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Variation in Morphology, Diet, and Venom Composition in Crotalus pyrrhus (Cope 1867)

Chip Cochran

LOMA LINDA UNIVERSITY
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Variation in Morphology, Diet, and Venom Composition in *Crotalus pyrrhus* (Cope 1867)

by

Chip Cochran

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the requirements for the degree
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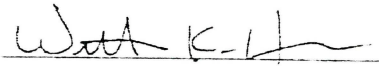

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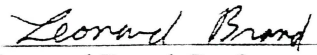

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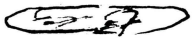

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ABBREVIATIONS

aPTT	Partial Thromboplastin Time
AU	Sonoran Desert Arizona Upland Subdivision
AV	Apple Valley
BCN	Baja California Norte
BNP	B-type Natriuretic Peptide
CD	Sonoran Desert Colorado Desert Subdivision
CHK	Chuckwalla Mountains
CNP	C-type Natriuretic Peptide
COP	Copper Mountains
CRiSP	Cysteine-rich Secretory Protein
CTL	C-type Lectin
CVF	Cobra Venom Factor
DFA	Discriminant Functions Analysis
DH	Deem Hills
DZA	Dulzura
ER	Elution Region
HAR	Harcuvar Mountains
Hb	Hemoglobin
HcT	Hematocrit
HL	Head Length
HW	Head Width
INR	International Normalized Ratio

kDa	Kilodalton
LAAO	L-amino Acid Oxidase
LACM	Los Angeles County Museum of Natural History
LCR	Sonoran Desert Lower Colorado River Subdivision
LSB	Little San Bernardino Mountains
LQ	La Quinta
McC	McCullough Range
MD	Mojave Desert
MS	Mass Spectrometry
MTX-A	Mojave Toxin Acidic
MTX-B	Mojave Toxin Basic
MUD	Muddy Mountains
NGF	Nerve Growth Factor
NR	Not Recorded In Medical Record
ORO	Orocopia Mountains
PC1	Principle Component 1
PIS	Pisgah Lava Flow
PLA2	Phospholipase A_2
PLT	Platelets
PMP	Phoenix Mountain Preserve
PR	Peninsular Ranges
PT	Prothrombin Time
PV	Palo Verde Mountains

SB	San Bernardino Mountains
SBCD	Sexual Body Component Dimorphism
SDAU	Sonoran Desert Arizona Upland
SDCD	Sonoran Desert Colorado Desert
SDLCR	Sonoran Desert Lower Colorado River
SDNMH	San Diego Natural History Museum
SJ	San Jacinto Mountains
SM	South Mountain
SP	Serine Protease
SSD	Sexual Size Dimorphism
SVL	Snout-vent-length
SVMP	Snake Venom Metalloprotease
TAM	Tinajas Altas Mountains
TEM	Temescal Mountains
TL	Tail Length
TR	Transverse Ranges
TrL	Trunk Length
VEGF	Vascular Endothelial Growth Factor
VUL	Vulture Mountains
WEA	Weaver Moutnains

ABSTRACT OF THE DISSERTATION

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in *Crotalus pyrrhus* (Cope 1867)

by

Chip Cochran

Doctor of Philosophy, Graduate Program in Biology
Loma Linda University, June 2019
Dr. William K. Hayes, Chairperson

Because rattlesnakes rely heavily on their venom for predation and defense, we can expect selection pressures from several sources to act on its composition, including, ontogeny, sex, and environmental variation. In this dissertation, I summarize the results of four studies of the southwestern speckled rattlesnake (*Crotalus pyrrhus*). First, I describe two cases of envenomation at separate Arizona localities (Tinajas Altas Mountains, Yuma County, and Phoenix Mountains, Maricopa County). Both patients experienced swelling, but neither demonstrated coagulopathy, thrombocytopenia, or hypofibrinogenemia. The latter patient required amputation of the distal portion of his middle finger. I also investigated variation in morphology, diet, and venom protein composition from 23 populations within six biogeographic regions across the species' United States range of Arizona, California, and Nevada. For morphological variation, snakes varied in in size among biogeographic regions, with the largest snakes occurring in the Mojave Desert, Transverse Ranges, and Peninsular Ranges of California and Nevada; the smallest snakes restricted to the Lower Colorado River subdivision of the Sonoran Desert; and snakes of intermediate size inhabiting the Colorado Desert (California) and Arizona Uplands subdivision of the Sonoran Desert. I also documented

sexual body component dimorphism, with females possessing larger heads and longer trunks than males relative to overall body size. The diet of *C. pyrrhus* consisted predominately of mammals (80.8%), in particular terrestrial squirrels (39.4%) and the heteromyid rodent genus *Chaetodipus* (26.9%). An ontogenetic shift occurred from primarily lizards to rodents, but neither biogeographic region nor sex significantly influenced prey class consumed. However, the Tinajas Altas Mountains population apparently relies on birds to a greater extent than other populations. For venom composition, I used reverse-phase liquid chromatography and mass spectrometry to define eight elution regions, each dominated by one to three protein families. Seven elution regions varied among the biogeographic regions and three by sex. The biggest distinction was between venoms of eastern (Arizona) and western (California/Nevada) snakes. We failed to detect Mojave Toxin (or a homolog) in any population. Collectively, these findings document differences in phenotypic and behavioral traits of *C. pyrrhus* and suggest that variable fitness landscapes provide different fitness optima.

CHAPTER ONE

INTRODUCTION

This dissertation concerns an important aspect of biology that is vital to both understanding the past and predicting the future: variation. We know from a large body of work that variation is the product and the substrate of change. Variation can be seen at all levels of complexity, and is best reflected in the term "biodiversity."

My work here originated with and narrates a personal journey that began with a friend who suffered envenomation from a rattlesnake. As frightening as the experience was, I also found it inspiring. I wanted to understand better the symptoms my friend suffered, which required an intimate look at the snake's venom composition. To better understand why venom varies, I needed to look at diet to see if the challenges of getting food, which can vary from one location to another as well as between the sexes and within an individual's lifetime, might somehow influence venom composition. To better understand how body size and sex influence diet and venom composition, I came to the realization that prior efforts to characterize size and shape in snakes have been flawed, and found a novel approach to better quantify morphological variation in these limbless creatures. The final narrative of this journey summarizes each of these topics in turn: I begin with the snakebite cases, and then delve into the diet and morphology before getting to the venom variation.

The outcome of this journey confirms my earliest suspicions: there is considerable variation in all aspects of biology, it seems, that bear on the issue of venom composition in rattlesnakes and how it influences the clinical symptoms of human envenomation.

Rattlesnake Morphology and the Venom Apparatus

Anyone familiar with rattlesnakes will recognize the two signature features: the triangular-shaped head in which the venom apparatus dwells, and the rattle that makes an audible sound to warn would-be predators and antagonists of the snake's lethal capacities. These structures showcase a remarkable suite of adaptations that have allowed rattlesnakes to evolve and occupy a wide diversity of ecosystems and niches.

Rattlesnakes vary considerable in size and shape. They are born with fangs and venom glands ready to serve the snake's interest, though the size and shape of these structures may differ among the 48+ recognized species of two genera (K. R. Beaman & Hayes, 2017); vary across the landscape occupied by a given species; differ subtly between the sexes; and change during the course of an individual's lifetime (e.g., Amarello et al., 2010; Beaupre, Duvall, & O'Leile, 1998; Klauber, 1997; Margres et al., 2015; Meik, Setser, Mociño- Deloya, & Lawing, 2012; Vincent, Herrel, & Irschick, 2004). The venom apparatus consists of a pair of venom glands in the dorsoposterior of the snake's head, a venom duct that connects each gland to a retractable, hollow, hypodermic needle-like fang attached to the anterior edge of each maxillary bone, and the associated nerves and muscles that regulate venom flow. Consistent with ontogenetic change in head size of all vertebrates, the relative size of the head and venom apparatus will be largest in neonate snakes and decline as the snake grows. Relative size of the head and venom apparatus may be similar or differ between the sexes, with either male-biased or female-biased head size dimorphism. The volume or mass of venom that a rattlesnake is capable of injecting during a predatory or defensive bite will depend largely on a

behavioral decision made by the snake (Hayes, 2008), but the capacity for venom delivery depends on overall size of these structures.

