl, 1971; Bücherl and Diniz, 1978; Candido and Lucas, 2004; Carvalho Ribeiro and Lira-da-Silva, 2009; D'Suze and Sevcik, 2010; Escobar et al., 2013; Kalapothakis and Chavéz-Olortegui, 1997; Miranda et al., 1970; Nagaraj et al., 2015; Schöttler, 1954; Whittemore et al., 1963; Yaqoob et al., 2016). Only one study (González-Sponga et al., 2001) found no effect of repeated extractions on venom yield. Several explanations have been proffered, relating to either the effect of extraction methods or captivity.

Consequences of extraction method on venom yield can result from both the method and frequency of extraction. Damage to the gland has been suggested with involuntary extractions (electrical extraction, Stahnke, 1978; Yaqoob et al., 2016), yet voluntary extractions can also result in reduced yields (Carvalho Ribeiro and Lira-da-Silva, 2009; Kalapothakis and Chavéz-Olortegui, 1997; Schöttler, 1954). The frequency and interval of extractions is particularly relevant to venom yield, as venom takes time to be regenerated (Alami et al., 2001; Nisani et al., 2012; 2007; Pimenta et al., 2003; Pucca et al., 2014). In scorpions, venom regeneration has been demonstrated to be asynchronous, with volume first (Nisani et al., 2012) to be restored followed by protein content over time (Nisani et al., 2012; Pimenta et al., 2003; Pucca et al., 2014; 2011). The types of protein expressed during venom regeneration are also asynchronous within the
time course of regeneration (Nisani et al., 2012; Pimenta et al., 2003). The amount and type of venom regenerated may also be influenced by the extraction method (Oukkache et al., 2013) and the degree of gland emptying (Nisani et al., 2012). Still more research would be necessary to evaluate these claims in scorpions. Venom yield and composition can also be affected by repeated extractions, which may result in reductions in volume and toxicity (Kalapothakis and Chavéz-Olortegui, 1997; Schöttler, 1954).

While the captive environment is likely to have a pronounced effect on venom productivity, and has been implicated as a contributing factor to reduced yields (Candido and Lucas, 2004; D'Suze et al., 2015b; Nagaraj et al., 2015), there is little information on potential mechanisms. There are several likely factors that could modulate venom production in captivity. As discussed previously, both diet and season may have impacts on venom yield. Although not well characterized in the literature, the captive environment itself likely plays a role in reduced venom yields through potential stress induced by crowding, inadequate housing, temperature, or humidity (Brenes and Gómez, 2016; Candido and Lucas, 2004; Whittemore et al., 1963; Yaqoob et al., 2016).

Senescence is also likely to influence longitudinal venom yields (D'Suze et al., 2015b). In the literature, the examples that provide data on repeated milkings all represent wild caught Buthid scorpions (Table 1). This family is characterized as having a shorter lifespan (3–5 years on average) than other scorpion families investigated (Lourenço, 2002; Polis and Sissom, 1990), and as such, senescence is likely a major contributor to the reduction of yield in repeated milkings.
**Natural Stings**

Absolute yield has been demonstrated to be quite variable, although how an individual scorpion apportions its venom may vary among individual stings (Mohammed, 1942; Yahel-Niv and Zlotkin, 1979; Zlotkin and Shulov, 1969). Manipulations in yield per sting can be controlled by venom flow rate and sting duration (van der Meijden et al., 2015), resulting in delivery of different volumes of venom. The dry mass per sting can also be variable at equal volumes delivered, resulting from sequential venom heterogeneity, which appears to be common within scorpions (Abdel-Rahman et al., 2009; Balozet, 1971; Inceoglu et al., 2003; Latifi and Tabatabai, 1979; Nisani and Hayes, 2011; Sarhan et al., 2013; Yaşmur et al., 2015; Yahel-Niv and Zlotkin, 1979; Zlotkin and Shulov, 1969), and has been characterized as a venom which progresses from transparent, through opalescent, to milky. Venom composition along this continuum is characterized by different protein contents and toxicities (Inceoglu et al., 2003; Yahel-Niv and Zlotkin, 1979). The proportions of each toxin type may also vary by species (Inceoglu et al., 2003; Yahel-Niv and Zlotkin, 1979; Zlotkin and Shulov, 1969). The yield per sting can be influenced behaviorally by threat level, with higher threat situations resulting in a greater yield per sting (Nisani and Hayes, 2011). Predatory context is also likely to affect venom delivery (Casper, 1985; Cushing and Matherne, 1980; Edmunds and Sibly, 2010; Rein, 1993; 2003; Sarhan et al., 2013), although this has not been evaluated in terms of yield.

**Individual Variation**

When examining different yields reported within the same species, it is easy to explain the intraspecific variation as an artifact of different extraction methods,
geographic location, or any of the other sources of variation that have been presented; however, this may not be the complete picture. In other organisms, the amount of individual variation in venom yield can be extreme (Abdel-Aal and Abdel-Baset, 2010; Cooper et al., 2014; Glenn and Straight, 1982; Klauber, 1997; Schöttler, 1951). This also appears to be true of scorpions, for which within-study differences can meet or exceed the between-study variations in average yield (Bücherl, 1953; Hafny et al., 2002; Yahel-Niv and Zlotkin, 1979). Even among the parthenogenetic T. serrulatus, inter-individual variations in venom yield are evident (Kalapothakis and Chavéz-Olortegui, 1997).

Conclusions and Future Directions

There are thousands of publications relating to the toxicity of scorpion venom, identifying its composition (Abdel-Rahman et al., 2014; 2016; Almaaytah and Albalas, 2014; Rendón-Anaya et al., 2015), mechanism of action (Adi-Bessalem et al., 2015; Laraba-Djebari et al., 2015; Quintero-Hernández et al., 2013; M. S. V. Santos et al., 2016), and biotechnological potential (Fratini et al., 2017; Harrison et al., 2014; Ortiz et al., 2015; Ortiz and Possani, 2015; X. Wang and G. Wang, 2016). The literature on venom yield, in stark contrast, remains limited. While venom is extracted to meet the growing needs of biomedical research, less attention is paid to the biology of the animal at its source, as such details often peripheral to the research goals of specific studies. This is an understandable situation with limited available funding. As a result, less is known about the biology and ecology of venom use in scorpions and other venomous taxa than might be expected.
Venom yield has been better characterized in spiders (Herzig, 2010; Herzig et al., 2002; 2008; Rocha-E-Silva et al., 2009b; Wiener, 1959) and, especially, snakes (Chippaux et al., 1991; de Roodt et al., 2016; Fix, 1980; Glenn and Straight, 1982; Hill and Mackessy, 1997; Mirtschin et al., 2002; 2006; Tare and Sutar, 1986), yet is still more advanced in scorpions than most other venomous taxa. Few studies have focused on specific factors that can influence venom yield, but the data from methodological and toxicological studies can still give insights into factors that affect venom yield.

Much of the variation in scorpion venom yield relates to phylogeny, as species differences in yield are frequently reported. This relationship, however, has not been well characterized statistically, and differences in body size and ecology have not been accounted for. Most of the available also data stems from a single family of scorpions which may not be representative of the other families. Current information suggests that body size accounts for the majority of the variation both within and between scorpion species.

The literature suggests several other factors that may exert influence, including geographic location, sex, diet, and season. Perhaps the most unexpected source of variation is the potential for circadian variation, which was even evident under involuntary extraction techniques. As in other taxa, individual variation has a large effect on venom yield, which is particularly influential in voluntary extraction techniques, as scorpions appear to be capable of metering venom during stinging. Individual variation may result from involuntary methods as well, making sample size an important variable for estimating yield values for a given species.
An influential source of variation in venom yield for experimental studies relates to the effect of captivity, including prior venom extraction history. Captivity in general, and number of prior venom extractions, result in reductions and can potentially bias studies of venom yield. Currently, no study has tried to disentangle these two sources of variation. It would be interesting to determine whether the effect of captivity could be mitigated by better husbandry, and whether repeated milkings could be carried out in a field situation without reductions in yield.

While toxicity is a key factor in evaluating medical potential of a species, venom yield is also integral in determining a species lethal potential and epidemiology. Scorpion size has already been tied to sting severity, but if geography, season, or circadian variation can also be verified in terms of yield, treatment protocols related to scorpionism could be modified. Clearly, future studies need to take into account the potential sources of variation that exist within this group when evaluating venom related questions.
<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Method</th>
<th>Nature of Study</th>
<th>Age Class</th>
<th>Measure</th>
<th>Venom Yield</th>
<th>Dry</th>
<th>Variation</th>
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<tr>
<td>B</td>
<td>Androctonus amoreuxi</td>
<td>TH</td>
<td>Characterization</td>
<td>–</td>
<td>PA</td>
<td>9.18 +/- 0.13 mg/ml</td>
<td>Interspecies</td>
<td>(Salama and Sharshar, 2013)</td>
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<td>Venom Flow rate</td>
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<td>AI</td>
<td>0.867 +/- 0.790 ul</td>
<td>Interspecies</td>
<td>(van der Meijden et al., 2015)</td>
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<td>PA</td>
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<td>–</td>
<td>(Wilson, 1904)</td>
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<td>Interspecies</td>
<td>(Hassan, 1984)</td>
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<td>Review</td>
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<td>8.9mg</td>
<td>–</td>
<td>(Phisalix, 1922)</td>
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<td>E</td>
<td>Toxicity, antivenom</td>
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<td>1.3mg</td>
<td>–</td>
<td>(Sergent, 1938)</td>
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<td>Characterization</td>
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<td>1.38mg</td>
<td>–</td>
<td>(Lucien Balozet, 1955)</td>
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<td>Review</td>
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<td>(Junqua and Vachon, 1968)</td>
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<td>2.45mg</td>
<td>–</td>
<td>(Junqua and Vachon, 1968)</td>
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<td>Purification</td>
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<td>1.5-2mg</td>
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<td>(Miranda et al., 1970)</td>
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<td>Review</td>
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<td>1.4mg</td>
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<td>(Lucien Balozet, 1971)</td>
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<td>AI</td>
<td>E = 2.4 +/-.1.1mg; M =0.74 to 0.56mg</td>
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<td>1.12mg</td>
<td>–</td>
<td>(van der Meijden et al., 2017)</td>
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<td>7.4 +/-0.1 ml/ml</td>
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<td>Androctonus crassicauda</td>
<td>TH, E</td>
<td>Venom Characterization</td>
<td>–</td>
<td>PA</td>
<td>All TH = 0.5mg, E= 0.3mg, size matched TH = 0.9 +/-0.1mg, E= 0.4 +/-0.2mg</td>
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<td>8.01 +/-0.002mg/ml</td>
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<td>Method</td>
<td>–</td>
<td>AI</td>
<td>7.14 +/-1.2ul</td>
<td>Repeated extractions</td>
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<td>AI</td>
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<td>Individual variation</td>
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<td>(Lafforgue, 1900)</td>
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**Other notes:**
- Mentions size, and repeated extracts
- Female = 846.2 ± 63.9 ug, Male = 425.3 ± 12.1ug per defensive sting. Calculated total Ave F = 4.61179mg, M = 2.16903.
- All TH = 0.1mg, E = 0.005mg; size matched TH = 0.1 ± 0.02mg, E = 0.4 ± 0.02mg.
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<td>E 0.6 mg, M 0.2-0.5 mg</td>
<td>AL</td>
<td>Extraction Method</td>
<td>(Mohammed, 1942)</td>
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<tr>
<td>B <em>Leiurus quinquestriatus</em></td>
<td>M</td>
<td>Method</td>
<td>Adult</td>
<td>22 +/- 0.5 ul</td>
<td>Extraction Method</td>
<td>(Zlotkin and Shulov, 1969)</td>
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<tr>
<td>B <em>Leiurus quinquestriatus</em></td>
<td>M</td>
<td>Venom Characterization</td>
<td>18.5-22 ul, 36 +/- 11 ul</td>
<td>PA</td>
<td>Individual variation, sequential</td>
<td>(Yael-Nir and Zlotkin, 1979)</td>
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<tr>
<td>B <em>Leiurus quinquestriatus</em></td>
<td>TH</td>
<td>Antivenom</td>
<td>0-1.5 mg</td>
<td>PA</td>
<td>Interspecies</td>
<td>(Hassan, 1984)</td>
<td></td>
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<tr>
<td>B <em>Mesobuthus eupeus</em></td>
<td>TH, E</td>
<td>Venom Characterization</td>
<td>All TH = 0.5 mg, E = 0.1 mg, size matched TH = 1.5 +/- 0.75 mg, E = 0.5 +/- 0.2 mg</td>
<td>PA</td>
<td>Extraction Method, Sequential</td>
<td>(Latifi and Tabataba'i, 1985)</td>
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<tr>
<td>B <em>Mesobuthus eupeus</em></td>
<td>E</td>
<td>Venom Characterization</td>
<td>37.47 +/- 4.2 mg/ml, 38.19 +/- 6.1 mg/ml</td>
<td>PA</td>
<td>Interspecies, Sex</td>
<td>(Ozkan et al., 2011)</td>
<td></td>
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<tr>
<td>B <em>Mesobuthus gibbosus</em></td>
<td>E</td>
<td>Venom Characterization</td>
<td>4 mg/ml (Min: 30 mg/ml - Max: 49 mg/ml)</td>
<td>PA</td>
<td>Interspecies, Sex</td>
<td>(Ozkan et al., 2011)</td>
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<tr>
<td>B <em>Mesobuthus tamulus</em></td>
<td>E</td>
<td>Method</td>
<td>4.9 +/- 0.31 ul</td>
<td>AL</td>
<td>Repeated extractions</td>
<td>(Yaqoob et al., 2016)</td>
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<tr>
<td>B <em>Odontobuthus dorai</em></td>
<td>TH, E</td>
<td>Venom Characterization</td>
<td>All TH = 1.7 mg, E = 0.6 mg, size matched TH = 1.5 +/- 0.75 mg, E = 0.5 +/- 0.2 mg</td>
<td>PA</td>
<td>Extraction Method, Sequential</td>
<td>(Latifi and Tabataba'i, 1985)</td>
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<tr>
<td>B <em>Odontobuthus odonturus</em></td>
<td>E</td>
<td>Method</td>
<td>5.2 +/- 0.37 ul</td>
<td>AL</td>
<td>Repeated extractions</td>
<td>(Yaqoob et al., 2016)</td>
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<tr>
<td>B <em>Orthochirus innesi</em></td>
<td>TH</td>
<td>Venom Characterization</td>
<td>1.7 +/- 0.3 mg/ml</td>
<td>PA</td>
<td>Interspecies</td>
<td>(Salama and Sharshar, 2013)</td>
<td></td>
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<tr>
<td>B <em>Parabuthus transvaalicus</em></td>
<td>E</td>
<td>Note</td>
<td>1.08 mg/g scorpion</td>
<td>PA</td>
<td>Interspecies</td>
<td>(Newlands, 1974)</td>
<td></td>
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<tr>
<td>B <em>Parabuthus transvaalicus</em></td>
<td>M</td>
<td>Venom Characterization</td>
<td>1.2 +/- 0.6 mg</td>
<td>AL</td>
<td>Sequential</td>
<td>(Inceoglu et al., 2003)</td>
<td></td>
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<tr>
<td>B <em>Parabuthus</em></td>
<td>M</td>
<td>Venom</td>
<td>Adult</td>
<td>Milk</td>
<td>Repeated</td>
<td>(Nisani et al., 2003)</td>
<td></td>
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<tr>
<td>Species</td>
<td>Type</td>
<td>Sex</td>
<td>Age</td>
<td>Toxicity</td>
<td>Yield</td>
<td>Size</td>
<td>Method</td>
<td>Notes</td>
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<tr>
<td>Parabuthus transvaalicus M</td>
<td>Regeneration</td>
<td>Adult female</td>
<td>AI</td>
<td>Milk1=37.23ug; Milk2=37.23ug and Milk1=69.87ug; Milk2=18.49ug (regenerated)</td>
<td>39.69 +/- 9.23ug; Milk2=37</td>
<td>High Threat = 1.38 +/- 0.15ul per sting; Low threat = 0.62 +/- 0.07ul per sting Day2<del>75 %, Day4</del>70 %, Day6<del>85 %, Day8</del>100%</td>
<td>Repeated (regenerated)</td>
<td>(Nisani et al., 2012)</td>
<td></td>
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<tr>
<td>Parabuthus transvaalicus M</td>
<td>Defensive behavior</td>
<td>Adult female</td>
<td>AI</td>
<td>Milk2=37.23ug; Milk1=69.87ug; Milk2=18.49ug (regenerated)</td>
<td>39.69 +/- 9.23ug; Milk2=37</td>
<td>High Threat = 1.38 +/- 0.15ul per sting; Low threat = 0.62 +/- 0.07ul per sting Day2<del>75 %, Day4</del>70 %, Day6<del>85 %, Day8</del>100%</td>
<td>Behavioral (high low threat), sequential</td>
<td>(Nisani and Hayes, 2011)</td>
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<tr>
<td>Parabuthus transvaalicus M</td>
<td>Regeneration</td>
<td>Adult Female</td>
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<tr>
<td>Parabuthus transvaalicus, and P. triradulatus Cap</td>
<td>Toxicity, antivenom</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>4.8mg</td>
<td></td>
<td>Inter species, no effect of season</td>
<td>(Grasset et al., 1946)</td>
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<tr>
<td>Rhopalurus laticauda E</td>
<td>Toxicity</td>
<td>–</td>
<td>AI</td>
<td></td>
<td>2.07ul</td>
<td></td>
<td>Interspecies</td>
<td>(Yeguez Cabeza, 2010)</td>
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<tr>
<td>Rhopalurus laticauda E</td>
<td>Yield</td>
<td>–</td>
<td>AI</td>
<td></td>
<td>2.07ul</td>
<td></td>
<td>Interspecies</td>
<td>(Cordova Aguir and Prino Valor, 2012)</td>
<td></td>
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<tr>
<td>Tityus bahiensis M</td>
<td>Toxicity</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>1.1-1.4mg</td>
<td></td>
<td>Inter species, Seasonal Suggested</td>
<td>(Magalhães, 1928)</td>
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<tr>
<td>Tityus bahiensis Heat, M. TH</td>
<td>Toxicity</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>36-92ug</td>
<td></td>
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<tr>
<td>Tityus bahiensis M.E</td>
<td>Scorpion maintenance and venom extraction</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>M=0.0765mg; E=0.23mg</td>
<td></td>
<td>Method, Interspecies</td>
<td>(Bücherl, 1953)</td>
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<tr>
<td>Tityus bahiensis M</td>
<td>Toxicity</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>Milk1=95ug; Milk2=38ug</td>
<td></td>
<td>Repeated, interspecies</td>
<td>(Schöttler, 1954)</td>
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<tr>
<td>Tityus bahiensis TH</td>
<td>Toxicity</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>0.21mg</td>
<td></td>
<td></td>
<td>(Diniz and Goncalves, 1956)</td>
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<tr>
<td>Tityus bahiensis E</td>
<td>Review</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>0.39mg</td>
<td></td>
<td></td>
<td>(Bücherl, 1971)</td>
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<tr>
<td>Tityus bahiensis E</td>
<td>Review</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>0.113mg; 1951-1953; 0.113mg; 1953, 0.113mg; 1963, 0.39mg</td>
<td></td>
<td>Repeated extractions</td>
<td>(Bücherl and Diniz, 1978)</td>
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<tr>
<td>Tityus bahiensis E</td>
<td>Yield</td>
<td>–</td>
<td>I</td>
<td></td>
<td>390ug</td>
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<tr>
<td>Tityus brasiliensis M</td>
<td>Yield</td>
<td>Adults and Juveniles</td>
<td>PA</td>
<td></td>
<td>33.02 +/- 20.23ug</td>
<td></td>
<td>Repeated</td>
<td>(Carvalho Ribeiro and Lira-da-Silva, 2009)</td>
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<tr>
<td>Tityus championi E</td>
<td>Method</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>0.39mg</td>
<td></td>
<td>Interspecies, Size</td>
<td>(Carvalho Ribeiro and Lira-da-Silva, 2009)</td>
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<tr>
<td>Tityus challanis E</td>
<td>Venom variability</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>78ug</td>
<td></td>
<td>Interspecies, Size, Repeated, captivity</td>
<td>(Brenes and Gómez, 2016)</td>
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<td>Tityus confusus TH, E</td>
<td>Toxicity, Region</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>TH (Jujuy) = 0.336mg; TH (Catamarca) = 0.161mg; E (Catamarca, Larioja) = 68.5ug</td>
<td></td>
<td>Extraction Method, Regional</td>
<td>(de Roodt et al., 2009)</td>
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<tr>
<td>Tityus costatus E</td>
<td>Yield</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>200ug</td>
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<tr>
<td>Tityus discrepans E</td>
<td>Toxicity</td>
<td>–</td>
<td>PA</td>
<td></td>
<td>0.5-1.5mg</td>
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<tr>
<td>Species</td>
<td>Sex</td>
<td>Venom Characterization</td>
<td>Size</td>
<td>AI</td>
<td>Venom Toxicity or Protein</td>
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<tr>
<td><em>Tityus discrepans</em></td>
<td>M</td>
<td>Venom Characterization</td>
<td>–</td>
<td>Al</td>
<td>0.54 +/- 0.10mg Interspecies</td>
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<tr>
<td><em>Tityus discrepans</em></td>
<td>E</td>
<td>Venom variability</td>
<td>–</td>
<td>PA</td>
<td>718.8ug Interspecies, Size, Repeated, captivity</td>
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<tr>
<td><em>Tityus falconensis</em></td>
<td>E</td>
<td>Venom variability</td>
<td>–</td>
<td>PA</td>
<td>487.5ug Interspecies, Size, Repeated, captivity</td>
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<td><em>Tityus funestus</em></td>
<td>E</td>
<td>Venom variability</td>
<td>–</td>
<td>PA</td>
<td>462.5ug Interspecies, Size, Repeated, captivity</td>
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<td><em>Tityus gonzalezponga</em></td>
<td>E</td>
<td>Characterization</td>
<td>–</td>
<td>Al</td>
<td>917ug Interspecies, Size, Repeated, captivity</td>
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<td><em>Tityus isabelceci</em></td>
<td>E</td>
<td>Characterization</td>
<td>–</td>
<td>Al</td>
<td>0.2-1.8mg protein no effect of mass, repeated milk, sex</td>
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<td><em>Tityus kaderkai</em></td>
<td>E</td>
<td>Characterization</td>
<td>Adult</td>
<td>Al</td>
<td>0.076mg (5.7, 4.4 and 2.8 mg of venom of 74, 56 and 39)</td>
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<tr>
<td><em>Tityus macrochirus</em></td>
<td>E</td>
<td>Characterization</td>
<td>–</td>
<td>Al</td>
<td>5.43ug (calculated from 1.81 mg/ml)</td>
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<tr>
<td><em>Tityus nororientalis</em></td>
<td>E</td>
<td>Characterization</td>
<td>–</td>
<td>Al</td>
<td>Male =2.77mg, Females = 1.18mg</td>
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<tr>
<td><em>Tityus nororientalis</em></td>
<td>E</td>
<td>Toxicity</td>
<td>–</td>
<td>Al</td>
<td>Male =2.30mg, Females = 0.98mg Sex</td>
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<tr>
<td><em>Tityus nororientalis</em></td>
<td>E</td>
<td>Venom Characterization</td>
<td>Adult</td>
<td>Al</td>
<td>Female = 0.99mg; Males = 2.39mg Sexual effect of volume, protein, toxicity and composition</td>
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<td><em>Tityus nororientalis</em></td>
<td>E</td>
<td>Toxicity</td>
<td>–</td>
<td>Al</td>
<td>1.45mg Interspecies</td>
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<tr>
<td><em>Tityus nororientalis</em></td>
<td>E</td>
<td>Yield</td>
<td>–</td>
<td>Al</td>
<td>1.64ug Interspecies</td>
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<tr>
<td><em>Tityus pachyurus</em></td>
<td>E</td>
<td>Method</td>
<td>–</td>
<td>PA</td>
<td>0.11mg Interspecies, Size</td>
<td></td>
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<tr>
<td><em>Tityus pachyurus</em></td>
<td>M</td>
<td>Venom Characterization</td>
<td>Adult</td>
<td>Al</td>
<td>0.688 +/- 0.2 mg (0.3-1mg per Individual milk)</td>
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<tr>
<td><em>Tityus perijanensis</em></td>
<td>M</td>
<td>Venom Characterization</td>
<td>–</td>
<td>Al</td>
<td>3.04 +/- 0.2mg (La orchila), 3.2+/- 0.1mg (ipika) Interspecific variation but no effect of distribution on toxicity, or electrophoretic fingerprint Interspecies,</td>
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<tr>
<td><em>Tityus serralatu</em></td>
<td>M</td>
<td>Toxicity</td>
<td>–</td>
<td>PA</td>
<td>53-136ug Method, Interspecies,</td>
<td></td>
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<tr>
<td><em>Tityus serralatu</em></td>
<td>M,E</td>
<td>Scorpion maintenance and venom</td>
<td>–</td>
<td>PA</td>
<td>M=0.053mg; E = 0.338mg</td>
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(Borges et al., 2004)
(D’Suze et al., 2015b)
(D’Suze et al., 2015b)
(D’Suze et al., 2015b)
(Bravo Salazar, 2010)
(González-Sponga et al., 2001)
(Escobar et al., 2013)
(Rincón-Cortés et al., 2016)
(Aguilera Rodriguez et al., 2010)
(Chadee Burgos, 2010)
(de Sousa et al., 2010)
(Yeguez Cabeza, 2010)
(Cordova Aguir and Pinto Valor, 2012)
(Brenes and Gómez, 2016)
(Barona et al., 2006)
(Borges and Rojasrunjaic, 2007)
(Magalhães, 1928)
(Bücherl, 1953)
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<td>252 +/- 29.85ug</td>
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<td>PA</td>
<td>1.9ul (0.8-3.8ul); 46.2 +/- 21.2ug/ul (15.1-73.1ug/ul)</td>
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<td>PA</td>
<td>3.33ul, Area 2 (Cerro do Batovi) 1.86ul</td>
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<td>Area 1 (Calculated from 3.34ug/ml) =11.16 ug, Area 2 (Calculated from 2.25ug/ml) = 3.79ug</td>
<td>(D. S. D. Santos et al., 2013)</td>
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<td></td>
<td>(Ramos and Escobar, 2007)</td>
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<td>(Antony Gomes and Aparna Gomes, 2004)</td>
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<td>0.937 +/- 0.710ul</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<td>PA</td>
<td>4.2mg</td>
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**Abbreviations**