Rattlesnake Diet

Numerous studies have examined the diet of rattlesnakes. In general, diet can be influenced by phylogeny, relative body size, and prey availability (Clark, 2002; Dugan & Hayes, 2012; Glaudas, Jezkova, & Rodríguez-Robles, 2008; Klauber, 1997; Taylor, 2001; Webber, Jezkova, & Rodríguez-Robles, 2016). Some species are generalists, consuming a wide range of ectothermic and/or endothermic invertebrates and/or vertebrates, whereas others are specialists. As gape-limited predators, the relative size of prey consumed will correspond to maximum gape, with larger snakes being capable of consuming larger prey than smaller snakes (Arnold, 1993; Rodríguez- Robles, Bell, & Greene, 1999; Shine, 1991). As a consequence, diet often changes during the course of ontogeny, with juveniles of many (but not all) species feeding preferentially on ectotherms, and shifting to larger, bulkier endotherms as the snake attains a larger size.

Venom: Definition, Phylogenetic Distribution, and Functional Roles

Venom comprises an adaptation widely distributed amongst animal phyla. Definitions for venom have unfortunately proved highly variable historically. Nelsen et al. (2014) evaluated prior definitions, and in an attempt for clarification, proposed the following definition: "Venom – a toxic substance (comprised of one or more toxins) causing dose-dependent physiological injury that is passively or actively transferred from one organism to the internal milieu of another organism *via* a delivery mechanism and

mechanical injury." Nelsen et al. (2014) also provided definitions for two other classes of biological secretions (poisons and toxungens), with poisons differing from venoms in that they are passively transferred (ingestion, inhalation, absorption) from one organism into another without a delivery mechanism, while toxungens differ from venoms in that while they are actively transferred *via* a delivery mechanism from one organism to the surface of another without mechanical injury.

Whereas venom use is a phylogenetically widespread adaptation with numerous familiar examples across the animal kingdom, including snakes, spiders, scorpions, and bees (Fry, Roelants, et al., 2009; Undheim, Fry, & King, 2015), many lesser-known animals and life forms also meet the criteria for being venomous. Blood-feeding animals like ticks, fleas, and even vampire bats have been found to possess secretions containing one, or some a combination, of vasodilatory components, inhibitors of ADP-induced platelet aggregation, platelet IIb-IIIa glycoprotein antagonists, thrombin inhibitors, fibrino(geno)lytic proteases, and plasminogen activators (Fry, Roelants, et al., 2009). Bacteriophages (González- Huici, Salas, & Hermoso, 2004; Rossmann, Mesyanzhinov, Arisaka, & Leiman, 2004), certain bacteria (Chatterjee, Chaudhury, McShan, Kaur, & De Guzman, 2013; Cornelis, 2010; Erhardt, Namba, & Hughes, 2010), and the ciliate *Dileptus gigas* (S. Miller, 1968; Visscher, 1923) all possess delivery mechanisms for delivering toxins, in the case of the bacteriophage its genome, into their respective targets.

Venom typically aids either in predation, defense, or both. Delivery mechanisms for the introduction of venom into the intended target are as varied as the animals that possess them, and include: barbs, beaks, harpoons, fangs/modified teeth, nematocysts,

pinchers, proboscises, spines, spurs, and stingers. Predatory venoms are variable in both composition and the physiological processes they disrupt (Fry, Roelants, et al., 2009), whereas defensive venoms are more compositionally streamlined (Casewell, Wüster, Vonk, Harrison, & Fry, 2013), possibly due to their primary function often being the induction of immediate and extreme pain (Chen, Chang, Lin, Chen, & Hwang, 2015; Church & Hodgson, 2002; De Graaf, Aerts, Danneels, & Devreese, 2009; Kiriake, Ishizaki, Nagashima, & Shiomi, 2017; Peiren et al., 2005; Ziegman & Alewood, 2015). Additional roles for venom use include territorial disputes. Anemones and some coral species, in addition to using venom for predation and defense, employ their venom, using specialized tentacles, in an attempt to protect/acquire additional territory from intra- and interspecific competitors (Williams, 1991). The male duck-billed platypus (*Ornithorhyncus anatinus*) employs its venomous spurs during intrasexual competition for mates and territory (Burrell, 1927; Martin & Tidswell, 1895). Ants, in addition to utilizing venom for predation and defense, have been reported to use venom as a form of communication and as a parasite control. Foraging fire ants (*Solenopsis invicta*) demonstrated context-specific air dispersal of venom and dispersed a greater amount of venom (up to 500 ng) in order to repel heterospecifics while foraging, than when dispensing venom onto the brood surface (~ 1 ng) where it is believed to function as an antiseptic (Obin & Vander Meer, 1985). Other species of ant secrete venom droplet(s) to the sting tip and use the volatile components from the extruded venom in various forms of communication (Buschinger, 1968, 1971; Michael Möglich, 1979; M Möglich, Maschwitz, & Hölldobler, 1974).

Venom: Evolution in Toxicofera and Caenophidia

Evolution of the venom system in the proposed clade Toxicofera (Vidal & Hedges, 2005), specifically whether or not it is due to a single early recruitment event and therefore a synapomorphy of the clade, has been a topic of much dispute (B. Fry et al., 2015; Fry et al., 2012; Fry et al., 2006; Fry, Vidal, Van der Weerd, Kochva, & Renjifo, 2009; Hargreaves, Swain, Logan, & Mulley, 2014; Kochva, 1978; Reyes-Velasco et al., 2015; Sweet, 2016).

In 2006, Fry and colleagues reported recovering nine toxin transcripts (AVIT, B-type natriuretic peptide (BNP), Cysteine-rich secretory proteins (CRiSPs), Cobra venom factor (CVF), Crotamine, Cystatin, Kallikrein, Nerve growth factor (NGF), and Vespryn) from oral gland cDNA libraries of members of the clade Toxicofera. The nine toxin types recovered from both lizard and snake oral gland cDNA libraries, coupled with fossil and molecular data, suggested a single early origin (200 mya) of the venom system of lizards and snakes, with the basal condition of the clade being serial, lobular, and non-compound protein-secreting glands present in both the mandibular and maxillary regions. Iguanian lizards retained this basal condition, whereas snakes restricted the protein-secreting function to the maxillary region, and anguimorph lizards restricted the protein-secreting function to the mandibular region (Fry et al., 2006).

Building upon their 2006 work, Fry et al. (2010) broadened their taxonomic sampling of anguimorph lizards and proposed two additional toxin types (lectin and hyaluronidase) as basal for the toxicoferan clade (Fry et al., 2010). Increased sampling of iguanian lizard and henophidian snake oral glands caused Fry et al. (2013) to revise the timing of toxicoferan toxin gene recruitment events. They proposed up to ten toxin gene

recruitment events at the base of the toxicoferan tree (CRiSP, crotamine, cystatin, C3/CVF, kunitz, L-amino oxidase (LAO), lectin, renin aspartate protease, veficolin, vespryn), and also showed that the mandibular, maxillary, and rictal glands simultaneously expressed identical transcripts (Fry et al., 2013).

In 2014, Hargreaves and colleagues published a pair of papers investigating venom evolution in reptiles. The first tested the hypothesis that venom proteins evolved via the duplication and recruitment of genes encoding body proteins into the venom gland (Hargreaves, Swain, Hegarty, Logan, & Mulley, 2014). The authors generated transcriptomic data for body tissues and salivary and venom glands from five species of venomous and non-venomous reptiles, including a gecko species (*Eublepharis macularius*) not placed within the proposed Toxicofera clade. Their results showed a diverse tissue expression pattern for gene families previously proposed to be "body" genes that had then subsequently undergone recruitment into the venom gland. These results lead Hargreaves et al. (2014) to conclude that the majority of snake venom toxins are likely derived from genes encoding existing salivary proteins that have been restricted to the venom gland after duplication. Later that year, Hargreaves and colleagues augmented the transcriptome data set by utilizing transcriptomes or RNA-Seq data published by other authors for a larger number of snake and lizard salivary glands and body tissues (Hargreaves, Swain, Logan, et al., 2014). Their transcriptomic analysis found that all of the 16 genes previously utilized to support the Toxicofera hypothesis were expressed in multiple body tissues and without higher expression levels in venom or salivary glands, leading the authors to suggest that the proposed basal Toxicoferan genes were most likely housekeeping genes. In addition, Hargreaves et al. (2014) cited this lack

of support for the Toxicofera hypothesis as reason to return to the previous hypothesis (Kardong, Weinstein, & Smith, 2009) that different lineages of reptiles evolved venom independently, once at the base of the advanced snakes, once in helodermatids, and possibly once in varanids.

Regardless of whether the evolution of venom in reptiles was a single occurrence, or if it arose independently a handful of times, it is well established that venomous snake species have since evolved toxic arsenals that allows them to disrupt various physiological processes of their prey and predators (Ainsworth et al., 2018; Debono et al., 2017; Fry et al., 2008; Holding, Biardi, & Gibbs, 2016; Jayanthi & Gowda, 1988; Pawlak et al., 2006; Sunagar et al., 2014). Gene duplications are believed to have played a major role in the diversification of snake venom proteins (Hargreaves, Swain, Hegarty, et al., 2014; Kordiš & Gubenšek, 2000; Wong & Belov, 2012), with neofunctionalization and subfunctionalization, following duplication, proposed as two major models by which adaptive selection or neutral forces can lead to the functional divergence of the duplicate copy (Wong & Belov, 2012), and accelerated rates of evolution have been detected in various snake venom gene duplicates (Deshimaru et al., 1996; Fujimi et al., 2003; Juárez, Comas, González-Candelas, & Calvete, 2008; Kordiš & Gubenšek, 2000; Ogawa, Chijiwa, Oda-Ueda, & Ohno, 2005; Župunski, Kordiš, & Gubenšek, 2003). Recently, however, selection for increased expression levels, not sequence diversity, following gene duplication was demonstrated to be the proximate evolutionary process leading to the origin and expansion of the myotoxin gene family in the eastern diamondback rattlesnake (*Crotalus adamanteus*; Margres, Bigelow, Lemmon, Lemmon, & Rokyta, 2017).

Gene duplications are likely not the only evolutionary mechanism that has led to the toxin diversification observed in snake venom. Gene duplication was found not to play a large role in the evolution of platypus venom (Wong, Papenfuss, Whittington, Warren, & Belov, 2011), and in addition to gene duplications, the molecular mechanisms implicated in producing the observed diversity of peptides in scorpion venoms include allelic polymorphism, alternative splicing, and transplicing (Zhijian, Feng, Yingliang, Xin, & Wenxin, 2006), and domain loss in Snake Venom Metalloprotease (SVMP) toxins has been found to play a key role in neofunctionalization (Casewell, Wagstaff, Harrison, Renjifo, & Wüster, 2011).

Snake Venom Variation

Snake venom composition varies at essentially all levels considered (Chippaux, Williams, & White, 1991), from differences in composition among families (Ainsworth et al., 2018; Debono et al., 2017; Kochva, Viljoen, & Botes, 1982; Sunagar et al., 2014), to interspecific differences present within a given genus (Ainsworth et al., 2018; Queiroz, Pessoa, Portaro, Maria de Fátima, & Tambourgi, 2008), intraspecific variation among populations (Calvete et al., 2011; Forstner, Hilsenbeck, & Scudday, 1997; Jayanthi & Gowda, 1988; Saravia et al., 2002; Straight, Glenn, Wolt, & Wolfe, 1991; Sunagar et al., 2014), intrapopulational variation (Anaya, Rael, Lieb, Perez, & Salo, 1992; Smiley-Walters, Farrell, & Gibbs, 2019), sexual variation (Daltry, Ponnudurai, et al., 1996; Daltry, Wüster, & Thorpe, 1996; Menezes, Furtado, Travaglia-Cardoso, Camargo, & Serrano, 2006), down to ontogenetic shifts within an individual (Andrade & Abe, 1999; S. P. Mackessy, 1988; S. P. Mackessy, Sixberry, Heyborne, & Fritts, 2006; Rokyta,

Margres, Ward, & Sanchez, 2017; Zelanis, Travaglia-Cardoso, & De Fátima Domingues Furtado, 2008). Some authors have argued venom diversity is the result of neutral evolutionary processes not subject to natural selection (Mebs, 2001; Sasa, 1999), whereas others have argued that strong natural selection has driven adaptation to particular prey species (Barlow, Pook, Harrison, & Wüster, 2009; Daltry, Wüster, et al., 1996; Gangur et al., 2017; Jackson et al., 2016; Richards, Barlow, & Wüster, 2012; Wüster, Daltry, & Thorpe, 1999).

Evidence for diet playing a major role in snake venom variation includes prey-specific toxins (Bénard-Valle, Carbajal-Saucedo, de Roodt, López-Vera, & Alagón, 2014; da Silva & Aird, 2001; Gibbs & Mackessy, 2009; S. P. Mackessy et al., 2006; Pawlak et al., 2006; Starkov, Osipov, & Utkin, 2007), prey resistance (Heatwole & Poran, 1995; Holding, Biardi, et al., 2016; Holding, Drabeck, Jansa, & Gibbs, 2016; Jansa & Voss, 2011; Perez, Pichyangkul, & Garcia, 1979; Poran, Coss, & Benjamini, 1987; Voss & Jansa, 2012), evidence for diet-related venom economy (Barlow et al., 2009), and the degeneration of the venom system after a shift to undefended and immobile prey (Fry et al., 2008; Li, Fry, & Kini, 2005). A coevolutionary arms race is often suggested as occurring between venomous predators and their prey (Casewell et al., 2013; Gibbs & Rossiter, 2008; Juárez et al., 2008; S. P. Mackessy, 2010b), with the evolution of increasing venom resistance in prey and novel venom composition in predators each exerting reciprocal selection pressures on the other, as encapsulated by Van Valen's Red Queen hypothesis (Van Valen, 1973).

Direct evidence of an escalating arms race, however, is currently lacking. Instead, recent evidence, in two different species of rattlesnake, points toward phenotype

matching as the mechanism influencing the coevolution and local adaptation of rattlesnake venom and resistance in prey (Holding, Biardi, et al., 2016; Margres, Wray, et al., 2017). Holding et al (2016) collected venom from adult northern pacific rattlesnakes (*Crotalus oreganus oreganus*) and blood serum from adult California ground squirrels (*Otospermophilus beecheyi*) at six high- (> 400 m) and six low-elevation (< 400 m) California localities. They then measured baseline SVMP activity of each snake's venom by itself and then once again after being incubated with the serum of *O. beecheyi* to determine the amount of a snake's SVMP activity lost to an individual squirrel's serum inhibitors. Their results indicated that *C. o. oreganus* is locally adapted to overcoming resistance of *O. beecheyi*, with snakes losing less SVMP activity when they were tested against sympatric or allopatric same-elevation squirrel serum. Margres et al (2017) collected hispid cotton rats (*Sigmodon hispidus*) and venom from adult eastern diamondback rattlesnakes (*C. adamanteus*) from island (St. George Island) and mainland (adjacent Florida panhandle) populations. Using reciprocal toxicity assays (LD_{50}), they found that both snake populations were adapted to overcoming local prey resistance, as island venom was significantly more effective on island prey than mainland prey and mainland venom was significantly more effective on mainland prey than island venom was.