B = Buthidae  
C = Caraboctonidae  
I = Iuridae  
S = Scorpionidae  
V = Vaejovidae  
M = Manual venom expression  
E = Electrical venom expression  
TH = Telson Homogenization  
Comp = Venom expression by compression  
Cap = Venom expression by Capillary  
PA = Pooled and averaged venom yield  
AI = Averaged individual measures of venom yield
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CHAPTER FOUR

VARIATION IN VENOM YIELD AND PROTEIN CONCENTRATION
IN THE DESERT HAIRY SCORPION, *HADRURUS ARIZONENSIS*

Gerad A. Fox, Allen M. Cooper, William K. Hayes

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24941 Stewart St., Loma Linda, CA, 92350, USA*
Abstract

Many animals benefit from possessing a toxic secretion that can be used for predation, defense, and other purposes. Any such secretion has accompanying metabolic and ecological costs for production and storage. Thus, as a limited commodity, the quantity of venom available to an organism and its protein complement should be optimized by selection. In scorpions, venom plays a significant role in prey capture, defense, and potentially mating; however, venom availability, which can constrain venom deployment, has received limited attention. To this end, we investigated how body size and other variables affect volume yield and protein concentration of electrically extracted venom in the North American scorpion *Hadrurus arizonensis*. Venom yield was strongly and exponentially related to overall body size, and weakly proportional to relative telson size, but was similar for the two sexes, independent of relative mass (body condition), and similar for the two milking groups (corresponding to season and/or duration in captivity). Compared to venom yield, venom protein concentration was much less dependent on overall body size, though there was a weak negative relationship. Protein concentration varied most among the milking groups (declining with duration in captivity and/or shift from fall to winter), and to a lesser extent between the sexes (greater in females than in males), with relative telson size and body condition having no measurable influence. When individual scorpions were subjected to repeated venom extractions at 21-day intervals, each extraction resulted in consistent volume yields, but reductions in protein concentration were evident over time. These findings offer meaningful insights regarding design of the scorpion venom delivery system, constraints on venom deployment, appropriate milking regimens for sustainable venom production, and medical risks and symptoms associated with scorpionism.
Introduction

How an organism interacts with its biotic environment is predicated on a suite of characteristics that define its activity and place in a biological community. Careful investigation of such defining characters elucidates how an organism occupies its niche. One unique character is the presence of venom and its delivery apparatus (Duda and Lee, 2009; Nelsen et al., 2014b; Sunagar et al., 2016). The deployment of this biochemical system can aid an organism’s survival through increased predatory efficiency via prey subjugation (Libersat, 2003; Pekár et al., 2014), tracking of prey released after envenomation (Chiszar et al., 2008; Saviola et al., 2013), and digestion (Cohen, 1995; Thomas and Pough, 1979). Venom also provides an effective deterrent against predators (Bohlen et al., 2011; 2010; Dutertre et al., 2014; Inceoglu et al., 2003; Siemens et al., 2006), and may even act as antimicrobial agents (Baracchi and Tragust, 2015; Obin and Vander Meer, 1985), or as pheromonal signals (Mateus, 2011; Pasteels et al., 1989; Post and Jeanne, 1984).

Although venom can enhance survival, it may also represent a significant expense, both metabolically (McCue, 2006; Nisani et al., 2007; but see Smith et al., 2014) and ecologically (Cooper et al., 2015; Hayes, 2008). It therefore seems likely that natural selection would act to modulate the production and stored supply of venom, which influence the quantity of venom available for deployment. As a limited commodity, venom availability and its use have been tied to factors relating to the organism’s internal state (Haight, 2012; 2008; 2002; Haight and Tschinkel, 2003; Klauber, 1997; Kuhn-Nentwig et al., 2016; Malli et al., 1993; Mirtschin et al., 2002; Nisani et al., 2012; Rocha-E-Silva et al., 2009b) and external influences (Cooper et al., 2015; Hayes, 2008; Hayes et al., 2002; Morgenstern and King, 2013; Wigger et al.,
How well venom availability is balanced or optimized among these factors will presumably affect evolutionary success.

The total venom available is defined as the yield. Venom yield can be measured as volume, wet mass, dry mass, or number of lethal doses. The relationship between volume or wet mass and dry mass depends on protein concentration, which can influence toxicity, and therefore should be measured as well. Obtaining venom has been accomplished by a variety of means that can be grouped into two categories: voluntary and involuntary. Voluntary methods rely on natural bites and stings, with the organism having full control of venom expulsion, and may best exemplify “normal” usage in predatory or defensive contexts. However, voluntary venom collection is limited by an organism's willingness to participate in the process and can only demonstrate amounts normally expended and not total capacity. Involuntary methods take control of expulsion away from the organism by use of artificial extraction (Bettini, 1978; Glenn and Straight, 1982). Venom can be expelled by electrical stimulation, glandular massage, or administration of induction chemicals (Hill and Mackessy, 1997). These methods attempt to demonstrate maximum availability, which may exceed functional availability and induce other artifacts that need to be considered (e.g., cell damage or contamination; Gopalakrishnakone et al., 1995; Kristensen, 2005; Perret, 1977a). Venom availability can also be estimated by in vivo imaging of the gland (e.g., computed tomography, magnetic resonance imaging; Undheim et al., 2015), venom gland excision with manual expression or trituration (Bettini, 1978; Bücherl, 1971), and classical histology of the gland (Bettini, 1978; Bücherl, 1971).
Scorpions comprise a good model system to evaluate the factors that influence venom availability and protein concentration: they can often be collected in large numbers (Polis, 2001; 1990a), maintained in captivity at low cost with relative ease (Brenes and Gómez, 2016; Bücherl, 1953a; Candido and Lucas, 2004; Gopalakrishnakone et al., 1995; Whittemore et al., 1963), and as invertebrates require minimal institutional oversight. Scorpions possess paired venom glands in the telson, which is the terminal segment of the tail-like metasoma (Hjelle, 1990). The telson includes a pointed tip, the aculus, which can be thrust into the soft tissues of a prey animal or potential predator and functions as a stinger by delivering venom into the target (Hjelle, 1990). In a few species, the venom can be delivered by spraying in addition to stinging (Newlands, 1974). Scorpions, like other venomous animals (Cooper et al., 2015; Hayes, 2008; Hayes et al., 2002; Nelsen et al., 2014a; Wigger et al., 2002), make decisions about whether to use their venom and how much venom to expulse with individual stings and sprays (Bub and Bowerman, 1979; Casper, 1985; Edmunds and Sibly, 2010; Nisani and Hayes, 2015; 2011; Rein, 1993). Because the amount of venom expended during stings and sprays is influenced in large part by the quantity of venom available, as well as the duration and rate at which venom is expulsed (van der Meijden et al., 2015), knowledge of venom yields can be helpful in understanding design of the venom delivery system, strategies of venom deployment, appropriate regimens for sustainable venom production, and medical risks and symptoms associated with scorpionism.

An estimated 2,000 scorpion species exist (Lourenço, 2015), yet researchers have examined venom yield in only a handful of these. Most of our knowledge derives from
studies that required venom collection from medically important species for biochemical analyses, with venom yields reported only incidentally. Consequently, our understanding of the factors that influence venom yield and venom protein concentration in scorpions remains remarkably deficient. We know much more about these parameters in other taxonomic groups, such as spiders (e.g., Herzig, 2010; Wong et al., 2016), centipedes (e.g., Cooper et al., 2014a), and snakes (e.g., Chippaux et al., 1991; Mirtschin et al., 2002).

Here, we characterize the factors associated with venom yield and venom protein concentration in the Desert Hairy Scorpion, *Hadrurus arizonensis*. The factors we examined included body size, relative telson size, body condition, sex, season and/or duration in captivity, and multiple venom extractions. The species occupies low- to mid-elevation desert flats, dunes, washes, and lower mountain slopes of the Mojave and Sonoran deserts of North America (Stahnke, 1945; Williams, 1970a; 1970b). It consumes a broad prey base, including both invertebrates and vertebrates (McCormick and Polis, 1982; Polis and McCormick, 1986), and the venom is critical for incapacitating larger prey (Bub and Bowerman, 1979). Medically, this species possesses a rather benign venom (Saunders and Johnson, 1970; Stahnke, 1945), but because it is the largest scorpion in North America, it presumably delivers large quantities of venom when stinging defensively. Males also employ a sexual sting during mating with females (Tallarovic, 2000; Tallarovic et al., 2000), and venom has been suggested to play a role in this behavior (Polis and Sissom, 1990; Yamaji et al., 2004). As in other scorpions, venom expulsion appears to be heterogeneous (Fox et al., 2009), with the initial secretion emerging clear and presumably potassium-rich, and then transitioning to an opaque
(milky), presumably protein-rich and more toxic secretion (Inceoglu et al., 2003; Nisani and Hayes, 2011; Yahel-Niv and Zlotkin, 1979). We further characterized this heterogeneity.

Materials and Methods

Scorpions

We collected *H. arizonensis* from two general locations (33.898354, -116.682936; 33.910966, -116.651685) in the western Sonoran Desert between the city of Cabazon and the town of Whitewater, Riverside County, California, USA. We captured scorpions at night during the months of July to October using ultraviolet light sources (Stahnke, 1972). Scorpions were housed individually in 17 × 15 × 7 cm (L × W × H) plastic containers with sand substrate and kept at 24–26 °C on a 12:12 hr light:dark cycle. We offered each scorpion a size-appropriate cricket (c.f. Edmunds and Sibly, 2010) every 3 weeks.

We measured six morphometric characters from each scorpion, including total length (anterior prosoma edge to posterior edge of metasoma segment 5); metasoma segment 1 width (MS1W); telson length, width, and height (all measures to nearest 0.1 mm using digital calipers); and mass (nearest 0.1 g). Because most characters, including total length, are sexually dimorphic in *H. arizonensis*, we used a neutral or unbiased measure, MS1W, as a proxy for overall body size (Fox et al., 2015). We derived measures for relative mass (body condition) and relative telson size (see section on statistical analysis). We determined sex of the scorpions by relative length and
arrangement of the pectines (Polis, 1990b). We excluded all individuals that appeared to be emaciated or gravid, but did not record the numbers.

**Venom Extraction and Venom Volume Determination**

To extract venom, we first immobilized scorpions in a restraining device (Gopalakrishnakone et al., 1995; Whittemore et al., 1963) with the telson protruding. Preliminary venom extractions using manual (voluntary) expression (Nisani et al., 2007; Nisani and Hayes, 2011) proved to be less effective than electrical stimulation; therefore, we obtained venom by electrical stimulation (12 V, 500 mA, DC) via forceps (Lowe and Farrell, 2011) applied to opposite sides of the telson with electrolyte solution added to increase conductivity. The number (generally 10–20) and duration (generally 0.3–5 sec) of shocks delivered varied among animals, with shocks continuing until venom expulsion ceased or venom became mucoid. We collected venom using graduated 5-μL Drummond® PCR micropipettes (0.246 mm radius; PGC Scientifics, Garner, NC, USA). We viewed the micropipette under a Carson Linen-Test Magnifier (Carson Optical Inc., Hauppauge, NY, USA) to determine venom volume. We calculated volume of venom (V) from the length of venom column in the micropipette (L) using the formula \( V = (L) \times (0.246^2) \times (3.14159) \). We also assessed venom samples visually during collection, noting whether they were clear, opalescent, or milky, indicative of venom heterogeneity (Nisani and Hayes, 2011; Yahel-Niv and Zlotkin, 1979). Individual venom samples were transferred to and stored in microcentrifuge tubes at −20 °C until protein quantification. All scorpions were extracted after a fast of 21–25 days to ensure replete venom glands (Boeve et al., 1995; Candido and Lucas, 2004; Gopalakrishnakone et al., 1995).
Single Venom Extractions

To assess the influences of body size and sex on venom yield and protein concentration, we obtained venom from two groups of scorpions: milking group 1 \((n = 156; \, 70 \, \delta \delta, \, 86 \, \varphi \varphi)\), collected August–September 2008 and milked February 2009 (5–6 months in captivity), and milking group 2 \((n = 53; \, 26 \, \delta \delta, \, 27 \, \varphi \varphi)\), collected August 2014 and milked October 2014 (2 months in captivity). Thus, the two milking groups differed in both season of venom collection and duration in captivity. None of the specimens had been subjected previously to venom extraction. Body size of scorpions from both groups combined ranged from 26.0–111.7 mm total length, and 0.14–8.95 g. We measured venom volume and protein concentration of individual samples.

Multiple Venom Extractions

To assess venom yield and protein concentration across multiple venom extractions, we subjected a third group of scorpions \((n = 27; \, 19 \, \delta \delta, \, 8 \, \varphi \varphi; \, 81.2–111.7 \, \text{mm total length, and 3.20–7.64 g mass})\) to five consecutive milkings separated by 21-day intervals. These scorpions were collected in August–September 2008, and milking took place from November 2008–February 2009 (after 2–6 months in captivity). All scorpions were fasted 21 days before each extraction, and then fed a cricket immediately after each milking. No scorpions were milked prior to initiation of the study. We recorded individual venom volumes, but because this analysis was preliminary to the larger study of individual venom samples (section on single venom extractions), we determined protein concentrations of the pooled venom at each milking. This group also was measured prior to the decision to use MS1W as the preferred reference character for body
size, so we used total length instead as the reference for overall body size. Total length appears to be a mildly dimorphic character in this species, as it is slightly larger in males than females, but it was demonstrated to display similar trends to MS1W as compared to the other reference characters previously evaluated (Fox et al., 2015).