Biomedical Implications of Snake Venom Variation

Snakebite is a major health problem causing considerable morbidity and mortality in many tropical and subtropical countries (Gutiérrez, Theakston, & Warrell, 2006; Harrison, Hargreaves, Wagstaff, Faragher, & Lalloo, 2009; Kasturiratne et al., 2008). In

North America, the risk of snakebite is far less with a high estimate of 4000 bites per year (Kasturiratne et al., 2008). Although rattlesnake envenomation is a serious health threat (Bhagat, Sharma, Sarode, & Shen, 2010; Bush & Siedenburg, 1999; Hardy, 1986; Hardy, Jeter, & Corrigan Jr, 1982; Jansen, Perkin, & Van Stralen, 1992; Massey et al., 2012; O'Neil, Mack, Gilchrist, & Wozniak, 2007; Russell, 1969; Tokish, Benjamin, & Walter, 2001; Yarema & Curry, 2005), quick access to quality healthcare in North America likely contributes to a mortality rate of only seven deaths a year (Kasturiratne et al., 2008), though at a substantial financial cost to the patient (Boyer, 2015).

At present, antivenoms represent the most efficacious treatment of snakebite. Antivenoms are derived through the hyperimmunization, typically of large mammals, until the desired antibody titer and neutralizing potency is reached after which the animal is bled for its plasma (reviewed by; Bénard-Valle et al., 2015). Because snake venom varies substantially among different taxa, antivenoms are typically designed for regional use, and produced from the venoms of species that are the most dangerous in those regions. In North America, two anivenoms are currently utilized for the treatment of native pitviper envenomations. One is produced by hyperimmunizing horses with the venoms of *C. durissus* and *Bothrops asper* by Instituto Bioclon in Mexico (Antivipmyn) and is a polyclonal antivenom $F(ab')_2$ fragment while the other is an affinity-purified Fab fragment of ovine origin (FabO), using venom from *C. adamanteus*, *C. atrox*, *C. scutulatus scutulatus*, and *Agkistrodon piscivorus piscivorus*, produced by Therapeutic Antibodies, Inc., London, England (reviewed by; Sánchez et al., 2003). Thus, a thorough understanding of species venom composition, including possible ontogenetic and geographic variation, is therefore crucial in improving antivenom efficacy and clinical

treatment protocols (Boldrini-Franca et al., 2010; Calvete, Sanz, Angulo, Lomonte, & Gutiérrez, 2009; Calvete et al., 2011; Fry, Winkel, Wickramaratna, Hodgson, & Wüster, 2003).

Snake venoms are also a potential source of new therapeutic drugs. Venom components of numerous animal species have convergently evolved to target physiological systems reachable by the bloodstream through a myriad of pathways (Fry, Roelants, et al., 2009; A. Harvey et al., 1998; Isbister, 2009). The high degree of target specificity and bioactivity demonstrated by venom toxins make them valuable scaffolds for drug development (Calvete, Sanz, Angulo, et al., 2009; Fry, Roelants, et al., 2009; A. Harvey et al., 1998; Ménez, 1998; Xu et al., 2006). Currently a number of FDA approved drugs have derived from snake venoms, including Captopril® to treat hypertension, Aggrastat® for treating angina, and Integrillin® for use in coronary angioplasty (Fox & Serrano, 2007; A. L. Harvey, 2014). Technological advances and the number of understudied snake species provide almost limitless potential for additional future medical breakthroughs based on venom toxins (B. G. Fry et al., 2015; McCleary, Kang, & Kini, 2015; Vonk et al., 2011).

Venom Variation Among Rattlesnakes

Venom variation in rattlesnakes (genera *Crotalus* and *Sistrurus*) is documented between genera (Boldrini-Franca et al., 2010; Calvete, Sanz, Cid, et al., 2009; Gibbs, Sanz, Sovic, & Calvete, 2013; Tan & Ponnudurai, 1991), among species (S. Mackessy & Castoe, 2016; S. P. Mackessy, 2008; Tan & Ponnudurai, 1991), geographically within a species (Forstner et al., 1997; Massey et al., 2012; Saravia et al., 2002; Sunagar et al.,

2014; Wilkinson, Glenn, Straight, & Sites Jr, 1991), within a given population of a species (Anaya et al., 1992; Smiley-Walters et al., 2019), and ontogenetically within an individual (Minton & Weinstein, 1986; Saravia et al., 2002). Rattlesnakes are the most studied group of snakes (K. Beaman & Hayes, 2008), so it should come as little surprise that the venom of this medically important group has received considerable attention when compared to other venomous snake groups. Each of the 48 recognized species and 83 recognized taxa (K. R. Beaman & Hayes, 2017) possess relatively complex venom, often producing over 100 protein spots, consisting of numerous identified components, when analyzed by two-dimentional gel electrophoresis (S. Mackessy & Castoe, 2016; S. P. Mackessy, 2008).

Levels of toxicity and metalloprotease activity are used to broadly characterize rattlesnake venoms as the two demonstrate an inverse relationship (S. P. Mackessy, 2008, 2010a). Type I venoms possess higher levels of metalloprotease activity and lower toxicity (>1.0 μg/g mouse body weight), whereas type II venoms possess higher toxicity (<1.0 μg/g mouse body weight) and lower levels of metalloprotease activity (S. P. Mackessy, 2008); however, the simplicity of this dichotomous classification system is not always reality (Massey et al., 2012). No clear pattern in distribution of neurotoxic and proteolytic venoms exists, so local adaptation has been the mechanism invoked to explain the variation (S. P. Mackessy, 2008).

The Southwestern Speckled Rattlesnake

The southwestern speckled rattlesnake, *Crotalus pyrrhus* (Cope, 1867), ranges from extreme southeastern Utah and southern Nevada, south through western Arizona,

southern California, and the northern panhandle portion of northwestern Sonora, into Baja California Norte, where it can be found as far south as the Vizcaíno region (Campbell & Lamar, 2004; Grismer, 2002; Klauber, 1936). Populations are also present on various Gulf of California Islands, including, El Muerto, and Smith (Campbell & Lamar, 2004; Grismer, 2002; Meik, Lawing, & Pires-daSilva, 2010; Meik, Schaack, et al., 2012; Meik, Streicher, Lawing, Flores-Villela, & Fujita, 2015). A saxicolous species, it is most commonly associated with the rugged, and rocky terrain of the mountains of the Mohave and Sonoran Deserts, where it is rarely encountered in the adjacent desert flats (Campbell & Lamar, 2004; Klauber, 1936; Meik, 2016). Of the biotic Communities in the region, following Brown (1994), *C. pyrrhus* is most commonly associated with the Lower Colorado River and Arizona Upland subdivisions of Sonoran Desertscrub, Mohave Desertscrub, Californian Chaparral, and Californian Coastal Scrub. The species is occasionally found in Interior Chaparral, and Great Basin Conifer Woodland (Campbell & Lamar, 2004; Meik, 2016), and it can even be found at the transition zone between Californian Chaparral and Sierran Montane Conifer Forest in the vicinity of Idyllwild, California (C. Cochran, pers. observ.).

A medium-sized rattlesnake species, displaying geographic variation in body size (Glaudas & Rodriguez-Robles, 2011; Meik et al., 2010; Meik, Schaack, et al., 2012; Moore, 1978), individuals are typically under 1000 mm SVL, with the longest measured specimen coming from an unspecified Peninsula range of southern California that possessed a total length slightly exceeding 1220 mm (Klauber, 1936). Highly variable in color and pattern, the species comes in an astonishing variety of colors, causing Klauber (1936) to remark, “This is the most variable of all the rattlesnakes in color and pattern; its

bewildering variety renders any considerable accuracy or consistency of description quite impossible."

An ambush predator and dietary generalist, *C. pyrrhus* predates upon a diverse array of prey types across its distribution. Small mammals and lizards appear to make up the majority of its diet (Klauber, 1936, 1997; Lowe, Schwalbe, & Johnson, 1986; Meik, Schaack, et al., 2012), though birds are also recorded as prey items with regularity (Klauber, 1936, 1997; Meik, 2016; Meik, Schaack, et al., 2012; A. H. Miller & Stebbins, 1964) and may be an important dietary component of the El Muerto island population (Meik, 2016).

Proteomic investigation of geographic variation in *C. pyrrhus* venom composition lags far behind that of *C. scutulatus* (Dobson et al., 2017; Glenn & Straight, 1978; Glenn & Straight, 1989; Glenn, Straight, Wolfe, & Hardy, 1983; Massey et al., 2012; Wilkinson et al., 1991) and *C. helleri* (Gren et al., 2016; Jurado et al., 2007; Sunagar et al., 2014), two species it shares large portions of its range with. This is a bit surprising considering the species' restriction to mountain ranges isolated by stretches of low desert, which makes it, in essence, an island species, and therefore an excellent model for better understanding the factors influencing intraspecific venom variation. The lack of attention paid to *C. pyrrhus* venom may be due to the fact that it rarely bites people (Bush, Green, Moynihan, Hayes, & Cardwell, 2002; Russell, 1969), as even in populated areas where it is known to be common, the snakes appear to successfully avoid detection by humans (Pitts, Hughes, & Mali, 2017), possibly due to their excellent camouflage.
Southwestern speckled rattlesnakes are classified as possessing a type I venom due to moderate lethal toxicity of crude venom toward inbred mice (2.5 μg/g) and relatively

high metalloprotease activity (S. P. Mackessy, 2008). Geographic variation in toxicity, select enzymatic activities, and genomic venom potential of *C. pyrrhus* has previously been reported (Glenn & Straight, 1985; Powell, Lieb, & Rael, 2008). Glenn and Straight (1985) described individual variation in lethal toxicity of crude venom (in Swiss-Webster mice) from Baja California Norte (BCN) specimens, and protease, esterase, and phosphodiesterase activity differences in *C. pyrrhus* originating from Utah and the BCN localities of Bahia de Los Angeles, San Felipe, and Smith Island. A study utilizing PCR and DNA sequencing to investigate the geographic distribution in various rattlesnake species of Mojave toxin, and its acidic and basic subunits, found individuals of *C. pyrrhus* from Maricopa County, Arizona, and California's San Bernardino County and Riverside County, tested positive for the Mojave toxin basic (MTX-B) subunit, yet it was absent in other individuals from the same counties (Powell et al., 2008). The presence of MTX-B in some populations of *C. pyrrhus* is an interesting finding, as Glenn and Straight (1985) failed to detect the subunit in individuals from Smith Island, San Felipe, and Bahia de Los Angeles using rabbit antibody to MTX-B. No individuals to date have tested positive for Mojave toxin acidic (MTX-A) subunit (Powell et al., 2008); however, both subunits are necessary for production of Mojave toxin, explaining why an anti-Mojave toxin antibody used by Powell et al. (2008) failed to detect Mojave toxin in venom samples of *C. pyrrhus*.

Study Objectives

The overall objectives of my research were: 1) to document the extent of geographic variation in venom composition in southwestern speckled rattlesnakes (*C. pyrrhus*) among six biogeographic regions across western Arizona, southern California,

and extreme southern Nevada, and 2) to gain insight into factors contributing to the observed venom variation.

The purpose of my first study was to describe two cases of envenoming by *C. pyrrhus* from two Arizona localities (Tinajas Altas Mountains, Yuma County, and Squaw Peak, Maricopa County). Both patients experienced swelling, but neither demonstrated coagulopathy, thrombocytopenia, or hypofibrinogenemia. The patient bitten on Squaw Peak developed hemorrhagic bullae and tissue damage in his bitten extremity, necessitating the amputation of the distal portion of his middle finger. The venome of the two populations upon visual inspection of 1D-gels and RP-HPLC chromatograms appeared to differ quantitatively between populations and among individuals within a population but not qualitatively (i.e., similar toxins were present, but the proportions of some varied).

My second study sought to examine geographic variation in the body size of *C. pyrrhus* across southern California, western Arizona, and southern Nevada and to determine if SSD and SBCD exist within the species. I demonstrated that overall body size varied significantly among six defined biogeographic regions, with the largest snakes occurring in the Mojave Desert, Transverse Ranges, and Peninsular Ranges of California and Nevada; the smallest snakes restricted to the Lower Colorado River subdivision of the Sonoran Desert; and snakes of intermediate size inhabiting the Colorado Desert (California) and Arizona Uplands subdivisions of the Sonoran Desert. Consistent with the findings of others we also documented SSD, with males attaining a greater length than females; however, our novel approach using geometric mean and principle component 1 as unbiased measures of overall body size allowed us to show SBCD also

exists, with females possessing relatively longer trunks and bigger heads. Our finding of female-biased trunk length is consistent with prior counts of trunk vertebrae in rattlesnakes and fecundity selection favoring longer trunks in females. Because essentially all prior studies of snake dimorphism have relied on trunk length or SVL, which are likely to be female-biased, essentially everything we know of sexual dimorphism in snakes needs reevaluation.

In my third study, I examined the diet and the possible influence of biogeographic region and ontogeny on prey class (ectotherms vs endotherms) consumed. The diet of *C. pyrrhus* consisted predominately of mammals (75%), in particular terrestrial squirrels (36.3%) and the heteromyid rodent genus *Chaetodipus* (30%). An ontogenetic shift occurred from primarily lizards to rodents, but neither biogeographic region nor sex had a significant impact on prey class consumed. However, the Tinajas Altas Mountains, Yuma County, Arizona population appears to rely on birds to a greater extent than other *C. pyrrhus* populations.

My fourth study examined the influence of biogeographic region and sex on protein composition in eight arbitrarily defined chromatographic elution regions. Venom samples from 151 snakes from 23 populations across six biogeographic regions were subjected to proteomic analysis and multivariate analyses used to compare venom composition. Venom composition varied biogeographically with snakes from the Mojave Desert possessing less protein content in elution region five (predominantly Serine Proteases, PLA2, SVMP, and C-type lectins) than other biogeographic regions. Discriminant analyses indicated a considerable distinction between venoms of eastern

(Arizona) and western (California/Nevada) portions of the species' range. We failed to detect Mojave Toxin (or a homolog) in any of our populations.

Collectively, these studies provide meaningful insights on the sources of variation that potentially influence venom composition and clinical symptoms of variation in a model rattlesnake species.

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CHAPTER TWO

CLINICAL SYNDROME AND VENOME DIFFERENCES BETWEEN TWO ARIZONA POPULATIONS OF THE SOUTHWESTERN SPECKLED RATTLESNAKE (CROTALUS PYRRHUS (COPE 1867))

Chip Cochran[1], Scott Hax[2,3], and William K. Hayes[1]

[1]Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, California 92354 USA

[2]NR-P, FP-C, C-NPT, Air Methods Corp, Mesa, AZ 84204

[3]Mesa Community College, Mesa, AZ 85202

Abstract

Envenomations by the southwestern speckled rattlesnake (*Crotalus pyrrhus*) are fairly rare. Previous descriptions in the literature do not include locality, an important factor in the clinical symptoms or syndromes of snakebites resulting from geographic variation in venom composition. Here, we describe two cases of envenoming by *C. pyrrhus* from two Arizona localities (Tinajas Altas Mountains, Yuma County, and Squaw Peak, Maricopa County). Both patients experienced swelling, but neither demonstrated coagulopathy, thrombocytopenia, or hypofibrinogenemia. The patient bitten on Squaw Peak developed hemorrhagic bullae and tissue damage in his bitten extremity, necessitating the amputation of the distal portion of his middle finger. Treatment for both consisted of medication for pain, isotonic crystalloid, and antivenom therapy with recovery in each case. The venome of the two populations upon visual inspection of 1D-gels and RP-HPLC chromatograms appeared to differ quantitatively between populations and among individuals within a population but not qualitatively.