Protein Quantification

We determined venom protein concentrations using the Coomassie method (Bradford, 1976) following the Thermo Fisher Scientific (Rockford, IL, USA) microplate protocol (1–25 μg/ml). Both the venom samples and bovine serum albumin (BSA) as the standard (4, 8, 12, 16, 20, and 25 μg/ml BSA) were diluted in nanopure water into the range of the assay. All assays were run in triplicate and resulted in large coefficients of determination ($r^2 > 0.96$), indicating high reliability of the method.

Statistical Analyses

We conducted statistical tests using SPSS 20.0 for Macintosh (Statistical Package for the Social Sciences, Inc., Chicago, 2011), with $\alpha = 0.05$. Prior to all statistical tests, we screened the data to verify compliance with parametric assumptions, and transformed variables if necessary. We removed a small number of outliers based on Mahalanobis distances (for multiple regression) or studentized residuals (for simple regression; Mertler and Vannatta, 2009).

We used regression analysis (Tabachnick and Fidell, 2013) to analyze the factors influencing venom volume and protein concentration from the single venom extractions, but to do so we generated several new variables. To create a single telson size variable,
we performed a principal component analysis (PCA) of telson length, width, and height, and used principal component 1 (hereafter telson size). The three telson measures contributed equally to the PC (factor loadings 0.993–0.996; percent variance explained = 99.0%). Because measures of telson size, mass, and MS1W were highly collinear, we ran separate simple linear regressions (not shown) of telson size and mass against MS1W as an independent variable to obtain unstandardized residual scores, interpreted as measures of relative telson size and relative mass, respectively (Mirtschin et al., 2002; Schulte-Hostedde et al., 2005). Relative mass can be considered a measure of body condition. A negative residual score for the regression of mass versus MS1W, for example, indicates a scorpion with a mass smaller than expected from its MS1W, whereas a positive residual score indicates a scorpion with a mass larger than expected from its MS1W. To better meet assumptions, we natural log-transformed (ln) the measures venom volume, MS1W, and mass. We then used separate multiple regression models to evaluate the effects of MS1W, relative telson size, relative mass, sex, and milking group on the dependent variables of venom volume and venom protein concentration (c.f. Cooper et al., 2014a). Sex and milking group, with two categories each, were treated as dummy variables. We included milking group as a factor because a difference clearly existed in venom protein concentration. We confirmed absence of multicollinearity using tolerance values and variance inflation factors. We computed estimated marginal means to compare group differences using analysis of covariance (ANCOVA) models (Tabachnick and Fidell, 2013) after confirming the assumption of homogenous regression slopes among groups.

To evaluate the shape of the relationship between venom volume and total length, we applied several curve-fitting models to the untransformed variables, including linear,
quadratic, exponential, and power models. For practical purposes, we used total length as well as MS1W in separate models as the independent variable, as the former is easier to conceptualize and because its use allows comparison of results with other studies of venom yield. Because sexual dimorphism in total length exists in this species (Fox et al., 2015), we constructed separate models for each sex.

For the multiple venom extractions, we used a repeated-measures ANCOVA model to examine the factors influencing ln-transformed venom volume. The model included milking number, ln-transformed total length, and sex as independent variables. For protein concentration, we used Kendall’s tau-b ($\tau_b$; Kendall, 1955) because of the small sample size ($n = 5$; Field, 2009) to test whether a directional change occurred across successive milkings.

We computed effect sizes in addition to the null hypothesis tests, as the former are biologically more meaningful, independent of sample size, and more readily compared among different data sets and different studies (Cohen, 1988; Nakagawa and Cuthill, 2007). We expressed effect sizes for bivariate correlations as Pearson ($r^2$) or Kendall’s ($\tau_b^2$) coefficients of determination, with values of $\sim 0.01$, $\sim 0.09$, and $\geq 0.25$ deemed small, moderate, and large, respectively (Cohen, 1988). For multiple regression, we obtained adjusted coefficients of multiple determination ($R^2_{\text{adj}}$) for the full models, and semipartial correlations ($sr^2$) for individual predictors (Cohen, 1988; Tabachnick and Fidell, 2013). These effect size estimators indicate the approximate proportion of variance in a dependent variable explained by an independent variable. For descriptive measures, we report mean $\pm 1$ S.E.
Results

Venom Heterogeneity

The initial venom expelled was clear, and this was followed by much larger quantities of opalescent venom (Fox et al., 2009). The transition was gradual and inconsistent among individuals, and therefore we did not quantify the change. Manual venom expression used in preliminary extractions showed color differences between initial and subsequent venom more clearly than electrical extraction.

Single Venom Extractions

Venom Volume

Venom volume from the milking of single-extraction scorpions (n = 207; 96 ♂♂, 111 ♀♀) averaged 45.9 ± 22.3 μL (range 0.4–108.3 μL) per individual. The multiple regression model, which included sex, milking group, ln(MS1W), relative mass, and relative telson size as predictors, significantly predicted (ln-transformed) venom yield ($F_{5,201} = 432.76, p < 0.001, R^2_{adj} = 0.913$). Correlations among the variables are shown in Table 1. Multicollinearity was not a problem. Two of the five variables contributed significantly to the model (Table 2). Venom volume was primarily predicted by ln(MS1W) ($p < 0.001, sr^2 = 0.880$), and to a lesser extent by relative telson size ($p < 0.001, sr^2 = 0.094$), with yield positively associated with both predictors. The unstandardized regression weight ($b$) for MS1W indicated a 3.32 μL increase in venom volume for every 1-unit increase in ln(MS1W). With all other variables held constant, there was no difference in venom yield among scorpions of varying relative mass ($p = 0.22, sr^2 = 0.008$), between males and females ($p = 0.82, sr^2 < 0.001$; estimated marginal
means at ln[MS1W] = 1.81 were 3.6 ± 0.03 ln[μL] for both sexes; mean difference and 95% CI = -0.01 [-0.10–0.08]), or between milking groups 1 and 2 (season/duration in captivity: \( p = 0.17, s r^2 = 0.009 \); estimated marginal means at same body length were 3.6 ± 0.03 vs. 3.6 ± 0.05 ln[μL], respectively; mean difference and 95% CI = -0.08 [-0.19–0.03]).

Table 1. Correlations (Pearson’s \( r \)) of variables in multiple linear regression model predicting venom volume yield in the scorpion *Hadrurus arizonensis* (\( n = 207 \)).

<table>
<thead>
<tr>
<th>Variable</th>
<th>MS1W</th>
<th>Relative telson size</th>
<th>Relative mass</th>
<th>Sex</th>
<th>Milking group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venom volume</td>
<td>0.949***</td>
<td>0.109</td>
<td>0.069</td>
<td>0.160*</td>
<td>-0.519***</td>
</tr>
<tr>
<td>MS1W (ln[mm])</td>
<td>--</td>
<td>-0.001</td>
<td>-0.001</td>
<td>0.155*</td>
<td>-0.559***</td>
</tr>
<tr>
<td>Relative telson size</td>
<td>--</td>
<td>0.381***</td>
<td>&lt;0.001</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>Relative mass</td>
<td>--</td>
<td>-0.223**</td>
<td>-0.124</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>--</td>
<td>--</td>
<td>-0.055</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milking group</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01; ***p < 0.001

MS1W = metasoma segment 1 width, used as an unbiased measure of overall body size
Relative telson size and relative mass (body condition) computed as unstandardized residual scores from separate simple linear regressions against MS1W
Sex coded as 0 = male, 1 = female
Milking group coded as 0 = Group 1, 1 = Group 2
Table 2. Multiple linear regression results for prediction of venom volume yield in the scorpion *Hadrurus arizonensis* (*n* = 207). Significant predictors indicated in bold.

<table>
<thead>
<tr>
<th>Model</th>
<th>B</th>
<th>SE b</th>
<th>β</th>
<th>p</th>
<th>Bivariate r</th>
<th>sr²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-2.447</td>
<td>0.165</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS1W (ln[mm])</td>
<td>3.322</td>
<td>0.086</td>
<td>0.968</td>
<td>&lt;0.001</td>
<td>0.949</td>
<td>0.880</td>
</tr>
<tr>
<td>Relative telson size</td>
<td>0.627</td>
<td>0.137</td>
<td>0.104</td>
<td>&lt;0.001</td>
<td>0.109</td>
<td>0.094</td>
</tr>
<tr>
<td>Relative mass</td>
<td>0.200</td>
<td>0.162</td>
<td>0.028</td>
<td>0.218</td>
<td>0.069</td>
<td>0.008</td>
</tr>
<tr>
<td>Sex</td>
<td>0.010</td>
<td>0.043</td>
<td>0.005</td>
<td>0.818</td>
<td>0.160</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Milking group</td>
<td>0.080</td>
<td>0.057</td>
<td>0.035</td>
<td>0.167</td>
<td>-0.519</td>
<td>0.009</td>
</tr>
</tbody>
</table>

*R² = 0.915, R²_adj = 0.913; standard error of estimate = 0.298
sr² is the squared semi-partial correlation
See Table 1 for description of variables

**Protein Concentration**

Venom protein concentration from the milking of single-extraction scorpions (*n* = 206; 94 ♂♂, 112 ♀♀) averaged 51.4 ± 15.8 μg/μL (range 10.3–95.7 μg/μL). The multiple regression model, which included MS1W (untransformed), relative telson size, relative mass, sex, and milking group as predictors, significantly predicted venom protein concentration (untransformed; *F*₅,200 = 14.48, *p* < 0.001, *R²_adj = 0.247), though with a much smaller effect size than the model for venom volume (*R²_adj = 0.913). Correlations among the variables are shown in Table 3. Multicollinearity was not a problem. Three of the five variables contributed significantly to the model (Table 4). Venom protein concentration was primarily predicted by season/duration in captivity (milking group: *p* < 0.001, sr² = 0.213), and to a lesser extent by sex (*p* = 0.003, sr² = 0.044) and MS1W (*p* = 0.034, sr² = 0.022). With all other variables held constant, the initial milking group (milked in February after 5–6 months in captivity) had a 54.1% more concentrated venom than that of the second group (milked in October after 2 months in captivity; estimated
marginal means at 6.33 mm MS1W were 56.4 ± 1.18 vs. 36.6 ± 2.22 μg/μL, respectively; mean difference and 95% CI = -19.82 [-25.14 – -14.49]). Females had a slightly (12.7%) more concentrated venom than males (estimated marginal means at 6.33 mm MS1W were 54.1 ± 1.33 vs. 48.0 ± 1.45 μg/μL, respectively; \( t_{200} = 3.03, p = 0.003 \); mean difference and 95% CI = 6.1 [2.12–10.03]). Venom protein concentration was negatively but somewhat trivially (considering effect size) associated with MS1W. The unstandardized regression weight (\( b \)) for MS1W indicated a 1.98 μg/μL decrease in protein concentration for every 1-mm increase in MS1W. Protein concentration was not influenced by relative telson size (\( p = 0.56, s_{r}^{2} = 0.002 \)) or body condition (relative mass: \( p = 0.39, s_{r}^{2} = 0.004 \)).

**Table 3.** Correlations (Pearson’s \( r \)) of variables in multiple linear regression model predicting venom protein concentration in the scorpion *Hadrurus arizonensis* (\( n = 206 \)).

<table>
<thead>
<tr>
<th>Variable</th>
<th>MS1W</th>
<th>Relative telson size</th>
<th>Relative mass</th>
<th>Sex</th>
<th>Milking group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein concentration</td>
<td>0.175**</td>
<td>0.069</td>
<td>0.089</td>
<td>0.202**</td>
<td>-0.461***</td>
</tr>
<tr>
<td>MS1W (mm)</td>
<td>--</td>
<td>-0.009</td>
<td>-0.003</td>
<td>0.147*</td>
<td>-0.558***</td>
</tr>
<tr>
<td>Relative telson size</td>
<td>--</td>
<td>0.362***</td>
<td>0.010</td>
<td>-0.153*</td>
<td></td>
</tr>
<tr>
<td>Relative mass</td>
<td>--</td>
<td>0.223***</td>
<td></td>
<td>-0.004</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td>-0.040</td>
</tr>
<tr>
<td>Milking group</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(*p < 0.05; \ **p < 0.01; \ ***p < 0.001\)

See Table 1 for description of variables
Table 4. Multiple linear regression results for prediction of venom protein concentration in the scorpion *Hadrurus arizonensis* (*n* = 206). Significant predictors indicated in bold.

<table>
<thead>
<tr>
<th>Model</th>
<th>b</th>
<th>SE b</th>
<th>β</th>
<th>P</th>
<th>Bivariate r</th>
<th>sr²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>65.682</td>
<td>6.317</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS1W (mm)</td>
<td>-1.981</td>
<td>0.929</td>
<td>-0.158</td>
<td>0.034</td>
<td>0.175</td>
<td>0.022</td>
</tr>
<tr>
<td>Relative telson size</td>
<td>-3.708</td>
<td>6.381</td>
<td>-0.039</td>
<td>0.562</td>
<td>0.069</td>
<td>0.002</td>
</tr>
<tr>
<td>Relative mass</td>
<td>6.619</td>
<td>7.676</td>
<td>0.058</td>
<td>0.390</td>
<td>0.089</td>
<td>0.004</td>
</tr>
<tr>
<td>Sex</td>
<td>6.071</td>
<td>2.005</td>
<td>0.191</td>
<td>0.003</td>
<td>0.202</td>
<td>0.044</td>
</tr>
<tr>
<td>Milking group</td>
<td>-19.815</td>
<td>2.700</td>
<td>-0.547</td>
<td>&lt;0.001</td>
<td>-0.461</td>
<td>0.213</td>
</tr>
</tbody>
</table>

R² = 0.266, R²adj = 0.247; standard error of estimate = 13.762

sr² is the squared semi-partial correlation

See Table 1 for description of variables

*Multiple Venom Extractions*

**Venom Volume**

We examined venom volume using a repeated-measures ANCOVA model, which included milking (five levels) and sex as within-subjects factors and ln(telson length) as a covariate. Because the data failed to meet the assumption of sphericity, we applied

- Greenhouse-Geisser adjustments to the degrees-of-freedom for within-subjects factors.

No interaction existed between milking and sex (F2.5,61.2 = 1.66, *p* = 0.19, partial η² = 0.065), or between milking and telson size (F2.5,61.2 = 0.39, *p* = 0.73, partial η² = 0.016).

While controlling for other variables, no differences occurred among the five venom milkings at 21-day intervals (means = 35.0 ± 2.74, 35.6 ± 3.10, 33.4 ± 3.12, 40.4 ± 3.16, and 42.1 ± 3.09 μL, respectively; F2.5,61.2 = 0.452, *p* = 0.69, partial η² = 0.018). Scorpion size (ln[total length]) was positively correlated with venom yield (F1,24 = 27.12, *p* <0.001, partial η² = 0.531), as expected, and males yielded a greater volume of venom than
females ($F_{1,24} = 4.01, p = 0.057$, partial $\eta^2 = 0.143$; estimated marginal means at 4.60 ln[telson length] were $3.6 \pm 0.07$ and $3.3 \pm 0.10 \ln[\text{volume}]$, respectively; mean difference and 95% CI = 0.2 [0.07–0.50]).

**Protein Concentration**

Protein concentration of the pooled venom samples appeared to decline across the five successive milkings (34.6, 30.4, 20.6, 25.7, 24.6 μg/μL). The decline was not significant given the small sample ($\tau_b = -0.60, p = 0.14$), but the effect size was substantial ($\tau^2_b = 0.360$). Protein concentration for the pooled samples (27.2 ± 2.4 μg/μL) averaged nearly half that of individual samples (51.4 ± 15.8 μg/μL).

**Relationship between Overall Body Size and Venom Yield**

We conducted two curve-fitting regressions to characterize the ontogeny of venom yield. First, we used MS1W as the covariate, which represented an unbiased measure of overall body size, but limited analysis to the single-extraction data. A power function best fit the relationship between untransformed venom volume ($V$) and MS1W for both males ($V = [0.090] \times L^{3.316}, r^2 = 0.961, n = 89$) and females ($V = [0.102] \times L^{3.282}, r^2 = 0.893, n = 102$), indicating an exponential increase in venom expenditure during scorpion growth (Fig. 1A). The measures of model fit for the power function were higher than those for linear (males: $r^2 = 0.685$; females: $r^2 = 0.559$), quadratic (males: $r^2 = 0.721$; females: $r^2 = 0.571$), and exponential (males: $r^2 = 0.942$; females: $r^2 = 0.878$) models. No difference existed between the sexes (See section on single extraction venom volume).
The second regression model used total length as the measure for overall body size to assess whether choice of covariate would influence interpretation of sex differences. After pooling data from the single-extraction scorpions and the first extraction from the multiple-extraction scorpions, the model again showed that venom yield increased exponentially in relation to scorpion size (Fig. 1B). A power function best fit the relationship between untransformed venom volume \( V \) and total length \( L \) for both males \( V = [1.672 \times 10^{-6}] \times L^{3.706}, r^2 = 0.959, n = 108 \) and females \( V = [1.464 \times 10^{-6}] \times L^{3.765}, r^2 = 0.837, n = 114 \). The measures of model fit for the power function were higher than those for linear (males: \( r^2 = 0.661 \); females: \( r^2 = 0.501 \)), quadratic (males: \( r^2 = 0.722 \); females: \( r^2 = 0.534 \)), and exponential (males: \( r^2 = 0.932 \); females: \( r^2 = 0.827 \)) models.
Figure 1. Venom yield as a function of body size (total length) in the scorpion *Hadrurus arizonensis* using two different covariates: (A) metasoma segment 1 width (MS1W) as an unbiased (preferred) measure of overall body size, and (B) total length as the more commonly used but biased measure of overall body size. Males are depicted by closed circles and solid line (n = 89 and 102 for the two models, respectively), and females are depicted by open circles and dashed line (n = 108 and 114, respectively). Exponential relationships between untransformed venom volume (V) and length (L) are best described by power functions (curves for A: males, $V = 0.090 \times L^{3.316}$, $r^2 = 0.961$; females, $V = 0.102 \times L^{3.282}$, $r^2 = 0.893$; curves for B: males, $V = (1.672 \times 10^{-6}) \times L^{3.706}$, $r^2 = 0.959$; females, $V = (1.464 \times 10^{-6}) \times L^{3.765}$, $r^2 = 0.837$). No difference existed between sexes with use of the unbiased (non-dimorphic) covariate ($p = 0.018$, Table 2).
Discussion

Our study of *H. arizonensis* comprises the most detailed assessment of the factors that influence venom yield and protein concentration in any scorpion species. We have established the utility of electrical venom extraction; confirmed the presence of venom heterogeneity; demonstrated the factors that influence venom yield and protein concentration; and demonstrated that scorpions can tolerate multiple venom extractions. In discussing the relevance of these findings, we draw from a limited body of research on scorpions. We therefore make comparisons not only to other scorpions, but also to other better-studied groups, specifically spiders (because they also belong to class Arachnida), centipedes, and snakes. We did not extensively survey the literature on other venomous groups.

At the outset, we believed it was important to analyze differences between the sexes using an appropriate measure of overall body size. Although *H. arizonensis* was thought to lack sexually dimorphic body parts other than the pectines (Stahnke, 1945; Tallarovic, 2000; Williams, 1970a), we found that numerous body components are indeed dimorphic, but identification as such depended on the reference character used for overall body size (Fox et al., 2015). We therefore analyzed the current data only after determining that MS1W was the least biased (non-dimorphic) indicator of size (Fox et al., 2015).

**Venom Collection**

By using repeated electrical stimulation of the venom glands, we believe we emptied the paired glands to the fullest extent possible. Electrical stimulation is
commonly relied on for venom collection from scorpions (Brenes and Gómez, 2016; Bücherl, 1971; Candido and Lucas, 2004; Lowe and Farrell, 2011; Whittemore et al., 1963; Yaqoob et al., 2016) and other animal groups (Barnes, 1967; Besson et al., 2016; Eskridge et al., 1981; Glenn et al., 1972; Kristensen, 2005; Lucas, 2015). However, different investigators often use alternative stimulation parameters, and manual (voluntary) as well as other methods of venom expression are still frequently relied on (de Roodt et al., 2012; Nisani et al., 2007; Salama and Sharshar, 2013; van der Meijden et al., 2015; Yahel-Niv and Zlotkin, 1979). Thus, varying methods of venom extraction will no doubt contribute to differences among studies.