Introduction

An estimated 10,000 snakebites result in emergency department visits across the United States annually (O'Neil, Mack, Gilchrist, & Wozniak, 2007). However, only approximately one-third of these admissions are due to bites from known venomous species (Kasturiratne et al., 2008; O'Neil et al., 2007), resulting in 5–7 deaths in the United States and Canada annually (Kasturiratne et al., 2008). A review of 163 snakebite admissions in southern Arizona over a 5-year period found that rattlesnakes were responsible for all but two envenomations, with the exceptions coming from exotic species kept as pets (Tokish, Benjamin, & Walter, 2001). These statistics are not

surprising, as Arizona possesses the greatest rattlesnake diversity in the United States (Campbell & Lamar, 2004), and the State's only other dangerously venomous snake, the Sonoran coral snake (*Micruroides euryxanthus*), is largely fossorial, and therefore rarely comes into contact with humans (Campbell, Lamar, & Brodie, 2004; Fowlie, 1965; Lowe, Schwalbe, & Johnson, 1986).

Rattlesnake envenomation typically manifests as pain, edema, hemorrhage, coagulopathy, and necrosis, sometimes resulting in permanent disfigurement and/or kidney failure (Arnold, 1982; Ownby, 1982). Systemic neurotoxicity resulting in respiratory paralysis is seldom seen in rattlesnake envenomations in the United States (Bush & Siedenburg, 1999; Jansen, Perkin, & Van Stralen, 1992; Massey et al., 2012), even though some species, or populations within species, have evolved neurotoxic venoms (Glenn & Straight, 1978; Mackessy, 1988; Massey et al., 2012; Sunagar et al., 2014; Wilkinson, Glenn, Straight, & Sites Jr, 1991). The only effective treatment for rattlesnake envenomation is antivenom administration (Arnold, 1982; Boyer et al., 2015). Snakebite patients in southern Arizona received an average of 19 vials of antivenom (range, 0 to 75 vials) during an average of 2.8 days in the hospital, with 1.6 days spent in an ICU setting (Tokish et al., 2001).

The southwestern speckled rattlesnake (*Crotalus pyrrhus* (Cope, 1867)) is a medium-sized species seldom exceeding a meter in length (Klauber, 1936, 1997a). The species ranges from Baja California Norte north into northwestern Sonora, southern California, western Arizona, southern Nevada, and extreme southwestern Utah (Campbell & Lamar, 2004; Klauber, 1936, 1997a). The species is most commonly associated with arid rocky environments within deserts, chaparral, coastal sage scrub, and piñyon-juniper

woodland (Campbell & Lamar, 2004; Klauber, 1936, 1997a), but can even occur in the transition zone between Californian Chaparral and Sierran Montane Conifer Forest in the San Jacinto Mountains (C. Cochran, pers. observ.)

The clinical syndrome of envenomation by the southwestern speckled rattlesnake has yet to be reported in detail. Hartnett (1931) provided the first, and still the most detailed, description of an envenomation. Klauber (1997b) remarked on three cases, one of which was the aforementioned Hartnett case, while another was referred to as "probably a *pyrrhus* bite." The third case description consisted of a single sentence: "In another *pyrrhus* bite, a sharp pain like that of a bee's sting was felt within about 45 seconds." Bush, Green, Moynihan, Hayes, and Cardwell (2002) included a single envenomation by *C. pyrrhus* in their study investigating the efficacy of CroFab® in treating the bites of *C. helleri*. Pain was experienced in all four envenomations, and was reported as being felt quickly (Klauber, 1997b), intensifying as time post-bite progressed (Hartnett, 1931), or associated with recurrent swelling (Bush et al., 2002; Klauber, 1997b). Whether or not the intensification of pain for some of the envenomations was due strictly to action of the venom or its effects in concert with the treatment methods of incision/suction and tourniquet is impossible to know. Swelling was reported for three of the cases. In a victim bitten in the left thumb, swelling progressed into the axilla and pectoral regions (Hartnett, 1931), while a patient bitten in the heel experienced considerable swelling in the calf and leg 4 hours post-bite (Klauber, 1997b), and a patient bitten in an unreported region experienced recurrence of progressive swelling despite scheduled dosing with CroFab® (Bush et al., 2002). Tachycardia, urticaria, and blebs covering the bitten hand were reported in the case reported by Hartnett, but it was unclear

if urticarial rash may have been related to an allergic phenomenon (Hartnett, 1931). The patient reported by Bush et al. (2002) suffered hypotension and severe tachycardia. Unfortunately, no locality data were provided for these case reports.

Apart from these case reports, Corbit and Hayes (2015) examined the clinical syndromes of 166 cases of envenomation from seven rattlesnake taxa in southern California, including those from 10 *C. pyrrhus* specimens. Of the six clinical symptoms measured as snakebite severity scores (Dart, Hurlbut, Garcia, & Boren, 1996), *C. pyrrhus* scored relatively high compared to other taxa for local wound, gastrointestinal, and hematological symptoms, and near average for pulmonary, cardiovascular, and neurological symptoms. Bites from *C. pyrrhus* were treated with a snake size-corrected average of 19.5 vials of antivenom, which exceeded that of all other species but was statistically similar in a model that considered all species.

Here, we describe two cases of envenomation following bites by *C. pyrrhus* from two distinct Arizona populations (Tinajas Altas Mountains, Yuma County, Arizona and Squaw Peak, Phoenix Mountains Preserve, Maricopa County, Arizona). We also obtained venom from multiple snakes at each location, and compared the venome (venom composition) of the two populations to determine whether differences in the clinical syndrome could be attributed to differences in the venome.

Case Reports

Case 1 - Tinajas Altas Mountains

On 9 May 2009, at approximately 0900 hours, a healthy 21-year-old male received a bite to his left thumb from an approximately 45-cm long southwestern

speckled rattlesnake (*C. pyrrhus*) in the Tinajas Altas Mountains of Yuma County, Arizona. Species identification was confirmed by independent herpetologists who examined a photo of the offending snake (Fig. 1). The patient self-administered diphenhydramine 50 mg orally, hoping that it might reduce the risk of allergic phenomenon, as he maintained a captive collection of a few *Crotalus* species and was concerned that a previous sensitizing event might increase his risk of developing an allergic response. After a 30-minute walk to the vehicles, swelling had progressed to the wrist. A friend drove the patient to Wellton, Arizona, where they arrived at 1009 hours. Swelling extended just proximal to the wrist at this time, and the patient was transported to Yuma Regional Medical Center where he was admitted at 1129 hours.

In the emergency department, the patient was hemodynamically stable and without clinical evidence of systemic toxicity. He had swelling of his entire left hand, extending midway up his left lower arm, and he found it difficult to use his left hand.

After he received hydromorphone for pain and an isotonic crystalloid bolus, Crofab® (Crotalidae Polyvalent Immune Fab [Ovine]) was eventually initiated in the emergency department. Initial laboratory studies are noted in Table 1. An initial fibrinogen was not obtained. Other than mild thrombocytopenia (initial platelet count 120,000 K/uL), documented hematologic parameters and all other recorded laboratory data were within normal limits.

Figure 1. Southwestern Speckled Rattlesnakes (*Crotalus pyrrhus*) responsible for each bite. Case 1 from the Tinajas Altas Mountains, Yuma County, Arizona (left) and Case 2 from Squaw Peak, Phoenix Mountains Preserve, Maricopa County, Arizona (right).

He was then admitted to the intensive care unit, and the regional poison center consulted. He received additional doses of antivenom until initial control of advancing of limb swelling was achieved. Repeat laboratory studies were finally obtained nine hours after initial doses of Crofab, and demonstrated an increase in platelet count to 160 k/uL. Serial laboratory data over the next four days (Table 1) demonstrated continued absence of coagulopathy, thrombocytopenia, and hypofibrinogenemia.

The patient was discharged on day four post-bite with marked reduction of extremity swelling and improved range of motion. He was instructed to follow up with his primary care provider, and had an unremarkable course with no long-term sequelae. Unfortunately, the medical record provided was not complete and does not indicate the total volume of antivenom administered.

Table 1. Relevant laboratory data recorded during hospitalization of a 21-year-old male (case 1) bitten by an adult *Crotalus pyrrhus* at Tinajas Altas Mountains, Yuma County, Arizona.

DATE/TIME	05/09 1145	05/09 2045	05/10 0015	05/10 0430	05/10 0850	05/10 1430	05/10 2030	05/11 0500	05/12 0500
POST-ENVENOMATION	2h 45 m	11h45m	12h30m	16h45m	23h50m	29h30m	35h30m	53H	77H
Hb (g/dL)	15.1	14.3	13.7	14.1	14.7	14.1	13.9	14.1	NR
HcT (%)	45.7	41.9	39.3	41.1	42.9	41.0	40.3	41.1	NR
PLT (K/uL)	120	169	162	150	147	160	155	126	NR
Fibrinogen (mg/dL)	NR	195	187	221	201	258	264	238	282
PT (secs)	14.4	15.2	15.3	13.9	13.8	14.2	14.0	13.7	NR
aPTT (secs)	38.2	38.4	40.6	38.4	28.2	38.1	37.9	26.8	NR
INR	1.10	1.18	1.19	1.06	1.05	1.08	1.07	1.04	1.03

aPTT = partial thromboplastin time; Hb = hemoglobin; HcT = hematocrit; INR = international normalized ratio; NR = not recorded in medical record; PLT = platelets, PT prothrombin time

Case 2 - Phoenix Mountain Preserve

A previously healthy 29-year-old male was bitten by an adult *C. pyrrhus* near the main trail to Squaw Peak in Phoenix Mountain Preserve, Maricopa County, Arizona while photographing the snake, at 0845 hours. Species identification was confirmed by independent herpetologists who examined a photo of the snake (Fig. 1). Experiencing immediate severe pain at the bite site, the victim abandoned further efforts to photograph the specimen and contacted the regional poison center while walking out of the park. He then proceeded straightaway to a tertiary medical center with a medical toxicology service and was evaluated by a medical toxicologist experienced with snakebite at 45 minutes post-envenomation.

The patient presented in the emergency department with a single fang puncture of his right distal third finger with dried blood surrounding the wound, but no active

bleeding. He was hemodynamically stable and without signs of systemic venom toxicity. He denied previous snakebite. Swelling of the entire right hand was noted, but distal perfusion, motor, and sensory function were preserved. Swelling extended just proximal to the wrist, and was obviously progressing during initial evaluation, a clinical indication for antivenom therapy. He was given fentanyl for analgesia and an intravenous bolus of isotonic crystalloid, and his right arm was elevated. Laboratory data (see Table 2) demonstrated no coagulopathy, thrombocytopenia, or hypofibrinogenemia. Hemoglobin and hematocrit were minimally elevated, which can be attributed to third-space losses of plasma volume that not uncommonly occur after Crotalinae envenomation, or to dehydration. This hemoconcentration reversed after previously mentioned IV fluid administration an infusion of isotonic crystalloid.

Table 2. Relevant laboratory data recorded during hospitalization of a 29-year-old male (case 2) bitten by an adult *Crotalus pyrrhus* at Squaw Peak, Phoenix Mountain Preserve, Maricopa County, Arizona.

DATE/TIME	**09/14 1000**	**09/14 1330**	**09/14 1400**	**09/14 1630**	**09/15 1030**	**09/17 0400**
POST- ENVENOMATION	2 Hrs	2.5 Hrs	6.5 Hrs	8.5 Hrs	26.5 Hrs	76 Hrs
Hb (g/dL)	17.2	16.7	NR	NR	26.6	16.7
HcT (%)	50.1	48.4	NR	NR	48.2	47.9
PLT (K/uL)	175	268	NR	282	280	266
Fibrinogen (mg/dL)	266	NR	243	255	316	484
PT (secs)	13.7	NR	14.9	14.5	14.2	13.5
aPTT (secs)	27	NR	31	31	32	NR
INR	1.1	NR	1.2	1.1	1.1	1.0

aPTT = partial thromboplastin time; Hb = hemoglobin; HcT = hematocrit; INR = international normalized ratio; NR = not recorded in medical record; PLT = platelets, PT prothrombin time

The patient consented to enrollment in a double-blinded phase III clinical trial (Bush et al., 2015) comparing the efficacy of Crofab® (Crotalidae Polyvalent Immune F(ab) [Ovine]; BTG, West Conshohocken, Pennsylvania), with Anavip® (Antivenin Crotalinae [pitviper] equine immune $F(ab)_2$; Instituto Bioclon, Mexico City, Mexico). Antivenom therapy was initiated, but the actual product delivered was blinded to the patient and treating clinicians.

The patient was transferred to the intensive care unit at an unspecified time where he remained hemodynamically stable and without signs of systemic venom toxicity. Progressive and severe local tissue injury, including significant hemorrhagic bullae formation, necessitated the administration of additional doses of antivenom (number not specified in chart) and continued supportive care. At 36 hours post-bite, in the face of worsening extensive right third finger hemorrhagic bullae, he was evaluated by a hand

surgeon with extensive experience treating rattlesnake bite victims. A decision was reached to proceed to the operating room emergently for digital dermotomy to interrupt hemorrhagic bleb and assess underlying tissue. Intraoperatively, he was found to possess an extensive hemorrhagic bulla with indeterminate viability of underlying soft tissue bed. The bulla extended under the subungal space, and avulsion of the nail plate was performed along with debridement. The finger was felt to be tense after debridement by the operating surgeon, who then performed an escharotomy. After recovering from anesthesia, the patient was transferred to an inpatient surgical floor for continued monitoring, wound management, and analgesia.

The patient was discharged from the hospital 3 days post-bite after receiving a total of 36 vials of antivenom subsequently identified as Anavip®. He never developed any hematologic derangement, subjective or objective evidence of systemic venom toxicity, or hemodynamic instability. After discharge, he was closely monitored by both the medical toxicology service and the hand surgery service. Unfortunately, the tissue bed at the bite site proved to be nonviable, necessitating finger amputation approximately 10 days post-bite.

Venom Composition

Pooled venom samples (10μg) from adult rattlesnakes captured in Phoenix Mountain Preserve (N = 3) and the Tinajas Altas Mountains (N = 3) were run on Novex NuPAGE® 4-12% Bis-Tris Mini Gels and MES SDS Running Buffer (20X) under reduced conditions following manufacturer's protocol (Invitrogen). Gels were then stained with SimplyBlue™ SafeStain (invitrogen™, Carlsbad, California) following

manufacturer protocol and then de-stained following manufacturer protocol with an additional overnight was in 3.3% NaCL to increase band sensitivity. Gels were photographed using a BioSpectrum™ 500 Imaging System W/LMS-26 Transilluminator, (analytikjena, Upland, California) (Figure 2). Upon visual inspection the Tinajas Altas population possesses bands of greater intensity at ~55 kDa, which correspond with the known weight of P-III SVMPs, ~22 kDa, which correspond to P-I SVMPs, ~15 kDa, which correspond to PLA2s, and ~10 kDa, which correspond to disintegrins.