We concur with others studying spiders (Celerier et al., 1993; Schanbacher et al., 1973) and centipedes (Cooper et al., 2014a; Dugon and Arthur, 2012) that applying saline solution to the forceps and telson proved vital in achieving consistent conduction of electricity to the venom gland muscles. Scorpion immobilization in a restraining device was also useful because the scorpions vigorously resisted CO₂ anesthetization often resulting in venom expression, we found it counterproductive to anesthetize the scorpions prior to extraction, and our restraining device was sufficient to minimize harm to both the scorpion and experimenter alike. In our experience, electrical stimulation proved more successful for *H. arizonensis* than manual (voluntary) venom expression, which has worked well previously in our lab with *Parabuthus transvaalicus* (Nisani et al., 2007; Nisani and Hayes, 2011).

Electrical stimulation has its drawbacks. Although the method generally produces higher yields than voluntary methods (di Tada et al., 1978; Tare and Sutar, 1986), neither the yields nor composition of the secretion necessarily equate with biological availability
or natural usage (Yahel-Niv and Zlotkin, 1979; Zlotkin and Shulov, 1969). Scorpions normally expulse only a fraction of available venom during individual stings and squirts (Nisani and Hayes, 2015; 2011; Yahel-Niv and Zlotkin, 1979; Zlotkin and Shulov, 1969), and may not be able to completely empty the glands apart from the presumably extreme muscle contraction caused by electrical stimulation. Venom composition also varies depending on the proportion of venom expended with each bolus, and how much was expended in prior recent usage (Nisani and Hayes, 2011; Zlotkin and Shulov, 1969).

Some scorpions provided unusually low venom yields, and these were excluded from analyses as outliers. Because venom yields decline preceding a molt in growing spiders (Herzig, 2010; Wiener, 1959) and with senescence in older spiders (Malli et al., 1993), similar phenomenon may exist in scorpions as well, and could therefore explain some of the low yields. In our study, repeated extractions at 21-day intervals resulted in no apparent harm to the scorpions and no changes in venom yield, but the protein concentration declined, as inferred from the large effect size (see sections on sex and on repeated venom extractions).

Despite any limitations to our methods, our results provide an estimate of venom quantity that is available to the species, which can be used as a baseline for evaluating venom deployment in natural contexts and interpreting the risks of scorpionism.

**Venom Heterogeneity**

Our study confirmed venom heterogenity in *H. arizonensis* (van der Meijden et al., 2015). Venom heterogeneity was evident from the progression of clear to opaque (milky) venom during the series of electrical shocks applied to the telson. The changes in
venom composition accompanying venom expulsion have been best characterized in another scorpion, *P. transvaalicus*, wherein the initial clear venom was rich in potassium ions, and the opaque venom that emerged subsequently was rich in protein (Inceoglu et al., 2003). Venom heterogeneity has been documented in the scorpion families Buthidae (Nisani et al., 2012; 2007; Nisani and Hayes, 2011; Sarhan et al., 2012; Yahel-Niv and Zlotkin, 1979; Zlotkin and Shulov, 1969), Scorpionidae (Abdel-Rahman et al., 2009; Gopalakrishnakone et al., 1995), and Vaejovidae (*Smeringurus* spp., G. A. Fox and W. K. Hayes, unpubl. data). Given the phylogenetic distribution of known occurrence, we suspect that venom heterogeneity may be present in all scorpions. Venom heterogeneity also exists in other arthropods (Morgenstern et al., 2012; Zobel-Thropp et al., 2013) and cone snails (Dutertre et al., 2014; 2010; Prator et al., 2014), but apart from one study of spitting cobras (Cascardi et al., 1999), it has not been reported in snakes.

From an analytical perspective, failure to fully empty the venom glands of animals with heterogeneous venom could influence measurement of protein concentration and characterization of venom composition. Venom heterogeneity also complicates measurement of venom expenditure if only a single antibody is used for an enzyme-linked immunosorbant assay (Morgenstern and King, 2013).

**Total Venom Yield**

Total venom volume in *H. arizonensis* averaged 45.9 μL, but varied substantially with body size, ranging from 0.4–108.3 μL. We did not measure dry mass, but calculations (volume × mean protein concentration) gave an average yield of 2.36 mg, and a range of 20.6 μg to 5.6 mg. Our mean value of 2.36 mg was less than that of the
only report of venom extraction in the genus: 4.2 mg in adult *H. hirsutus* (Meadows and Russell, 1970). The difference could result from discrepancies between the species, methodology, and/or body size. A likely explanation stems from the venom yield in *H. hirsutus* being derived from whole dry venom, whereas we evaluated protein concentration of wet venom after it had been centrifuged to remove potential cellular debris and mucoid proteins. Several of our initial comparisons between centrifuged and non-centrifuged samples resulted in a near doubling of protein concentration in non-centrifuged samples (unpublished data).

For perspective, we can compare the maximum quantities of venom volume (108.3 μL) and dry mass (5.6 mg after centrifugation) from a large adult *H. arizonensis* to yields reported from other venomous animals. The yield by volume that was obtained in this study was greater than any other scorpion species we could find (e.g., *Tityus gonzalespontgui*; 13.1–39.2 μL, Bravo Salazar, 2010; *P. transvaalicus*; 39.69 ± 9.23 μL, Nisani et al., 2007; *Lieurus quinquestriatus*; 18.5–52 μL, Yahel-Niv and Zlotkin, 1979), although most of the species that have been examined are of smaller size than *H. arizonensis*. Similarly, venom mass was comparable to the highest yields reported for species that are of far greater toxicity (e.g., *Tityus zulianus*: 3.4mg, D’Suze et al., 2015; *P. transvaalicus*: 4.8mg, Grasset et al., 1946; *Androctonus australis*: 8–9mg, Phisalix, 1922; *L. quinquestriatus*: 1.92–6.49mg, Yahel-Niv and Zlotkin, 1979), but again, the larger size of *H. arizonensis* biases the comparison. Maximum venom yield in *H. arizonensis* was comparable to or greater than the largest mean venom yields we found for a spider (*Pamphobeteus nigricolor*: 5.7 mg dry mass without centrifugation, Estrada-Gómez et al., 2013; *Vitalius dubius*: 3.3 mg dry mass presumably after centrifugation, Rocha-E-Silva et
al., 2009b), though there are larger species yet to be examined. Our values also dwarfed
the largest yield reported for a centipede (*Scolopendra subspinipes*; 7.3 μL Cooper et al.,
2014a). Maximum volume and dry mass in *H. arizonensis* were equivalent to those from
juvenile rattlesnakes of the *Crotalus oreganus/helleri/concolor* complex that measure 40–
45 cm and 30–35 cm, respectively (Mackessy, 1988; Mackessy et al., 2003).

**Factors Influencing Venom Yield**

We identified two factors that significantly influenced venom yield, and both
were size-related. We discuss these, along with the other factors we measured that
appeared to be independent of venom yield.

**Body Size (Ontogeny)**

The most important factor influencing venom yield in *H. arizonensis* was body
size, which explained roughly 88.0% of the variation (*s^2* value) in the single-extraction
multiple regression model. Using a comparative approach, three studies have shown that
average venom yield for a given species corresponds to the body size of the species (six
species of genus *Tityus*: D’Suze et al., 2015; five species of four genera: van der Meijden
et al., 2015; three species from two genera: Whittemore et al., 1963) but our study is the
first (as a follow-up to Fox et al., 2009) to examine the shape of the relationship between
body size and venom yield within a species. The relationship for *H. arizonensis* was
exponential, and best described by a 3.28- order (females) to 3.32-order (males) power
function (Fig. 1A).
We suspect that most or all scorpion species exhibit an exponential relationship between body size and venom yield, but the shape of the relationship appears to vary among even closely related animals. In spiders, venom volume has been found to correspond linearly to prosoma length (Cupiennius salei: Vapenik and Nentwig, 2000) and carapace volume (Atrax sutherlandi; Wong et al., 2016); wet mass linearly to body mass (V. dubius; Rocha-E-Silva et al., 2009a); dry mass exponentially to body mass (P. nigricolor; Estrada-Gómez et al., 2013) and by fourth-order power function to prosoma length (Phoneutria nigriventer; Herzig et al., 2004); and volume or mass to size in general (Coremiocnemis tropix; Herzig, 2010; Loxosceles reclusa; Morgan, 1969; Pterinochilus sp.; Perret and Freyvogel, 1973). The different relationships among species could result from species variation in the anatomical location and size of the venom gland (Herzig, 2010), species variation in nutritional needs during ontogeny and after attaining adulthood (Herzig, 2010), and the diverse reference characters used for overall body size (see Fox et al., 2015; Suter and Stratton, 2011). In the centipede Scolopendra polymorpha, the relationship between venom volume and body length was linear rather than exponential (Cooper et al., 2014a). Studies of snakes generally reveal an exponential relationship of venom yield with body length (Glenn and Straight, 1982; Huang and Mackessy, 2004; Mackessy, 1988; Mackessy et al., 2003; Mackessy and Baxter, 2006; McCue, 2006; Mirtschin et al., 2002), but several have reported a linear relationship (Abdel-Aal and Abdel-Baset, 2010; de Roodt et al., 1998; Kochva et al., 1982; McCleary and Heard, 2010).
Relative Telson Size

We found that venom yield in *H. arizonensis* was also significantly related to relative telson size, which explained roughly 9.4% of the variation in the single-extraction analysis. The bivariate correlation was not significant (Table 1), so the relationship became apparent only when other variables were controlled for in the multiple regression model. It seems intuitive that individuals having proportionally larger telsons would also produce more venom, as this structure houses the paired venom glands. Similar relationships between venom yield and relative size of the structure(s) housing the venom gland(s) have been reported for the centipede *S. polymorpha* (forcipule length but not width; Cooper et al., 2014a) and the elapid snake *Pseudonaja textilis* (head length but not width; Mirtschin et al., 2002).

Variation in relative telson size clearly exists within *H. arizonensis*, but whether the causes are genetic and/or ecophenotypic remain unknown. More importantly, the variation could lead to functional differences in both venom availability and venom usage that could become optimized by selection (Herbert and Hayes, 2008), and therefore merits further study.

Body Condition

Venom yield appeared to be independent of body condition (relative body mass), which explained well under 1% of the variation in our single-extraction analysis. Relative body mass has been used as a measure of an animal’s nutritional state and fitness (Jakob et al., 1996; Schulte-Hostedde et al., 2005). In the only two studies that have addressed the relationship in arachnids, nutrition did not affect venom yield in the spiders *C. salei*.
(starved 4 and 8 weeks; Vapenik and Nentwig, 2000) or C. tropix (ratio of opisthosoma length to prosoma length; Herzig, 2010). However, especially poor-nourished (with possibly insufficient energy for venom production) and well-nourished individuals (with possibly decreased need of venom) of C. tropix were less likely to yield any venom during extraction (Herzig, 2010). For the centipede S. polymorpha, body condition had a small but significant positive association with venom yield (Cooper et al., 2014a).

Considering the sheer volume of publications produced, snake venom researchers should be the most familiar with nutritional effects. Klauber (1997), who extracted venom from many rattlesnakes (genera Crotalus and Sistrurus), believed that well-fed snakes would yield greater volumes of venom. Kochva (1960), however, reported that factors such as food consumption, ecdysis, and pregnancy did not affect venom yields in the viper Daboia palaestinae. Venom yields from repeated extractions of the rattlesnake Crotalus atrox nevertheless appeared to be greater in fasted snakes than force-fed snakes (Glenn et al., 1972), which was suggested to be the result of more rapid venom replenishment in the fasted snakes. A three-fold difference in venom yields between two populations of tiger snakes (Notechis scutatus) was attributed to a diminished body condition from drought in one population (Fairley and Splatt, 1929). The only study that has examined actual venom usage in relation to nutrition found that food-deprived Crotalus viridis rattlesnakes expended less venom in biting rodent prey than recently-fed snakes (Hayes, 1993).

We conclude that body condition within the normal range of variation exerts a minimal influence on venom yields. Because relative mass is not expected to influence size of the venom glands, we suspect it would influence venom yield via degree of filling
of the gland, representing a differential investment in venom production (Cooper et al., 2014a).

Sex

Venom yield in *H. arizonensis* appeared to be independent of sex, which explained well under 1% of the variation in the single-extraction analysis. Our multiple regression model controlled for overall body size so as to compare the sexes at equivalent size. Had we used a reference character other than MS1W, such as total length, metasoma length, or prosoma length—all of which are sexually dimorphic—our conclusions could have been different (Fox et al., 2015).

Few prior studies have compared the venom yields of male and female scorpions. Yields were greater in males of *Tityus nororientalis* (Aguilera Rodriguez et al., 2010; Chadee Burgos, 2010; de Sousa et al., 2010), similar in *Tityus isabelcecilia* (González-Sponga et al., 2001), and greater in females of *Centruroides limpidus* (Cid Uribe et al., 2017). However, all of these studies described SSD in the species studied, but failed to provide measures of body size, so it remains unclear whether sex differences existed when compared at a similar body size. Miller et al. (2016) reported that defensive venom expenditure from single stings of *Centruroides vittatus* was greater in females than males; however, females averaged larger in size than males, and when venom yield was treated as a percentage of a scorpion's mass, no difference in venom yield existed despite a large effect size ($r^2 = 0.38$). Both sexes exhausted their venom after an average of five stings.

A larger number of studies have examined sexual differences in the venom yields of spiders. Because adult female spiders are generally larger than adult males, sex
comparisons are often confounded with body size in the literature, so we consider here only those studies that controlled for body size or included sufficient details for reasonable inference. Females appear to have larger yields in the mygalomorphs (infraorder with parallel fangs) *C. tropix* (Herzig, 2010) and *Missulena pruinosa* (Herzig et al., 2008), and in the araneomorphs (infraorder with fangs that cross medially) *C. salei* (Malli et al., 1993), *L. reclusa* (Morgan, 1969), and *Tegenaria agrestis* (Binford, 2001). Although female-biased venom yields have been stated as a “general rule” for spiders (Herzig, 2010), males have been reported to have larger yields in the mygalomorph *V. dubius* (Rocha-E-Silva et al., 2009a), and in the araneomorph *P. nigriventer* (Herzig et al., 2002). No difference existed between the sexes in the mygalomorphs *Atrax robustus* (Wiener, 1959) and *Scoda griseipes* (reported statistics ambiguous; Celerier et al., 1993). Herzig (2010) offered a rationale for why female mygalomorph spiders might have larger yields. After reaching adulthood, males seek mates and experience reduced food intake, so they maintain venom production at a lower level than females, which need higher levels of food intake to produce eggs, construct the egg-sac, molt, and then continue the reproductive cycle with other males in subsequent years, living considerably longer than the males. Many scorpion species have exceptional longevity (several years to several decades: Lourenço, 2002; Polis and Sissom, 1990; Warburg, 2011) compared to most spiders and other terrestrial arthropods, so sex differences in longevity may be less likely to promote intersexual variation in morphology and life history traits. Nevertheless, the range of longevity within the group is broad enough that we could predict sexual differences in venom yield to be more profound in short-lived species.
Sexual differences in behavioral deployment of venom have been described in scorpions. Both sexes use their venom for predation and defense, but males of some species—including *H. arizonensis* (Tallarovic, 2000; Tallarovic et al., 2000)—deploy venom in a sexual sting during courtship (Angermann, 1957; 1955; Benton, 1973; Francke, 1979; Garnier and Stockmann, 1972; Jiao and Zhu, 2010; Mirza and Sanap, 2009; Polis and Sissom, 1990; Toscano-Gadea, 2010), and females of *C. vittatus* exhibit greater reliance than males on the use of stings for defense (Carlson et al., 2014; Carlson and Rowe, 2009; Miller et al., 2016). In spite of any sexual differences in behavior (Tallarovic, 2000; Tallarovic et al., 2000) or venom composition (C. Sarfo-Poku and W. K. Hayes, unpubl. data) that might exist in *H. arizonensis*, selection has apparently favored similar quantities of venom availability (relative to body size) in males and females.

**Season and/or Duration in Captivity**

We found no difference in venom yield between the two milking groups of scorpions, which explained well under 1% of the variation in the single-extraction analysis. The two groups differed in both season (October versus February) and duration in captivity (2 months versus 5–6 months) when extractions were conducted, and therefore the two variables were confounded. If venom yield varies seasonally in *H. arizonensis*, or if captive conditions somehow influence venom yield, we were unable to document these effects (assuming the absence of an interaction between the two variables, which could have cancelled out any differences).
A number of studies have reported declines in scorpion venom yield during captivity, but the declines invariably resulted after multiple extractions (Bücherl, 1971; Bücherl and Diniz, 1978; Candido and Lucas, 2004; D'Suze et al., 2015; Kalapothakis and Chavéz-Olortegui, 1997; Schöttler, 1954; Whittemore et al., 1963; Yaqoob et al., 2016), with no study showing an independent effect of captivity. Balozet (1971) nonetheless remarked, without supporting evidence, that venom yields decline in captivity relative to wild scorpions. Venom yield might vary with season, but one group of researchers that milked the venom from 15,926 scorpions of three genera representing two families remarked that no seasonal effect was evident (Grasset et al., 1946). In a study with more rigorous analyses, the venom yield of both male and female *Tityus discrepans*, milked at 19–42 day intervals, declined for a period of time in captivity (between days 96–215), returning to higher levels on the last extraction (day 215), suggesting a seasonal component since body mass did not change (D'Suze et al., 2015). More compelling evidence for seasonal variation in venom yield has been reported in two genera of spiders, with *Atrax infensus* yields higher in winter than summer or fall (Atkinson, 1981), *A. robustus* highest in spring but similar summer through winter (Sheumack et al., 1984; Wiener, 1959), *A. sutherlandi* higher in winter than autumn (Wong et al., 2016), *Phoneutria fera* higher in winter than summer (Schenberg and Lima, 1966), and *P. nigriventer* higher in summer than winter (Bücherl, 1953b). No obvious trend or explanation can be inferred from the spiders, particularly when species within the same genus have contrasting patterns. Perhaps some of the seasonal variation observed could be attributed to other causes, such as investigator experience with the milking procedure. In rattlesnakes, a larger body of evidence suggests that venom yields are
greater in summer than in winter or spring, and positively associated with maintenance temperature in captivity (Glenn and Straight, 1982).

Season and duration in captivity can also be confounded with age, which could affect venom yield through eventual senescence. Declining yields attributable to presumed senescence of older female spiders has been reported in *C. salei* (Malli et al., 1993). In a study of *V. dubius*, the heaviest females also showed reduced venom yields (Rocha-E-Silva et al., 2009a), but this might have resulted from reduced need for venom production (Herzig, 2010) or an unspecified reproductive state rather than senescence. Regarding reproduction, the presence of egg sacs did not influence venom yield in females of the spider *P. nigriventer* (Herzig et al., 2002), but the effect of male sperm (spiders) or spermataphore (scorpions) production has not been examined. We doubt that senescence had any effect on venom yield in our study. *Hadrurus* species require several years to attain adulthood (Quijano-Ravell et al., 2011; Tallarovic, 2000), and can reportedly live more than 25 years, but authors who cite Stahnke (1966) in support of this longevity have done so incorrectly. Nevertheless, the 3–4 month difference in our study between milking groups probably represents a small window within even an adult scorpion’s lifetime.

**Repeated Venom Extractions**

As mentioned earlier, venom yields remained consistent across the five successive venom extractions, which suggests that electrical stimulation at the interval we used (21 days) can be repeated over time to accumulate larger venom samples from individual scorpions. Body size in both datasets proved to be the most important factor explaining
venom yields. But in apparent contrast to the single-extraction study, males in the multiple-extraction study yielded a significantly greater volume of venom than females. The difference between the sexes, however, was likely an artifact of using total length (the only measure of overall body size obtained during for this dataset) as a measure of overall body size. In a detailed analysis of sexual dimorphism in *H. arizonensis*, discriminant function analyses based on the measurements of 14 body components suggested that total length is significantly longer in males than females (Fox et al., 2015), and therefore comprises a biased reference character for overall body size. Females, accordingly, would be expected to have more venom when relying on total length as a measure for body size. The use of an unbiased reference character for body size (MS1W) in the single-extraction study provides a more valid comparison, suggesting, again, that no difference in venom yield exists between the sexes.

**Other Variables**

Additional variables that we did not investigate may also influence venom yield in *H. arizonensis* and other scorpions. Importantly, any exploration of these possibilities must take into account the sources of variation we have identified, which may be confounding, especially body size. We briefly consider five variables: phylogenetic constraints, environmental variation, circadian variation, diet, and venom composition.

Phylogenetic constraints and selection arising from environmental variation (i.e., geographic variation) can lead to different venom yields in species similar in body size and morphology. *Tityus confluens* in Argentina, for example, showed a two-fold difference between two populations in protein content of telson homogenate (de Roodt et
al., 2009). We have also found venom yield differences in two morphologically similar but allotopic species of Smeringurus (Chapter Five).

Because of its short-term nature, circadian variation seems to be an unlikely influence. However, electrically-stimulated venom yields from Heterometrus indus were greatest when conducted at night, least in daylight, and intermediate when crepuscular (Tare et al., 1992). Latency to venom appearance and rate of venom expulsion were likewise influenced by time of day. This study implies a circadian variation in venom gland muscle receptivity to venom expulsion.

Diet can influence the morphology of prey subjugation structures (e.g., Řezáč et al., 2008), prey-capture behavior (Cooper et al., 2015; Edmunds and Sibly, 2010; Wullschleger and Nentwig, 2002), and venom composition (Binford, 2001; Pucca et al., 2014) of arachnids, and therefore might affect venom yield as well. Although prey choice is influenced by availability, which reflects environmental variation, prey choice also has a strong experiential component, and could affect venom availability via feeding rate (frequency of venom use) and replenishment rate, as different amounts of venom are often required to subdue different prey species (Cooper et al., 2015; Edmunds and Sibly, 2010; Wullschleger and Nentwig, 2002). Tityus serrulatus scorpions fed size-equivalent meals of either crickets (Grillus sp.) or cockroaches (Nauphoeta cinerea) gave different venom yields after 30 days of food deprivation (with a 1.9-fold greater yield following cockroach consumption; Pucca et al., 2014). The discrepancy might have resulted from different levels of venom depletion to procure the two prey species.

Differences in venom composition, presumably influenced (genetically and possibly epigenetically) by local prey and predator species, could potentially influence
venom yield. For arachnids, a reciprocal relationship between toxicity and venom yield has been hypothesized among taxa (limited support from three taxa: van der Meijden et al., 2015), between the sexes to overcome the small volume of venom in the smaller sex (supported from seven species of four genera, but also contradicted in four species representing three genera; G. A. Fox and W. K. Hayes, unpubl. data), and even ontogenetically within individual species to overcome the small volume of venom possessed by young arachnids (limited support from instars 6–10 of C. salei: Malli et al., 1993).

**Venom Protein Concentration**

Because proteins are largely responsible for the toxicity of many venoms, researchers often report the protein concentration or content of venom samples. Greater amounts of protein in a given secretion can lead to greater toxicity. However, comparisons among studies can be plagued by several important considerations. First, protein concentration and content will vary depending on extraction method (Oukkache et al., 2013) and the extent to which the glands are emptied (McCleary and Heard, 2010). Second, not all of the solid (dried) material represents toxins, as cellular debris inevitably will be present in the secretion. Some researchers centrifuge venom samples to remove the insoluble material, which will reduce the protein concentration and dry mass measured; some researchers characterize the crude sample that is secreted and deployed naturally by the animal; and other researchers neglect to mention whether the samples were centrifuged. Third, the amount of protein in a sample is usually reported in one of two forms that are not equivalent but often used interchangeably: as the protein
concentration (i.e., percentage of volume, or weight per volume [w/v]: μg/μL or mg/mL), or as the protein content (i.e., percentage of solids, or weight-to-weight ratio [w/w] in μg/mg). The two measures should not be conflated. To state, for example, that the venom of males had a higher protein content than females (w/w) does not imply that males had a higher protein concentration (μg/μL) as well. Fourth, protein concentration and content vary substantially during venom replenishment (e.g., Boeve et al., 1995; Cooper et al., 2014b; Nisani et al., 2007; Perret, 1977b), such that recent venom expulsion—whether natural or from milking—can influence the measured protein in a sample. Finally, nutrition and hydration likely influence the amount of protein in a secretion, but these variables are difficult to quantify, especially for recently captured scorpions, and further study is needed to understand their effects.

Although these considerations illustrate the need for caution when comparisons are made among studies, the same methods typically are used within a single study, and therefore group comparisons (e.g., age classes, sexes) and associations with variables within a study (e.g., body size) should be valid.

The mean protein concentration of 51.4 μg/μL (range 10.3–95.7 μg/μL) in *H. arizonensis* (for single-extraction samples) was within the range of mean values reported for various species of scorpions (7.2–85.2μg/μL; de Roodt et al., 2012; Inceoglu et al., 2003; Nisani et al., 2007; Ozkan et al., 2011), spiders (2–300μg/μL; Celerier et al., 1993; de Oliveira et al., 1999; Friedel and Nentwig, 1989) and snakes (36–370μg/μl; Kopper et al., 2013; Mackessy and Baxter, 2006), but below those of two species of *Scolopendra* centipedes (113–165μg/μL; Cooper et al., 2014a). Protein measurements in *H. arizonensis* venom samples showed substantial (9.3-fold) variation, which exceeded
reports we found for the centipedes *S. polymorpha* (3.5-fold) and *S. subspinipes* (2-fold; Cooper et al., 2014a), the spider *Eurypelma californicum* (3.7-fold; Savel-Niemann and Roth, 1989), and the coralsnake *Micrurus tener* (3.7-fold; Kopper et al., 2013). Although taxonomic variation in protein concentration of venom may well exist, we assume that some of the variation can be attributed to different methods and sample sizes.

*Factors that Influence Venom Protein Concentration*

We found that several measured variables statistically influenced protein concentration in *H. arizonensis*, and these differed somewhat from those that influenced venom yield.

**Body size**

We uncovered a weak but significant negative relationship between venom protein concentration and body size in *H. arizonensis*, which explained roughly 2.2% of the variation in the single-extraction multiple regression model. The difference could have resulted from differential evaporation during the transfers and handling of venom samples, with the smaller samples from small scorpions experiencing higher levels of evaporation. Very few studies have examined this relationship in arthropods. The spider *C. salei* showed a slight but non-significant increase in protein concentration during ontogeny (Malli et al., 1993), whereas the centipede *S. polymorpha* showed a significant increase (Cooper et al., 2014a). For snakes, protein concentration and/or content increased during ontogeny in some species (Furtado et al., 1991; Lourenço et al., 2013; Mackessy and Baxter, 2006; Meier and Freyvogel, 1980), but remained the same or
decreased in others (Antunes et al., 2010; Furtado et al., 1991). Given the absence of any consistent pattern among these animal groups, further study is needed to confirm and understand why smaller scorpions possibly have a higher concentration of protein in their venom than adults.

**Sex**

Venom protein concentration was 12.7% greater in females than males, with sex explaining roughly 4.4% of the variation in the single-extraction analyses. Again, few studies have examined this relationship in arthropods. In spiders, protein concentration was greater in males of *S. griseipes* (Celerier et al., 1993), greater in females of *Loxosceles intermedia* (de Oliveira et al., 1999), and equal in both sexes of *Tegenaria agrestis* (Binford, 2001). No sex differences in protein concentration were observed in the centipede *S. polymorpha* (Cooper et al., 2014a). For snakes, venoms of the sexes were similar for protein concentration in *Bothrops jaracara* (Saad et al., 2012) and protein content in *Crotalus concolor* (Glenn and Straight, 1977). Again, the lack of any consistent pattern suggests that further study is needed to understand why female scorpions have a higher concentration of protein in their venom than males.

**Season and/or Duration in Captivity**

Venom protein concentration in *H. arizonensis* differed between the two groups of single-extraction scorpions tested at different times, which explained a surprisingly large proportion of the variation, roughly 21.3%. Protein concentration was 54.1% greater in the group 1 specimens milked in February after 5–6 months in captivity.
compared to the group 2 specimens milked in October after 2 months in captivity. This finding suggests either seasonal variation, effects of long-term captivity, or both, as the two variables were confounded. Seasonal variation could be linked to a gradual shift in protein secretion and accumulation from the late summer (August–October) mating season (Tallarovic, 2000; Tallarovic et al., 2000)—when we collected the scorpions—to the prolonged period of inactivity during winter. We captured our scorpions during the mating season, when energy and resources might be diverted away from venom production, but our specimens remained active and feeding at moderate temperatures during the winter. The effects of captivity could reflect accumulated changes in nutrition (relative to removal from the wild) and/or hydration (not in burrows). To our knowledge, the effects of season and captivity on protein concentration have not been explored formally in other arthropods, in centipedes, or in snakes. Future work could easily tease apart these effects on venom protein concentration.

Repeated Venom Extractions

Protein concentration of the pooled venom from the first milk of multiple venom extractions (34.6 μg/μL) was comparable to that of individual samples from the single extractions (56.4 and 36.6 μg/μL for groups 1 and 2, respectively). However, in contrast to venom volume, which remained consistent across the five consecutive milkings in *H. arizonensis*, the venom protein concentration declined over time. We infer the decline from the large effect size for repeated milkings, which explained 36.0% of the variation in the multiple-extractions analysis. The repeated venom extractions occurred over a 12-
week period spanning several seasons, so there was confounding of this variable, as well,
with season and duration in captivity.

Apart from possible changes related to season and duration in captivity, there are
two more possible explanations for the decline in protein concentration. First, complete
protein regeneration may require more than the 21-day interval we used between
successive milkings. The phenomenon of protein regeneration lagging behind venom
replenishment has been observed in a number of animals, including scorpions (Nisani et
al., 2007), spiders (Boeve et al., 1995; Perret, 1977c), centipedes (Cooper et al., 2014b),
and a number of snakes (Brown et al., 1975; Klauber, 1997; Kochva, 1960; Schenberg et
al., 1970; Willemse et al., 1979). Several studies reported much more rapid protein
replenishment in two snake species (Currier et al., 2012; Marsh and Glatston, 1974), but
the differences among studies may have resulted from different levels of venom gland
depletion. Longer intervals between extractions in our study might have avoided the
decline in protein concentration. Second, the decline could have resulted from injury to
the venom glands resulting from electrical stimulation. Sissom et al. (1990) suggested
that scorpions can only be milked, on average, four times before the muscles of the gland
stop responding to electrical stimulation. In some cases, electrical milking may even kill
the animal (Nisani et al., 2012; Sahayaraj et al., 2006). However, the five electrical
venom extractions did not reduce volume yield in our scorpions, and repeated electrical
stimulation had negligible effects on venom yield in the spider Coremiocnemis tropix
(Herzig, 2010) and in several snakes (Marsh and Whaler, 1984; McCleary and Heard,
2010). Clearly, further study is needed to explore these potential influences on venom
concentration.
Other Variables

Protein concentration in the venom of *H. arizonensis* was not affected by relative telson size or body condition, each of which explained well under 1% of the variation in the single-extraction analysis. In spite of the negligible effect of body condition in our study, it may be profitable to explore the potential effects of nutrition on hydration by experimentally creating groups exposed to very different conditions.

Relevance of Venom Yield and Protein Concentration

Collectively, our findings offer meaningful insights regarding design of the venom delivery system, strategies of venom deployment, appropriate regimens for sustainable venom production, and medical risks and symptoms associated with scorpionism. We briefly elaborate on these.

A growing body of evidence suggests that the quantity of venom available to an animal can influence decisions about venom deployment (Cooper et al., 2015; Hayes, 2008; Hayes et al., 2002; Hostettler and Nentwig, 2006; Wullschleger and Nentwig, 2002). Scorpions normally use only a fraction of available venom in their glands when stinging or spraying. Because of physical constraints on venom expulsion rates (van der Meijden et al., 2015), and increased vulnerability when a stinger is engaged in venom expulsion (Rowe and Rowe, 2006), larger scorpions can deliver more venom during what is typically a very brief sting or spray episode (Nisani and Hayes, 2015; van der Meijden et al., 2015). Having larger quantities of venom available opens opportunities for procuring prey that are larger or more resistant to venom, and confers greater levels of protection against predators and antagonists. By allocating varying proportions of venom...
among individual stings (Nisani and Hayes, 2011), scorpions can conserve the metabolic costs of venom replenishment, particularly for smaller prey, or deliver larger quantities of venom per sting, or even multiple stings. If scorpions are like spiders (Hostettler and Nentwig, 2006; Wullschleger and Nentwig, 2002), they might be aware of how much venom is available for use, and will make decisions accordingly. Knowledge of venom availability may relate not only to total venom supply, but also incremental depletion from recent sting use (Hostettler and Nentwig, 2006; Wullschleger and Nentwig, 2002). The mating season may present special circumstances for venom maintenance, quality, and allocation, as male scorpions of at least some species (including H. arizonensis) deliver repeated stings to the female during courtship (Tallarovic et al., 2000), and females may divert energy to egg production.

The quantity of venom available to a scorpion has important implications for venom production for research and commercial purposes, including antivenom preparation. Again, scorpion size is clearly important for obtaining the largest yields, but the interval between repeated milkings is also critical to maintain suitable protein levels. Regardless of whether venom yields are similar for male and female scorpions, their venom composition may differ in key toxins (e.g., D'Suze et al., 2015; de Sousa et al., 2010; Rodríguez-Ravelo et al., 2015; Yamaji et al., 2004), such that venom should ideally be procured from both sexes unless specific toxins are desired.

The incidence of scorpionism remains under-reported worldwide. Several scorpion-related factors influence the envenomation event and subsequent prognosis, including species, size, condition of the telson at the time of envenomation, number of stings and/or the quantity of venom injected, season, and temperature (Chowell et al.,
2005; de Roodt et al., 2003; Dehesa-Dávila, 1989; Dehesa-Dávila and Possani, 1994; Santos et al., 2016). While most of these elements have not been fully explored, the quantity of toxins injected into the human victim is strongly correlated with clinical symptomology (Ghalim et al., 2000; Krifi et al., 1998). Thus, our findings reinforce the view that, within a given species, the largest scorpions are the most dangerous. Relative toxicity of the venom is also important, as many of the most dangerous species are relatively small, yet any scorpion over 5 cm total length should be handled cautiously (Chippaux and Goyffon, 2008).

Although we have identified some factors that significantly influence venom yield and protein concentration in *H. arizonensis*, substantial individual variation exists, as can be seen in Fig. 1A. This variation can serve as the substrate for selection arising from the factors that determine likely venom use, including prey size, prey type, feeding frequency, defensive encounters, and mating needs (sexual sting use). Future studies should examine whether venom yield in scorpions varies depending on these factors. The scorpion *Pandinus imperator*, for example, discontinues predatory use of venom as adults (Casper, 1985), and may exhibit a venom yield–body size relationship that differs dramatically from other scorpions that use venom for predation throughout their life.

**Conclusions**

We relied largely on multiple linear regression to investigate a number of factors that potentially influence venom yield and venom protein concentration in the scorpion *H. arizonensis*. We showed that these two properties of venom were subject to very different influences. We expected venom yield, as a volumetric measure, to be highly
dependent on the size of the organism, which was evident especially for an unbiased measure of overall body size (MS1W), but also for relative size of the telson that harbors the paired venom glands. Perhaps in part because of this strong scaling relationship, venom yield appeared to be independent of other variables we examined, including sex, season/duration in captivity, and body condition. Venom protein concentration, as a biochemical property of the secretion itself, was much less dependent on overall body size, though there was a weak negative relationship. Protein concentration instead varied the most between the two milking groups (increasing with duration in captivity, and/or greater in winter than fall), and to a lesser extent between the sexes (greater in females than in males). Relative telson size and body condition had no measurable influence on protein concentration. Repeated venom milkings showed that consistently large venom yields could be obtained over an extended period of time, but that protein regeneration requires more time than volume replenishment.

Much of what we know regarding venom yield in scorpions has been reported incidentally while procuring venom to be used for other objectives. As a consequence, few studies have addressed specific hypotheses. Pertinent ancillary details have often been omitted, such as scorpion body size, whether venom samples were centrifuged, and pertinent statistical details, including measures of variance and tests of central tendency. As a result of this neglect, few generalizations can be made from existing literature on scorpion venom yields, and much remains to be learned. If future researchers would devote more attention to these details, we would have a larger dataset to test simple evolutionary and phylogenetic hypotheses, such as the relationships among venom yield,
venom toxicity, body size, telson size, and pedipalp size. The dataset could also be mined for additional insights on scorpionism.
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CHAPTER FIVE

DESIGN OF COMPLEX WEAPON SYSTEMS:
SEXUAL, ONTOGENETIC, AND INTERSPECIFIC VARIATION IN
WEAPONRY OF TWO ALLOTOPIC SMERINGURUS SCORPIONS

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Abstract

Scorpions possess two multicomponent, multifunctional, integrated weapons systems. Anteriorly, they possess a grasping system comprised of a pair of pedipalps ending in chelae that seize and manipulate prey, ward off potential predators, and secure potential mates. Posteriorly, they wield a venom delivery system consisting of a tail-like metasoma with a stinger at the tip of the terminal segment (telson) that can be thrust into prey items, predators, or mates to inject venom. Given the complexity of these systems, we hypothesized that weaponry design would be subject to selective forces arising from differences in usage between the sexes, during ontogeny, and among closely-related species occupying different habitats, resulting in measurable variation. We examined two widespread species of Smeringurus scorpions: *S. mesaensis*, a psammophilous species that can occur at high densities, and *S. vachoni*, a generalist or lithophilic species existing at lower densities. Sexual body component dimorphism existed in physical weaponry, and was most exaggerated for adults of both species. Males trended toward more robust chela, especially in *S. vachoni*. Metasoma length averaged longer in males, with *S. mesaensis* demonstrating greatest divergence. The telson housing the chemical weapon stores was larger in females of both species, as was venom volume. Venom availability increased exponentially during ontogeny for both species. Although adults were of similar size, *S. vachoni* possessed significantly larger venom stores than *S. mesaensis*. Differential allocation of resources toward weaponry, both within and between these species, likely results from different selective regimes. Female-biased venom supply is associated with survival and increased reproductive demands, whereas male investment in the chela and metasoma could represent greater priority in securing mates. In the dense populations of *S. mesaensis*, adult males seldom live beyond a single breeding season,
and the exaggerated metasoma length may help ward off cannibalistic females. The robust and modified chela of male *S. vachoni* may aid in securing mating opportunities where fewer opportunities exist. Our findings highlight the multiple factors that influence weapons design in scorpions, and underscore the functional importance of these complex systems that are relied upon in varying roles and contexts.

**Introduction**

Various animal groups have independently evolved diverse weapon systems (Casewell et al., 2013; Emlen, 2008; Stankowich, 2012). Scorpions have taken this theme further than many groups in possessing two weapon systems that are multicomponent, multifunctional, and integrated (Benton, 2001; Coelho et al., 2017; van der Meijden et al., 2013). Anteriorly, they possess a pair of pedipalps ending in chelae that are useful for grasping and manipulating prey, warding off potential predators, and holding potential mates. Posteriorly, they wield a venom delivery system consisting of a stinger at the end of a tail-like multisegmented metasoma that can be thrust into a predator, prey item, or mate to inject venom. Venom is ejected through the stinger from the paired venom glands and ducts housed within the telson, the terminal segment of the metasoma.

Scorpion weapons function through the selective environments of predation (Casper, 1985; Quinlan et al., 1995; Rein, 1993), defense (Coelho et al., 2017; Heatwole, 1967; Nisani and Hayes, 2011), and reproduction (Benton, 2001; Stockmann, 2015); as such, their structure and function should vary between the sexes, ontogenetically, and across species when different life history traits, habitat requirements, and phylogenetic affiliations exist. An association often occurs, for example, between the structure of the
pedipalps/chelae, the metasoma/telson, and the venom toxicity of scorpions. Species more reliant on venom tend to have more gracile pedipalps and chelae that can operate quickly but possess a more elaborate (longer or thicker) metasoma and/or telson that delivers larger quantities of, and/or more toxic, venom (Leeming, 2003; Mebs, 2002; Newlands, 1969; Stockmann, 2015). Species less reliant on venom, in contrast, tend to have more robust pedipalps and chelae, and a less well-developed venom delivery system. In these latter species, venom may not be used for the majority of predatory and defensive encounters, at least in adults (Casper, 1985; Cushing and Matherne, 1980).

Many scorpions exhibit varying degrees of sexual dimorphism that can influence weapons design. Interssexual differences may be manifest as sexual size dimorphism (overall body size, SSD) and sexual body component dimorphism (SBCD; Fox et al., 2015). Variation at either level may result from different selective regimes and life history patterns between males and females (Blanckenhorn, 2005; Shine, 1989). The most universally dimorphic character in scorpions is elaboration of the pectines in male scorpions (Polis and Sissom, 1990; Stockmann, 2015), which has been associated with enhanced chemoreception (Gaffin and Brownell, 1997a; 1997b) for mate tracking (Melville et al., 2003; Miller and Formanowicz, 2010; Steinmetz et al., 2004; Taylor et al., 2012) and mechanoreception (Kladt et al., 2007) to identify appropriate substrates for spermatophore deposition (Abushama, 1968; Alexander, 1957; Jiao and Zhu, 2009; Melville, 2000; Tallarovic et al., 2000). Other dimorphic characters are less consistent than pectine differences, but several trends are common. In many species, females are larger overall (female-biased SSD), particularly in prosoma and mesosoma size (Polis, 1977; Polis and Sissom, 1990; Stockmann, 2015), presumably as a result of fecundity.
selection (Benton, 2001; Brown, 2001; Brown and Formanowicz, 1996; 1995; Formanowicz and Shaffer, 1993; Francke, 1981; Lourenço et al., 1996; Outeda-Jorge et al., 2009; Smith, 1990). Male chelae are often modified to better grasp and hold female chelae during the promenade au deux of courtship (Benton, 2001; 1992; Booncham et al., 2007; Kovařík, 2011; Peretti et al., 2001). Males also tend to have longer metasomas (Polis and Sissom, 1990; Stahnke, 1957a), which may result from sexual selection associated with signaling during courtship (Alexander, 1959; Benton, 2001), or the male’s sexual sting (Tallarovic et al., 2000; Toscano-Gadea, 2010); however, some of the most extreme intersexual differences are in species that do not engage in sexual stinging (Centruroides, Teruel et al., 2015), and species that do sting sexually often have shorter metasomas (Benton, 2001; Polis and Sissom, 1990). Behavioral and physiological variation may also be associated with dimorphism, as sexual differences in defensive behavior (Carlson et al., 2014; Miller et al., 2016; Shaffer et al., 1996), venom yield (Aguilera Rodriguez et al., 2010; Chadee Burgos, 2010; de Sousa et al., 2010; Miller et al., 2016), and venom composition (D’Suze et al., 2015; Miller et al., 2016; Ozkan et al., 2011; Rodríguez-Ravelo et al., 2015; Schwartz et al., 2008) have been documented in several species.

Females may also be selective during courtship (Chantall-Rocha and Japyassú, 2017; Nobile and Johns, 2005; Peretti and Carrera, 2005), whereby male size and persistence can affect reproductive success (Benton, 2001; 1992). Male size also matters in species that exhibit mate guarding, with the larger male monopolizing the female until she is ready to mate (Benton, 2001; 1993a; 1992). If total length is used to identify SSD males are typically identified as the larger sex because males tend to have longer
metasomas than females and this is the major contributor to the difference in SSD (Polis and Sissom, 1990; Stahnke, 1957a).

The complex body components of scorpions vary morphologically in ways that influence the design and effectiveness of their weapons, including the venom delivery system (Carlson et al., 2014; Coelho et al., 2017; van der Meijden et al., 2013; 2010; 2012). Yet scorpions remain under-represented in studies of their venom biology (reviewed by Nisani and Hayes, 2011) compared to other taxa, notably snakes (Fry, 2015; Hayes, 2008; Mackessy, 2010) and spiders (reviewed by Cooper et al., 2015).

Although venom can be highly beneficial to an organism, it is not without cost (McCue, 2006; Nisani et al., 2007; but see Smith et al., 2014); thus, venom composition, availability, and deployment should be finely tuned to the organism’s life history to facilitate survival (Gangur et al., 2017; Hayes, 2008; Morgenstern and King, 2013; Sunagar et al., 2016; Wigger et al., 2002). Although selection can act on any or all of these attributes, the large majority of studies have examined venom composition, but stop short of determining whether variation corresponds to functional differences (Diz and Calvete, 2016; Sunagar et al., 2016). Venom constituents have been examined in only about 100 of the 1,500-2,000 known scorpion species, with most of the characterized toxins described from fewer than 50 species of the medically important family Buthidae (Abdel-Rahman et al., 2016; Smith and Alewood, 2015). Comparisons of venom constituents among species are further complicated because scorpion venoms consist of highly heterogeneous mixtures of toxic and non-toxic components (Simard and Watt, 1990). The proteinaceous (toxic) component of a single individual’s venom may include more than 100 different molecules, most of which remain to be characterized for all but a
few species (Abdel-Rahman et al., 2016; 2014; Rodríguez de la Vega et al., 2010; Smith and Alewood, 2015). Thus, venom availability may provide a better starting point for comparative studies of how selection might influence the venom delivery system. Venom availability is subject to both anatomical and physiological constraints (e.g., secretion, storage, and activation), and represents a more fundamental and easily studied property of the system. Venom availability also relates to variation in venom composition, venom deployment, prey preference, and risk of predation. Venom availability, or “yield,” is measured as volume, wet mass, dry mass, or number of lethal doses available.

The primary purpose of this study was to examine intersexual, ontogenetic, and interspecific variation in the weaponry of two closely related Smeringurus scorpion species. More specifically, we compared the chemical arsenal (venom yield) and physical arsenal (size and shape of chela, metasoma, and telson) of the psammophilic (sand-dwelling) S. mesaensis with the more generalist, or lithophilic (rock-associated), S. vachoni. In terms of ecology (Polis, 1986; 1980a; 1979; Polis and Farley, 1979a; Polis et al., 1985; 1989; 1986) and life history (Polis and Farley, 1980; 1979b), S. mesaensis may be the best characterized of any scorpion species, whereas comparable studies of S. vachoni are lacking.

To disentangle the potentially confounding influences of intersexual, ontogenetic, and interspecific effects on weapon systems design, we needed to begin with a rigorous assessment of sexual dimorphism to determine the best character to use as a measure of overall body size. Sexual differences have been reported for both species (Haradon, 1983; Polis, 1986; Polis and Farley, 1979a; Stahnke, 1961), though their extent, and the distinctions between SSD and SBCD, remained unclear. We used an approach to identify
SSD and SBCD developed previously for lizards (Kratochvíl et al., 2003; Scharf and Meiri, 2013) and another scorpion genus (Fox et al., 2015). This method seeks to statistically establish a non-dimorphic body component that can then be used as an unbiased (or least biased) measure of overall body size. The choice of reference character for overall body size matters in the direction and interpretation of dimorphism, as use of a biased character can lead to erroneous conclusions (Fox et al., 2015).

**Materials and Methods**

*Subjects and Measurements*

We captured scorpions at night during the months of May and June 2016 and June 2017 using ultraviolet light sources. We collected 111 *S. mesaensis* from an area near Ocotillo, Imperial County, California, and 47 *S. vachoni* from scattered locations in Imperial County, Riverside Counties, and San Bernardino Counties along the California side of the border with Arizona (Fig. 1). These two species are broadly sympatric but allotopic across the desert regions of southern California and extending across the Colorado River into Arizona. Scorpions were fed and housed individually in 17 × 15 × 7 cm (L × W × H) plastic containers with sand substrate and kept at 20–24 °C on a 12:12 hr light:dark cycle. We determined sex of the scorpions by relative length and arrangement of the pectines (Polis, 1990).

We measured 12 morphological characters from each scorpion to the nearest 0.01 mm using digital calipers (ST Industries, St. James, Minnesota). The characters included chela length, width, and height; prosoma length and width (at median eye); metasoma segment 1 length (MS1L) and width (MS1W); metasoma segment 5 length (MS5L) and
width (MS5W); and telson length, width, and height. Sexual dimorphism has been reported in both species, and differences are visible to the eye (Fig. 2). As described in the analysis section, we used an unbiased measure of overall body size as our operational measure of SSD, and we evaluated SBCD for each character.

Figure 1. Collection localities for Smeringures mesaensis (Sm) and S. vachoni (Sv) specimens from southern California. The two species are broadly sympatric across the desert regions illustrated.

We assigned age class (juvenile or adult) to individuals by examining scatterplots of known dimorphic characters (primarily metasoma length and chelae size) plotted against an assumed neutral character for overall body size (MS1W; Fox et al., 2015) to visually identify the two distinct best-fit lines differing in elevation (y-intercept) that
characterized earlier instars (juveniles; lesser y-intercept) and the final instar (adults, greater y-intercept). The y-intercept difference between juveniles and adults result from the enhanced sexual dimorphism of the final instar. We also considered the chelae of *S. vachoni*, which as adults show increased keelation in both males and females and a gap between the scalloped manus and tarsus in the male (Haradon, 1983; Stahnke, 1961). We used our best judgment for assigning age class to individuals within the narrow range of overlap for overall size between the two best-fit lines.

**Figure 2.** Representative images of adult female (top row) and male (bottom row) specimens of *Smeringurus mesaensis* (right column) and *S. vachoni* (left column).
Our sample for *S. smeringurus* included 30 male and 27 female juveniles, and 19 male and 35 female adults. Our sample for *S. vachoni* included 11 male and 10 female juveniles, and 9 male and 17 female adults.

**Venom Extraction**

To facilitate venom extraction, we first immobilized the scorpions in a restraining device with the telson protruding to allow access for electrical stimulation. We applied saline to the telson to increase conductivity and applied repeated brief taps with electrically charged forceps (9 V, 100 mA, DC) to elicit venom expulsion. The number (generally 10–20) and duration (generally 0.3–2 sec) of shocks delivered varied among animals, with shocks continuing until venom expulsion ceased. We collected venom using graduated 5-μL Drummond® PCR micropipettes (0.246 mm radius; PGC Scientifics, Garner, NC, USA). The length of the venom column in the pipette was measured using digital calipers (ST Industries, St. James, Minnesota). We calculated volume of venom (V) from length of the venom column in the micropipette (L) using the formula $V = (L) \times (0.246^2) \times (3.14159)$. We also assessed venom samples visually during collection, noting whether they were clear, opalescent (cloudy), or milky (white), corresponding to the unidirectional sequence (clear to opalescent to milky) for heterogeneous venom expression (Nisani and Hayes, 2011; Yahel-Niv and Zlotkin, 1979; Zlotkin and Shulov, 1969) in a number of scorpion families (Chapter Three). Individual venom samples were transferred to and stored in microcentrifuge tubes at −20 °C. All scorpions were extracted after a fast of 21–25 days to ensure replete venom glands (Boeve et al., 1995; Candido and Lucas, 2004; Gopalakrishnakone et al., 1995).
Statistical Analyses

We conducted all analyses using SPSS ver. 20 for Macintosh (Statistical Package for the Social Sciences, Inc., Chicago, 2011), with alpha set to 0.05. Before each analysis, we screened the data to identify and remove outliers. We identified outliers via scatterplots, Mahalanobis distances, leverage values, and measures of influence (Barnett and Lewis, 1994). We removed a maximum of three outliers for any analysis involving adults, and a maximum of five for any analysis involving all individuals. As an intuitive indicator of the magnitude of group differences, we computed the percent difference (Lovich and Gibbons, 1992; Smith, 1999) for all morphological characters using the mean difference of each group (e.g., [male – female]) divided by the average of both groups (e.g., 0.5 [male + female]). However, we report absolute differences for measures of venom yield.

We used the approach of Fox et al. (2015) to assess SBCD separately in each species. First, we conducted a t-test (Field, 2015) on each of the 12 morphological characters of adults to identify characters showing relatively small or negligible dimorphism. We used only adult scorpions because dimorphism is most exaggerated in this age class. Second, we entered the least dimorphic characters from the t-tests into a discriminant function analysis (DFA; Tabachnick and Fidell, 2013) to ascertain the most suitable (least biased) reference character for overall body size. We used this character not only for evaluating SBCD for each character, but also to assess SSD. For the DFA, we preferred a smaller set of variables (rather than an omnibus DFA) because of the need to reduce parameterization with the smaller dataset (from adults only), so we used a single character for relative chelae size, created by subjecting the three characters for chelae (length, width, height) to principal components analysis (PCA; Tabachnick and
Fidell, 2013) to derive principle component 1 (PC1[chelae]). Third, using the preferred body size character as a covariate and sex as a between-subjects factor, we subjected the remaining 11 characters to univariate analyses of covariance (ANCOVAs; Tabachnick and Fidell, 2013) to characterize SBCD. For each ANCOVA, we tested the assumption of homogeneous regression slopes by pre-testing for an interaction between sex and the covariate for body size, and then removing the interaction term, if non-significant, from the final model. We calculated percent differences among groups based on estimated marginal means.

To evaluate morphological differences between the two species, we conducted a separate DFA for each sex that included all scorpions and all morphological characters. However, to reduce parameterization, we again used PCA to derive a single character for relative chelae size (PC1[chelae]), and similarly derived a single character for relative telson size using the three telson characters (PC1[telson]). Because relative chelae size and telson size differed between the species in the DFA models for both sexes, we conducted separate univariate ANCOVAs to assess interspecific differences for each of the three chelae and telson dimensions (length, width, height) including models for prosoma (length and width), MS1L, and MS5L, all using the preferred body size character (MS1W) as the covariate, separately for juveniles and adults of each sex.

To examine the variables that influence venom yield (ln-transformed), we used an omnibus ANCOVA model that included adult scorpions only (to better test for sex differences), species and sex as independent variables, and the preferred variable for body size and relative telson size as covariates. To characterize the ontogenetic relationship
between body size and venom yield, we conducted curve-fitting regressions (Tabachnick and Fidell, 2013) for each species using all scorpions and untransformed data.

We computed effect sizes as $r^2$ for $t$-tests and curve-fitting regression, eta-squared ($\eta^2$) for DFAs (computed as $1 - \text{Wilks' } \Lambda$), and partial $\eta^2$ for factorial ANCOVAs (Field, 2015; Tabachnick and Fidell, 2013). These all indicate approximate percent of variance explained in the dependent variable by an independent variable or interaction, with small, medium, and large effects corresponding loosely to values of $\sim 0.01$, $\sim 0.09$, and $\geq 0.25$ for $r^2$, and $\sim 0.01$, $\sim 0.06$, and $\geq 0.14$ for $\eta^2$, respectively (Cohen, 1988). Because partial $\eta^2$ is upward-biased when multiple variables are included in a model (Field, 2015; Tabachnick and Fidell, 2013), we adjusted values when they summed to $>1.0$ by dividing each partial $\eta^2$ value by the sum of all values. Following Nakagawa (2004), we chose not to control for experiment wise-error because doing so overemphasizes the importance of null hypothesis testing when effect sizes are more meaningful (i.e., they are more independent of sample size and more readily compared among different data sets and studies), and unacceptably increases the probability of making type II errors (Cohen, 1988; Moran, 2003; Nakagawa and Cuthill, 2007).

**Results**

**Morphology**

**Sexual Dimorphism and Intersexual Differences in Weaponry**

Separate $t$-tests for adults of each species revealed significant differences between the sexes in six characters for *S. mesaensis* and two characters for *S. vachoni* (Table 1), although differences in sample size likely contributed to the fewer dimorphic characters
in *S. vachoni*. In both species, females had approximately 5% larger prosomas (length and width). In *S. mesaensis*, males had 10–11% longer metasoma 1 and 5 segments, and females had 7–9% larger telsons (width and height, but not length). In *S. vachoni*, the moderate-to-large effect sizes suggest that males similarly had longer metasoma 5 segments (6%), and females similarly had larger telsons (6–7% in width and height, but not length).

We used separate DFA models for adults of each species, using the four characters likely to be least dimorphic based on *t*-tests (PC1[chelae], MS1W, MS5W, telson length), to identify a preferred (least biased) character for body size (Table 2). To reduce model parameterization, we combined the three chelae characters into a single principle component. For *S. mesaensis*, the non-significant model (Wilks’ Λ = 0.89, $\chi^2 = 6.12$, df = 4, $P = 0.191$, $\eta^2 = 0.11$) suggested that any of the four characters would have been suitable as a reference character for body size. For *S. vachoni*, MS1W was the least discriminating variable within a significant model (Wilks’ Λ = 0.48, $\chi^2 = 15.98$, df = 4, $P = 0.003$, $\eta^2 = 0.52$), and therefore the best reference character for body size. For consistency between species, we used MS1W as the reference character for overall body size in further analyses. Based on this character, no difference existed between sexes in overall size (SSD) of *S. mesaensis* ($r^2 = 0.01$), but moderate (though non-significant) female-biased SSD may be present in *S. vachoni* ($r^2 = 0.10$; Table 1).
Table 1. Comparisons (mean ± 1 SE) of morphological characters between adult male and female *Smeringurus mesaensis* and *S. vachoni*, including t-test results and effect sizes ($r^2$).

<table>
<thead>
<tr>
<th>Component</th>
<th>S. mesaensis ($N = 19$ males, $35$ females)</th>
<th>S. vachoni ($N = 9$ males, $17$ females)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prosoma L</td>
<td>8.3±0.07, 8.9±0.11</td>
<td>9.2±0.13, 9.7±0.12</td>
</tr>
<tr>
<td>Prosoma W</td>
<td>6.6±0.06, 6.9±0.08</td>
<td>7.4±0.11, 7.7±0.11</td>
</tr>
<tr>
<td>Chela L</td>
<td>15.0±0.12, 15.3±0.17</td>
<td>16.9±0.36, 17.0±0.22</td>
</tr>
<tr>
<td>Chela W</td>
<td>4.0±0.50, 3.9±0.05</td>
<td>4.9±0.17, 4.7±0.09</td>
</tr>
<tr>
<td>Chela H</td>
<td>5.5±0.07, 5.3±0.08</td>
<td>6.6±0.23, 6.4±0.11</td>
</tr>
<tr>
<td>MS1L</td>
<td>5.4±0.06, 4.9±0.08</td>
<td>5.9±0.26, 5.7±0.11</td>
</tr>
<tr>
<td>MS1W</td>
<td>3.7±0.03, 3.7±0.05</td>
<td>3.9±0.05, 4.0±0.06</td>
</tr>
<tr>
<td>MS5L</td>
<td>11.8±0.10, 10.6±0.16</td>
<td>12.8±0.45, 12.1±0.24</td>
</tr>
<tr>
<td>MS5W</td>
<td>2.7±0.03, 2.7±0.03</td>
<td>2.8±0.04, 3.0±0.06</td>
</tr>
<tr>
<td>Telson L</td>
<td>9.4±0.07, 9.6±0.13</td>
<td>9.6±0.21, 10.0±0.14</td>
</tr>
<tr>
<td>Telson W</td>
<td>2.5±0.03, 2.7±0.04</td>
<td>3.5±0.10, 3.7±0.07</td>
</tr>
<tr>
<td>Telson H</td>
<td>2.3±0.03, 2.5±0.04</td>
<td>3.1±0.08, 3.3±0.07</td>
</tr>
</tbody>
</table>

L = length, W = width, H = height (mm); MS1 = metasoma segment 1; MS5 = metasoma segment 5.

% Diff = percent difference between the sexes, relative to males (males larger is positive value; females larger is negative value).
Table 2. Standardized canonical coefficients for morphological characters used in discriminant function analyses to distinguish, separately for each species, between males and females of *Smeringurus mesaensis* and *S. vachoni*.

<table>
<thead>
<tr>
<th>Body Component</th>
<th><em>S. mesaensis</em></th>
<th><em>S. vachoni</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1(Chelae)</td>
<td>-1.331</td>
<td>-2.021</td>
</tr>
<tr>
<td>Telson length</td>
<td>1.671</td>
<td>1.54</td>
</tr>
<tr>
<td>Metasoma segment 1 width</td>
<td>-0.532</td>
<td>0.094</td>
</tr>
<tr>
<td>Metasoma segment 5 width</td>
<td>0.213</td>
<td>0.815</td>
</tr>
</tbody>
</table>

PC1(Chelae) = principle component 1 derived from principal components analysis of the three chelae characters (length, width, height).

The least discriminating characters have the lowest values, and represent the preferred (least biased) measures of overall body.

Separate univariate ANCOVAs for adults of each species, using MS1W as the covariate, provided more appropriate assessments of SBCD because they controlled for overall body size (Table 3, Fig. 3). Some results differed notably from direct comparisons of body size between the sexes (*t*-test outcomes). Prosoma size proved to be 5–6% larger (width and length, based on estimated marginal means at 3.7 mm MS1W) in females of *S. mesaensis*, but only 3% longer and of similar width in females of *S. vachoni* (at 4.0 mm MS1W). Chelae were more robust in males of *S. mesaensis* (2.7% shorter) and *S. vachoni* (7% wider and higher). Metasomas were longer in males of both species, with MS1L and MS5L being 7–10% longer in males of both *S. mesaensis* and *S. vachoni*. (Two male *S. vachoni* individuals showed a combination of juvenile and adult characteristics, evident in the bivariate scatterplots [not shown], resulting in significant interactions between the two metasoma segment lengths and sex, suggesting allometric slope differences between the sexes). Telsons were larger in female *S. mesaensis* (3–10%, depending on dimension), but no SBCD existed in telson size of *S. vachoni*, which contrasted to direct comparisons of the sexes (effect sizes for *t*-tests).
Table 3. Analysis of covariance (ANCOVA) results and effect sizes (partial $\eta^2$) for each morphological character tested for sexual body component dimorphism (SBCD) using MS1W as the reference character (covariate) for overall body size.

<table>
<thead>
<tr>
<th>Body Component</th>
<th>$F$</th>
<th>$P$</th>
<th>Partial $\eta^2$</th>
<th>Marginal means ± 1 S.E.</th>
<th>Percent Difference (M to F)</th>
<th>$F$</th>
<th>$P$</th>
<th>Partial $\eta^2$</th>
<th>Marginal means ± 1 S.E.</th>
<th>Percent Difference (M to F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prosoma L</td>
<td>40.7</td>
<td>&lt;0.001</td>
<td>0.44</td>
<td>8.2 ± 0.7</td>
<td>8.8 ± 0.5</td>
<td>-5.6</td>
<td>4.3</td>
<td>0.05</td>
<td>9.4 ± 0.11</td>
<td>9.7 ± 0.08</td>
</tr>
<tr>
<td>Prosoma W</td>
<td>32.7</td>
<td>&lt;0.001</td>
<td>0.39</td>
<td>6.5 ± 0.06</td>
<td>6.9 ± 0.04</td>
<td>-6.22</td>
<td>1.49</td>
<td>0.24</td>
<td>7.5 ± 0.09</td>
<td>7.6 ± 0.06</td>
</tr>
<tr>
<td>Chela L</td>
<td>7.91</td>
<td>0.007</td>
<td>0.13</td>
<td>14.9 ± 0.12</td>
<td>15.3 ± 0.09</td>
<td>-2.66</td>
<td>0.88</td>
<td>0.36</td>
<td>17.2 ± 0.25</td>
<td>16.9 ± 0.18</td>
</tr>
<tr>
<td>Chela W</td>
<td>1.18</td>
<td>0.282</td>
<td>0.02</td>
<td>4.0 ± 0.04</td>
<td>3.9 ± 0.03</td>
<td>1.29</td>
<td>8.49</td>
<td>0.01</td>
<td>5.0 ± 0.10</td>
<td>4.6 ± 0.07</td>
</tr>
<tr>
<td>Chela H</td>
<td>3.86</td>
<td>0.055</td>
<td>0.07</td>
<td>5.5 ± 0.06</td>
<td>5.4 ± 0.04</td>
<td>2.69</td>
<td>7.38</td>
<td>0.01</td>
<td>6.8 ± 0.13</td>
<td>6.3 ± 0.10</td>
</tr>
<tr>
<td>MS1L</td>
<td>35.8</td>
<td>&lt;0.001</td>
<td>0.41</td>
<td>5.4 ± 0.07</td>
<td>4.9 ± 0.05</td>
<td>10.14</td>
<td>4.4</td>
<td>0.05</td>
<td>6.1 ± 0.16</td>
<td>5.6 ± 0.12</td>
</tr>
<tr>
<td>MS5L</td>
<td>60.8</td>
<td>&lt;0.001</td>
<td>0.55</td>
<td>11.6 ± 0.10</td>
<td>10.7 ± 0.07</td>
<td>8.67</td>
<td>12.3</td>
<td>0.35</td>
<td>13.2 ± 0.29</td>
<td>11.9 ± 0.21</td>
</tr>
<tr>
<td>MS5W</td>
<td>0.19</td>
<td>0.67</td>
<td>0</td>
<td>2.7 ± 0.03</td>
<td>2.7 ± 0.02</td>
<td>-0.59</td>
<td>0.54</td>
<td>0.47</td>
<td>2.9 ± 0.03</td>
<td>2.9 ± 0.02</td>
</tr>
<tr>
<td>Telson L</td>
<td>5.14</td>
<td>0.028</td>
<td>0.09</td>
<td>9.3 ± 0.11</td>
<td>9.6 ± 0.08</td>
<td>-3.09</td>
<td>0.46</td>
<td>0.5</td>
<td>9.8 ± 0.16</td>
<td>9.9 ± 0.11</td>
</tr>
<tr>
<td>Telson W</td>
<td>43.1</td>
<td>&lt;0.001</td>
<td>0.46</td>
<td>2.5 ± 0.03</td>
<td>2.7 ± 0.02</td>
<td>-8.21</td>
<td>1.13</td>
<td>0.3</td>
<td>3.5 ± 0.06</td>
<td>3.6 ± 0.05</td>
</tr>
<tr>
<td>Telson H</td>
<td>53.6</td>
<td>&lt;0.001</td>
<td>0.51</td>
<td>2.3 ± 0.03</td>
<td>2.5 ± 0.02</td>
<td>-10.14</td>
<td>0.89</td>
<td>0.36</td>
<td>3.2 ± 0.07</td>
<td>3.3 ± 0.05</td>
</tr>
</tbody>
</table>

L = length; W = width; H = height; MS1 = metasoma segment 1; MS5 = metasoma segment 5.
Percent difference compares estimated marginal means between the sexes (computed at 3.7 mm MS1W for *S. mesaensis* and 4.0 mm MS1W for *S. vachoni*), relative to males (males larger is positive value; females larger is negative value).
Figure 3. Sexual body component dimorphism (SBCD). Results of analyses of covariance (ANCOVAs; Table 3) as percent difference in estimated marginal means between the sexes (y-axis) for each morphological character of *Smeringurus mesaensis* and *S. vachoni*. Percent difference was calculated as \(((\text{male marginal mean} - \text{female marginal mean})/(\text{male marginal mean} - \text{female marginal mean})/2))\times 100. Bars with an asterisk (*) indicate characters exhibiting significant male-biased (above zero) or female-biased (below zero) sexual body component dimorphism (SBCD). Arrows indicate comparisons for which a significant interaction between sex and body size existed (i.e., heterogenous regression slopes). Estimated marginal means were computed at 3.7 mm MS1W for *S. mesaensis* and 4.0 mm MS1W for *S. vachoni*; see Table 3).
Interspecific Differences in Weaponry

To compare the body size for adults of the two species, we conducted a $t$-test of MS1W, which demonstrated a significant difference between the two species ($t_{78} = 4.77$, $P < 0.001$, $r^2 = 0.23$). *Smeringurus mesaensis* (mean ± 1 S.E.: 3.7 ± 0.03 mm) averaged smaller than *S. vachoni* (4.0 ± 0.05 mm; 7.1% difference; 95% CI of difference between means: 0.16–0.39 mm).

Separate DFAs for all individuals (to increase sample size) of each sex, using eight characters (including PC1[chelae] and PC1[telson] to reduce parameterization), confirmed significant differentiation between the two species for both males (Wilks’ $\Lambda = 0.39$, $\chi^2 = 59.85$, df = 8, $P < 0.001$, $\eta^2 = 0.61$, $N = 69$) and females (Wilks’ $\Lambda = 0.37$, $\chi^2 = 82.69$, df = 8, $P < 0.001$, $\eta^2 = 0.63$, $N = 88$). Standardized canonical coefficients and their signs (Table 4) suggested that the most discriminating characters were, in order, telson size (larger in *S. vachoni*), MS5L (longer in *S. mesaensis*), a prosoma character (longer in male *S. mesaensis*, wider in female *S. mesaensis*), and chela size (larger in *S. vachoni*).

Univariate ANCOVAs (for adults of each sex) were used to better examine species differences while controlling for overall body size with percent differences calculated from estimated marginal means shown in Fig. 4 ($P$-values and effect sizes not provided), led us to conclude that even when accounting for body size *S. vachoni* was proportionally larger then *S. mesaensis* for all characters tested other then telson length, which resulted in a non-significant model for both sexes. The differences between males were more pronounced then those for females in all characters tested other then chela height. Several characters, Met1L, Met5L, and telson length in males resulted in
significant interactions between species and body size (i.e., heterogenous regression slopes).

Table 4. Standardized canonical coefficients for morphological characters used in discriminant function analyses to distinguish, separately for each sex, between individuals of *Smeringurus mesaensis* and *S. vachoni*.

<table>
<thead>
<tr>
<th>Body Component</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1(Chela)</td>
<td>2.368</td>
<td>-1.000</td>
</tr>
<tr>
<td>Prosoma length</td>
<td>-2.971</td>
<td>0.274</td>
</tr>
<tr>
<td>Prosoma width</td>
<td>1.321</td>
<td>-1.330</td>
</tr>
<tr>
<td>Metasoma segment 1 length</td>
<td>-1.259</td>
<td>0.052</td>
</tr>
<tr>
<td>Metasoma segment 1 width</td>
<td>-1.363</td>
<td>0.688</td>
</tr>
<tr>
<td>Metasoma segment 5 length</td>
<td>-3.669</td>
<td>-4.940</td>
</tr>
<tr>
<td>Metasoma segment 5 width</td>
<td>0.347</td>
<td>-0.748</td>
</tr>
<tr>
<td>PC1(Telson)</td>
<td>5.391</td>
<td>7.336</td>
</tr>
</tbody>
</table>

PC1(Chelae) and PC1(Telson) = principle component 1 derived from separate principle components analyses of the three chelae characters (length, width, height) and three telson characters (length, width, height), respectively.

The most discriminating characters have the highest values.
Figure 4. Species differences in weaponry. Results of analyses of covariance (ANCOVAs) as percent difference in estimated marginal means between the species (y-axis) separated by sex for chela and telson characters of *Smeringurus mesaensis* and *S. vachoni*. Percent difference was calculated as \((S. mesaensis \text{ marginal mean} - S. vachoni \text{ marginal mean})/((S. mesaensis \text{ marginal mean} - S. vachoni \text{ marginal mean})/2)\) \times 100. Bars with a pound symbol (#) indicate characters exhibiting no significant species difference for that body component, and arrows indicate comparisons for which a significant interaction between species and body size existed (i.e., heterogenous regression slopes). Estimated marginal means were computed at 3.78 mm MS1W for males and 3.80 mm MS1W for females.
Ontogenetic Variation in Weaponry

When plotted against overall body size (MS1W), ontogenetic trajectories for all dimensions (length, width, height) of weapon characters (chelae, Met1, Met5, telson) appeared to be linear within each of the eight groups formed by sex × species × age class. However, scatterplots for many characters exhibited obvious shifts in elevation (y-intercept) for regression lines of juveniles (lesser y-intercept) and adults (greater y-intercept), as illustrated in Fig. 5. Considering the significant intersexual and interspecific differences, and apparent age class differences, we chose not to run regression analyses to characterize allometric relationships (Fox et al., 2015) because of the small samples within each of the eight groups that would be needed to run these tests. Preliminary tests Utilizing ANCOVA models suggested that for juveniles most of the body characters measured resulted in non-significant models suggesting a high degree of morphological similarity between the species when testing the sexes together or separately (Data not included). Given the adult species differences (see the section on interspecific differences in weaponry) the ontogenetic shifts that must occur would be interesting but require a larger data set. The characters that appeared distinctive as juveniles (Chela width and telson measures) fit with the known species differences.
Figure 5. Representative case of the ontogenetic increase in a body component between juveniles and adults in *Smeringurus vachoni* for chelae height relative to body size (metasoma segment 1 width, MS1W). Differences in elevation (y-intercept) of the best-fit regression lines indicate enhanced sexual dimorphism of the final instar and identification of juvenile and adult individuals.
**Venom Yield**

Intersexual, Ontogenetic, and Interspecific Variation in Weaponry

Venom yield obtained through electrical stimulation of all *S. mesaensis* (*n* = 114; 53 ♂♂, 61 ♀♀) averaged 3.8 ± 3.0 μL (range 0.1–10.3 μL) per individual. Adults (*n* = 53; 19 ♂♂, 34 ♀♀) yielded 6.5 ± 1.7 μL (range 2.6–10.3 μL), with males delivering 5.8 ± 1.1 μL (range 4.1–8.9 μL) and females 6.9 ± 1.8 μL (range 2.6–10.3 μL). *Smeringurus vachoni* averaged larger volumes (*n* = 44; 19 ♂♂, 25 ♀♀), producing 11.6 ± 6.8 μL (range 0.7–26.4 μL). Adults (*n* = 25; 9 ♂♂, 16 ♀♀) yielded 16.5 ± 4.6 μL (range 7.7–26.4 μL), with males delivering 14.0 ± 3.3 μL (range 7.9–18.8 μL) and females 17.9 ± 4.7 μL (range 7.7–26.4 μL).

Our omnibus model of ln(venom yield) for adults, using species and sex as independent variables and ln(MS1W) and relative telson size (residuals of ln[PC1(telson)]) regressed against ln[MS1W]) as covariates, provided significant main effects with no interactions. We included relative telson size, in part, to understand its importance to venom yield regardless of species and sex. Significant differences in venom yield existed between the species (*F*<sub>1,69</sub> = 48.14, *P* < 0.001, adjusted partial η<sup>2</sup> = 0.29), with *S. vachoni* averaging 22.6% (among males, absolute difference based on estimated marginal means at ln[MS1W] = 1.33 and residuals of ln[PC1(telson)] = -0.015) and 26.0% (among females) larger yields than *S. mesaensis*. The sexes also differed significantly (*F*<sub>1,69</sub> = 4.50, *P* = 0.038, adjusted partial η<sup>2</sup> = 0.04), but the effect size was rather small, with females averaging just 2.4% (within *S. mesaensis*) and 5.2% (within *S. vachoni*) larger yields than males. Venom yield also increased significantly with body size (*F*<sub>1,69</sub> = 97.74, *P* < 0.001, adjusted partial η<sup>2</sup> = 0.42) and with relative telson size.
(F\textsubscript{1,69} = 35.65, P < 0.001, partial η\textsuperscript{2} = 0.24). The importance of relative telson size to species and sex differences becomes more apparent after its removal from a second model. The main effects were similar in this second model, but at an equivalent body size (ln[MS1W] = 1.33), the difference in venom yield between the species became greater (averaging 39.7% and 48.1% more in S. vachoni for females and males, respectively), and the difference between the sexes became much greater for S. mesaensis (females 11.7% greater) but remained essentially unchanged for S. vachoni (females 5.4% greater).

We examined the shape of the relationship between venom yield and scorpion size (MS1W) for each of the two species after pooling data from the sexes because sex differences were small relative to species differences. Curve-fitting regression with untransformed data showed that venom yield increased exponentially in relation to scorpion size (Fig. 6). A power function best fit the relationship for both S. mesaensis (venom yield = 0.050 x MS1W\textsuperscript{3.68}, r\textsuperscript{2} = 0.96, N = 110) and S. vachoni (venom yield = 0.034 x MS1W\textsuperscript{4.45}, r\textsuperscript{2} = 0.93, N = 43). Measures of model fit for the power function were higher than those for alternative models, including linear (S. mesaensis and S. vachoni, respectively: r\textsuperscript{2} = 0.85 and 0.74), quadratic (r\textsuperscript{2} = 0.90 and 0.78), and exponential (r\textsuperscript{2} = 0.94 and 0.90) models.

**Venom Heterogeneity**

We noted venom heterogeneity in the expressed venom of both species. Most samples progressed visually from clear to opalescent, clear to opalescent to milky, clear to milky, or opalescent to milky. However, some remained opalescent or milky throughout. None showed a reversal in sequence.
Venom yield as a function of body size (metasoma segment 1 width, MS1W) for the scorpions *Smeringurus mesaensis* and *S. vachoni*. Sexes were pooled because differences were minor compared to species differences. Venom yields for both *S. mesaensis* (closed circles, solid line) and *S. vachoni* (open circles, dashed line) represented an exponential relationship between volume (V) and body size (MS1W) best described by power functions (*S. mesaensis*: $V = (0.05) \times MS1W^{3.68}$, $r^2 = 0.96$, $n = 110$; *S. vachoni*: $V = (0.034) \times MS1W^{4.45}$, $r^2 = 0.93$, $n = 43$).

**Discussion**

To our knowledge, this study represents the first rigorous comparisons of sexual and ontogenetic influences on the weaponry (pedipalps/chelae and venom delivery system) of closely-related scorpion species. After identifying a suitable measure of overall body size (MS1W), we demonstrated remarkable variation in weaponry design at
all three levels examined: intersexual (as SBCD), ontogenetic, and interspecific. We discuss each of these in turn.

**Sexual Dimorphism and Intersexual Differences in Weaponry**

Our analyses comparing MS1W suggest that SSD is absent in *S. mesaensis*, but hint (based on effect size) that female-biased SSD may be present in *S. vachoni*. The existing literature claims that SSD exists in both species (Polis, 1986; Polis and Farley, 1979b; Stahnke, 1961; 1957b), with females attaining a larger body size based on mass, or dimensions of the prosoma and mesosoma. Assessing the overall size difference between sexes becomes complicated when some body components are larger in males, and other body components are larger in females. As we have shown here and elsewhere (Fox et al., 2015), the prosoma, mesosoma, and other body components may represent biased measures of overall body size. Body mass is also unreliable (Brown, 2001; van der Meijden et al., 2013; 2012) because it fluctuates with nutrition and, in females, reproductive status. We are confident that our approach, which led us to choose MS1W as the preferred character for overall body size, provides a salient interpretation (operational definition) of SSD in these two species. However, alternative approaches could be justified as well, such as use of the first principal component from a PCA that includes all characters, which generally characterizes body size (Bookstein, 1989; Zelditch et al., 2004).

Our analyses provide much stronger evidence for the presence of SBCD in both species, with the pattern matching that described for other scorpion taxa. Prosomal size was proportionally larger in females of both species, fitting the concept of fecundity
selection that enhances reproductive capacity in scorpions (Benton, 2001; Brown and Formanowicz, 1996; 1995; Formanowicz and Shaffer, 1993; Smith, 1990). Chela size and metasoma lengths were proportionally greater in males of both species, which is typical of many scorpions, though selective regimes may differ for the two structures, which we address next.

In several scorpion families, the chelae of adult males appear to be designed, at least in part, to grasp the female chelae during the promenade au deux that precedes mating in most, if not all, scorpions (Benton, 2001; Polis and Sissom, 1990). The distinctive chela shape is most apparent in adults, suggesting an important role for mating (Benton, 1993b). The entire chelae is visually dimorphic in some species, but more subtly dimorphic in others, with depressions in the tibia that accommodate the female’s chelae, or scalloping of the cutting edge of the tibia and tarsus to provide a better grip (Benton, 2001; 1992; Booncham et al., 2007; Kovařík, 2011; Peretti et al., 2001). Of the two species we examined, S. vachoni has more dimorphic chelae, demonstrating both the increased scalloping of the cutting surface and greater robustness in width and height, but not length. Smeringurus mesaensis has less dimorphic chela, with more subtle scalloping and males having a slightly shorter length than females.

The adaptive significance of male bias in metasoma segment lengths, though pervasive in scorpions (Polis and Sissom, 1990), remains less clearly defined; however, it has been suggested to affect sexual signaling via visual (length, movement during pre-courship behaviors: Alexander, 1959; Gaffin and Brownell, 1992), tactile (grasping the metasoma: Alexander, 1959; Ross, 2009; or clubbing Peretti, 1993; 1991; Polis and Farley, 1979a; Polis and Sissom, 1990; Tallarovic et al., 2000) or chemical means (sexual
sting: Jiao and Zhu, 2010; Peretti, 1993; Tallarovic et al., 2000), and via telson glands (González et al., 2015; Olivero et al., 2017; Peretti, 1993). Telson SBCD also occurs commonly in scorpions, but the direction of bias can vary. Prior descriptions *S. mesaensis* suggested that males have larger, or more robust, telsons (Stahnke, 1961); however, we found the opposite with females having proportionally larger telsons in both species.

We were surprised to identify sexual differences in venom yield, which existed even when controlling for relative telson size. Females of both species (more so for *S. mesaensis*) possessed a larger venom supply, which suggests that their venom glands occupied a larger portion of the telson than those of males. We did not detect this difference in juveniles (data not shown) because the difference likely emerges with sexual maturation. Greater venom availability in adult females may be related to allocation of resources toward survival and offspring provisioning. Demographic analyses of *S. mesaensis* suggest disproportionate mortality of adult males, which likely results from increased vagrancy in search of receptive females during the breeding season (Polis, 1986; 1980a; 1977). Males seldom survive more than one breeding season, whereas females may survive several (Polis and Farley, 1980). The sexual sting known in other scorpion species has not been observed in males of *S. mesaensis* (Polis and Farley, 1979a), and it remains unknown whether this form of venom use exists in other *Smeringurus* species which may further limit the necessity for venom availability. Males, therefore, may invest more in mobility and spermatophore production, whereas females invest more in venom to acquire sufficient nutrition for developing embryos and provisioning of offspring, which can result in a 10–60 percent increase in body mass (Brown, 2001; 2003; Myers, 2001; Warburg, 2011).
Sexual differences in venom yield, while less common in the literature than sexual differences in venom composition have been reported in other scorpions. In both *Centruroides limpidus* and *C. vittatus*, females exhibit greater venom yields than males (Cid Uribe et al., 2017; Miller et al., 2016). For *C. vittatus*, females had twice the venom available and were more likely to utilize it for defense compared to their male counterparts, although they delivered a similar percentage of total yield per sting, and had similar proportional volumes when controlling for body size (Miller et al., 2016). Females do not always have greater venom stores. In the South American species *Tityus nororientalis*, males seem to have larger venom stores (Aguilera Rodriguez et al., 2010; Chadee Burgos, 2010; de Sousa et al., 2010), yet a similar species, *Tityus isabelcecilae*, had no sexual difference in venom yield (González-Sponga et al., 2001), as was the case with *H. arizonensis*, another scorpion sharing habitat with both Smeringurus species we examined (Chapter Four).

Clearly, much remains to be learned about sexual differences in the venom yields of scorpions. Because sexual differences in venom composition have been identified in several scorpion families (Abdel-Rahman et al., 2009; D'Suze et al., 2015; de Sousa et al., 2010; Miller et al., 2016; Ozkan et al., 2011; Rodríguez-Ravelo et al., 2015; Schwartz et al., 2008; Yamaji et al., 2004), we should expect selection to favor differences in venom yield as well. Our findings provide the first example documented within the family Vaejovidae. We urge future investigators to adopt more rigorous approaches in controlling for body size when examining sexual differences in venom yield.
Ontogenetic Variation in Weaponry

Sexual dimorphism in scorpions, while identifiable throughout development in some body components (especially the pectines), becomes most apparent across multiple body components (including the weapon structures) at the transition from the penultimate to adult instar (Brown, 1998). This change was very abrupt in some characters of *Smeringurus* (Fig. 5). Scorpions generally become mature after a fixed number of instars (believed to be 7–8 in *S. mesaensis*; Fox, 1975; Francke and Sissom, 1984; Polis and Farley, 1979b), but growth rates can vary among individuals, creating variation in size at each instar and the potential for differences in reproductive success between small and large adults (Benton, 2001). In several scorpion genera, individuals may transition into adulthood from different instars, resulting in early- and late-developing adults (Francke and Sissom, 1984) with differential expression of sexually dimorphic body components. These individuals represent a tradeoff between developmental time and adult size in an attempt to maximize reproductive opportunities (Benton, 2001; 1992; 1991). In males, early-developing individuals tend to be smaller and less physically dimorphic than late-developing males (Benton, 1991; Francke and Sissom, 1984; Teruel et al., 2015), but may gain mating opportunities by being reproductively active over a longer period of time than late-developing males (Benton, 2001; 1991), especially in short-lived species.

In our study, two *S. vachoni* males exhibited a combination of juvenile and adult characters, resulting in a statistical interaction between sex and relative metasoma segment lengths, suggesting allometric differences in slope between the sexes. These males had less-than-expected metasoma lengths based on their body size (MS1W). One other adult male also exhibited a combination of adult and juvenile traits, but had an insufficient effect to influence the models. We offer three plausible interpretations for
these unusual specimens. First, adult males may exist as two distinct instars, as noted above. Second, the variation among males might reflect population differences, as \textit{S. vachoni} scorpions were collected from several locations; however, other males from the same localities grouped as expected. Third, the results may reflect insufficient sampling, with intermediate individuals lacking. A larger survey of adults from these population would be necessary to better evaluate these alternative explanations.

Similar to the trend found in \textit{Hadurus arizonensis} (Fox et al., 2009), venom yield increased exponentially with body size in both species of \textit{Smeringurus} (Fig. 6). Body size explained the largest amount of variation in venom yield, which is consistent with most venomous animals, including other scorpions (D'Suze et al., 2015; van der Meijden et al., 2015; Whittemore et al., 1963), spiders (Estrada-Gómez et al., 2013; Herzig et al., 2008; Morgan, 1969; Perret and Freyvogel, 1973; Rocha-E-Silva et al., 2009; Vapenik and Nentwig, 2000; Wong et al., 2016), and snakes (Glenn and Straight, 1982; Mackessy, 1988; Mackessy and Baxter, 2006; Mackessy et al., 2003; Mirtschin et al., 2002). Our model also suggested a strong correlation between venom yield and relative telson size in adults of both species, and juveniles demonstrated a similar trend (data not shown). The paired venom glands occupy a large portion of the telson (see Hjelle, 1990), so we would expect this relationship if we were successful in consistently expressing all accessible venom from the glands.

\textit{Interspecific Differences in Weaponry}

Morphological differences between \textit{S. mesaensis} and \textit{S. vachoni} are largely consistent with prior descriptions (Haradon, 1983; Stahnke, 1961; 1957b). When using
MS1W as a measure of body size, adult S. vachoni averaged slightly larger than S. mesaensis, but the difference was small and could reflect population variation in average adult size, which has been reported in S. mesaensis (McCormick and Polis, 1986). As expected (Haradon, 1983; Stahnke, 1961), the chela and telson were more robust (greater width and height, shorter length) in both sexes of S. vachoni. The proportionally larger telson of S. vachoni conferred greater venom availability compared to S. mesaensis. More unexpected and with no obvious explanation, the prosoma and metasomal segments were proportionally longer in S. mesaensis.

We assume that weapons design in scorpions is closely linked to ecology, which influences population density and demographics, prey species and abundance, predator species and abundance, and mating behaviors. We should therefore expect to see species differences in weapons design, and possibly geographic variation, depending on local selective regimes. The most ecologically salient feature distinguishing these two species appears to be habitat specialization. Smeringurus mesaensis is a typical psammophile, whereas S. vachoni is more lithophilic (Fet et al., 1998; Graham et al., 2017; Haradon, 1983). The psammophilic specialization of S. mesaensis may be derived (Fet et al., 1998), as the other three species in the genus, and presumably the common ancestor, are also associated with rocky terrain (Graham et al., 2017; Haradon, 1983). Psammophily has likely resulted in unique morphological, physiological, and behavioral adaptations (Fet et al., 1998). Population density also appears to differ between the two species, which presumably influences mating tactics and frequency of cannibalism. In our experience, having surveyed both species at numerous locations in both Arizona and California (G. A. Fox and W. K. Hayes, unpubl. data), S. vachoni nowhere attains the high densities
documented for many *S. mesaensis* populations (Polis and Farley, 1980; Polis and McCormick, 1986). Although prey and predators have been described for *S. mesaensis* (McCormick and Polis, 1986; Polis, 1986; 1979), comparable studies are lacking for *S. vachoni*, but we assume that differences must exist in the different habitats. Differences in population density could result in higher levels of cannibalism in *S. mesaensis* (Polis, 1980b), leading to selection for the proportionally longer metasomal segments and telson. The proportionally larger prosoma (in length, but not width) could result from fecundity selection, but we would expect *S. mesaensis* to experience k-selection (Polis, 1990; Polis and Farley, 1980) at the higher densities, and therefore smaller litters. Further study is needed to examine these possibilities.

**Venom Heterogeneity**

Our study confirmed venom heterogeneity in *S. mesaensis* (van der Meijden et al., 2015) and demonstrated its presence in *S. vachoni*. Venom heterogeneity was evident from the progression of clear to opaque (milky) venom during the series of electrical shocks applied to the telson, although higher flow rates in some individuals coupled with low volumes may have obscured this trend. Changes in venom composition accompanying venom expulsion have been best characterized in another scorpion, *Parabuthus transvaalicus*, wherein the initial clear venom is rich in potassium ions, and the opaque venom that emerges subsequently is rich in protein (Inceoglu et al., 2003). In addition to Vaejovidae (*Smeringurus*), venom heterogeneity has also been documented in the scorpion families Buthidae (Inceoglu et al., 2003; Nisani et al., 2007; 2012; Nisani and Hayes, 2011; Sarhan et al., 2012; Yahel-Niv and Zlotkin, 1979; Zlotkin and Shulov,
Venom heterogeneity also exists in other arthropods (Morgenstern et al., 2012; Zobel-Thropp et al., 2013) and cone snails (Dutertre et al., 2010; 2014; Prator et al., 2014), but apart from one study of spitting cobras (Cascardi et al., 1999), it has not been reported in snakes.

Conclusions

In this study, we have documented remarkable variation in weaponry design subject to sexual, ontogenetic, and interspecific influences. Sexual differences were evident for all weapon components in both species, including the chela (females longer, males more robust), metasoma (males longer), telson (females larger), and venom yield (females greater). Ontogenetic variation was likely for most weapons, though small sample sizes for morphological measures constrained statistical analyses; however, morphological differences between the sexes became most pronounced after the last instar, and venom yield increased exponentially with overall body size. Interspecific differences were apparent for all weapon components, including chela (more robust in *S. vachoni*), metasoma (longer in *S. mesaensis*), telson (greater width and height in *S. vachoni*, but longer in *S. mesaensis*), and venom yield (greater in *S. vachoni*). The data we present, along with recent phylogeographic information on the genus and ecological differences between the species, suggest that *Smeringurus* scorpions are ideally suited for studying the factors that influence weapons design.
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CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, I have characterized the weapons systems of two representative scorpion genera, and I have tested hypotheses that relate to the design of their weapons. Scorpions possess two integrated multifunctional weapons systems. Anteriorly, they possess a grasping system comprised of a pair of pedipalps ending in chelae that seize and manipulate prey, ward off potential predators, and secure potential mates. Posteriorly, they wield a venom delivery system consisting of a tail-like metasoma with a stinger at the tip of the terminal segment (telson) that can be thrust into prey items, predators, or mates to inject venom. Given the complexity of these systems, I hypothesized that weaponry design would be subject to selective forces arising from differences in usage between the sexes, during ontogeny, and among closely-related species occupying different habitats. In this concluding chapter, I begin by revisiting the rationale by which my studies progressed; I summarize the findings of each chapter; and I offer some thoughts regarding the directions that future research could proceed.

Concept Development and Progression

To begin characterizing the weapon systems of scorpions, I first grappled with the issue of overall body size and sexual dimorphism, as both of these attributes must be taken into account when comparing weapons design among different groups. Sexual dimorphism in scorpions can exist in virtually every body part, and virtually all prior studies of scorpions have failed to properly control for overall body size when comparing specific body components. Borrowing a statistical approach used previously for lizards,
and using a scorpion species believed to be sexually monomorphic (*Hadrurus arizonensis*), I was able to identify a relatively unbiased measure of overall body size—equivalent in males in females—and used this to control for body size in subsequent analyses.

Next, I evaluated the venom delivery system of *H. arizonensis* to determine whether sexual differences exist, particularly in venom yield. Finally, using the morphometric, venom extraction, and statistical approaches developed for *H. arizonensis*, I examined both weapons systems—venom delivery and the pedipalps/chelae—in two sister species of the genus *Smeringurus* inhabiting different environments.

**Conclusions from Individual Studies**

In Chapter two, I implemented a statistical model to evaluate the presence of sexual dimorphism in a traditionally-defined monomorphic species, *H. arizonensis*. To aid in the evaluation of sexual dimorphism, I introduced a new term into the literature to differentiate between the effects of dimorphism on overall body size (sexual size dimorphism, SSD, which enjoys widespread use) and dimorphism that can exist for specific characters that may be under sexual selection (sexual body component dimorphism, SBCD, which is a new term and frequently conflated with SSD). Using a statistical approach borrowed from lizard researchers, I confirmed the absence of SSD in *H. arizonensis*, but showed that SBCD exists in essentially every body component of this cryptically dimorphic species. In doing so, I demonstrated the benefits of utilizing a minimally biased character to control for body size differences between the sexes compared to the potentially spurious correlations that can result from using a biased
character when evaluating both SBCD and static allometric relationships. The pattern of SBCD in *H. arizonensis* mirrored that seen in other more obviously dimorphic scorpions, with static allometry trending towards isometry in most characters. My findings are consistent with the conclusions of others that fecundity selection likely favors a larger prosoma in female scorpions, whereas sexual selection may favor other body parts being larger in males, especially the metasoma, pectines, and possibly the chela. For this scorpion and probably most other organisms, the choice of reference character profoundly affects interpretations of SSD, SBCD, and allometry. Based on analyses from *H. arizonensis*, and an evaluation of the literature for examples of dimorphic characters in scorpions, I suggested that metasoma segment 1 width should be a largely unbiased character for use in controlling for body size in many scorpion species.

In Chapter three, I evaluated the literature on venom yield in scorpions. Because venom may represent a significant metabolic expense, natural selection should act to optimize its production and supply. However, venom supply (i.e. venom yield) may be subject to numerous internal (e.g., genetics, age, sex, body size, health, reproductive state, recent usage, regeneration rate, production costs) and external (e.g., season, temperature, humidity, prey availability, prey size, prey susceptibility to venom) influences. While improved analytical techniques and decreased costs are permitting the characterization of many more species, only five families have been examined, and only about 70 of the roughly 2,140 scorpion species, mostly within family Buthidae. Apart from ontogeny (body size), few factors can be identified that substantially influence venom availability in scorpions. Unfortunately, reported values for venom yield are often tangential to the primary investigation and highly biased toward medically relevant
species, and therefore lack sufficient detail for rigorous comparisons of yield. Future research is needed that specifically addresses venom yield and its influences to provide a better understanding of venom supply and expression in scorpions.

In Chapter four, I investigated how ontogeny, sex, and other variables affect volume yield and protein concentration of electrically extracted venom in *H. arizonensis*. Venom yield was strongly and exponentially related to overall body size, and weakly proportional to relative telson size, but was similar for the two sexes, independent of relative mass (body condition), and similar for the two milking groups (corresponding to season and/or duration in captivity). Compared to venom yield, venom protein concentration was much less dependent on overall body size, though there was a weak negative relationship. Protein concentration varied most among the milking groups (declining with duration in captivity and/or shift from fall to winter), and to a lesser extent between the sexes (greater in females than in males), with relative telson size and body condition having no measurable influence. When individual scorpions were subjected to repeated venom extractions at 21-day intervals, each extraction resulted in consistent volume yields, but reductions in protein concentration were evident over time.

In Chapter five, I examined two widespread species of *Smeringurus* scorpions: *S. mesaensis*, a psammophilous species that can occur at high densities, and *S. vachoni*, a generalist or lithophilic species existing at lower densities. I showed that SSD is probably absent in both species. However, sexual body component dimorphism existed in physical weaponry, and was most exaggerated for adults of both species. Males trended toward more robust chela, especially in *S. vachoni*. Metasoma length averaged longer in males, with *S. mesaensis* demonstrating greatest divergence. The telson housing the chemical
weapon stores was larger in females of both species, as was venom volume. Venom availability increased exponentially during ontogeny for both species. Although adults were of similar adult size, *S. vachoni* possessed significantly larger venom stores than *S. mesaensis*. Differential allocation of resources toward weaponry, both within and between these species, likely results from different selective regimes. Female-biased venom supply is associated with survival and increased reproductive demands, whereas male investment in the chela and metasoma could represent greater priority in securing mates. In the dense populations of *S. mesaensis*, adult males seldom live beyond a single breeding season, and the exaggerated metasoma length may help ward off cannibalistic females. The robust and modified chela of male *S. vachoni* may aid in securing mating opportunities where fewer opportunities exist.

Collectively, my findings highlight the multiple factors that influence weapons design in scorpions, and underscore the functional importance of these complex systems that are relied upon in varying roles and contexts. My findings also offer meaningful insights on the constraints on behavioral deployment of venom, appropriate milking regimens for sustainable venom production, and medical risks and symptoms associated with scorpionism.

**Future Directions**

My initial study of sexual dimorphism invites re-evaluation of the way researchers examine sexual dimorphism in various body components of animals. To my knowledge, only a handful of studies have properly disentangled SSD and SBCD when evaluating the latter. Clearly, researchers need to broaden their consideration of an
appropriate reference for overall body size, and exercise caution in interpreting findings. Although other approaches may be valid, I highly recommend use of discriminant function analysis to identify the least-biased reference character for overall body size. Alternative approaches may work, such as use of principal component 1, particularly if it is the only component generated. Many notions based on earlier analyses of SBCD will need to be reconsidered, as has been the case for head size dimorphism in lizards (Braña, 1996; Kratochvíl et al., 2003; Scharf and Meiri, 2013). I am presently examining another scorpion species, and the lab in which I have done my work is currently applying my approach to the analysis of SSD and SBCD in snakes, which are especially challenging due to the limited number of body components.

As I concluded in my review of venom yield in Chapter three, much remains to be learned about the factors that influence venom yield. Considering the importance of venom volume to envenomation capacity and physiological effects on a target organism, the factors that influence venom yield might be more informative about weapons design than studies of venom composition. Well-designed studies should be undertaken to evaluate potential trade-offs between venom investment and body condition, and between venom investment and reproductive state. The effects of venom supply, which can vary with recent usage and regeneration rate, might also influence venom deployment during feeding (as documented for spiders, Hostettler and Nentwig, 2006; Wullschleger and Nentwig, 2002) and defense. Constraints on venom production should also be examined for external factors that influence metabolism, such as season, temperature, and humidity. We need more studies of different species to apply the comparative approach to several hypotheses regarding venom delivery system design. With such studies we could learn,
for example, whether the suspected negative association between anterior (pedipalp/chelae) and poster (venom) weapons systems would hold up. In other words, do scorpion species that rely more on crushing their prey have diminished envenomation capacity, and those that rely more on venom have diminished pedipalp/chelae function? And how might an ontogenetic shift away from venom use and toward pedipalp/chelae use in some species influence investment in venom supply?

Although future work should be done with other organisms, my work with the two species of *Smeringurus* invites follow-up study. Why do the two species differ so substantially in weapons design? In Chapter five, I hypothesized that the different habitats occupied would have different selective regimes, but at this point we have a good understanding of population density, prey species consumption, and significant predators for only one of these species (the psammophile *S. mesaensis*). Clearly, comparable data need to be collected for *S. vachoni*.
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