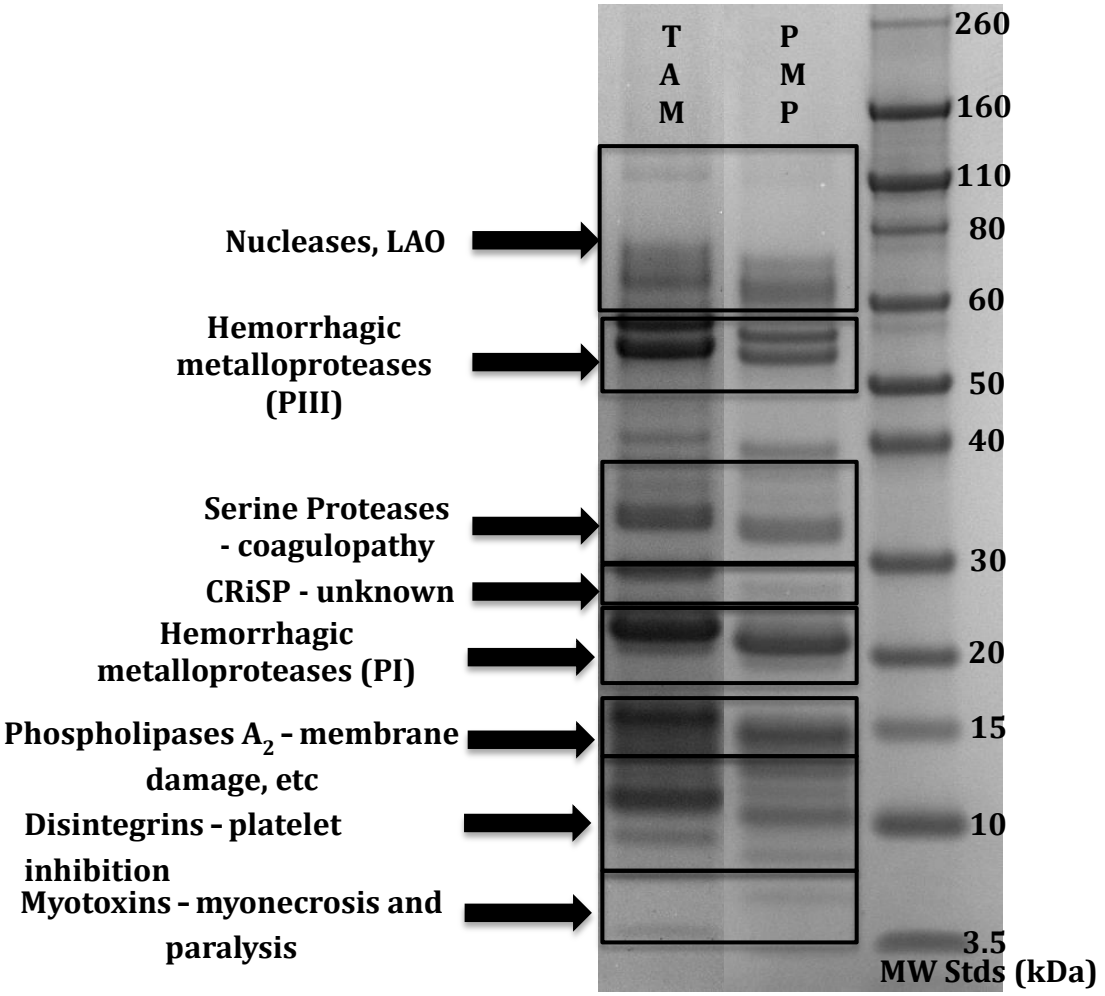

Figure 2. SDS-PAGE analysis (composite gel) of venoms from two populations of *Crotalus pyrrhus*. Relevant portions of individual gels were aligned using protein standards run on each gel. Approximate molecular masses are given on the right, and protein classes of major constituents, and their major actions, are given on the left. It is apparent that many proteins are shared between the two populatons. CRiSPs = cysteine-rich secretory proteins; LAAO = L-amino acid oxidase; PMP = Phoenix Mountains Preserve; TAM = Tinajas Altas Mountains

Venom samples of individual snakes (Phoenix Mountains = 11, Tinajas Altas = 21) were also subjected to reverse-phase high-pressure liquid chromatography (RP-HPLC) following methods described in Sunagar et al. (2014). Resulting chromatograms (Figure 3) were visually inspected for differences in time of peak elution and size of eluted peaks. There were no noticeable peaks at a given elution time that were present in

one population but absent in the other. Size of eluted peaks at a given time varied in both populations with both possessing some individuals that eluted a large peak at approximately 26 milliliters, a time that likely corresponds to SVMPs based off MS results from another *C. pyrrhus* population (Hayes, unpubl. data).

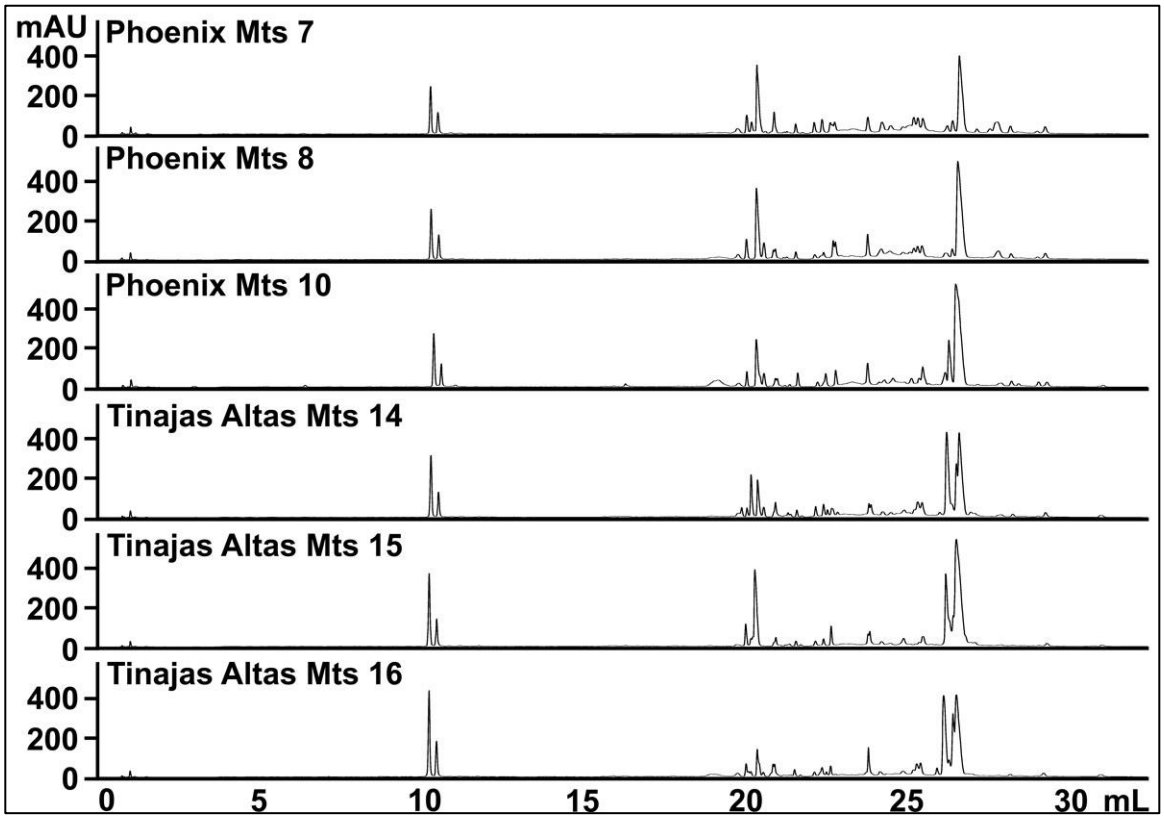

Figure 3. Representative RP-HPLC chromatograms illustrating similarity in the venom of southwestern speckled rattlesnakes (*Crotalus pyrrhus*) from two Arizona populations (Phoenix Mountains Preserve, Maricopa County, and Tinajas Altas Mountains, Yuma County).

Discussion

Swelling was prominent in both cases and appears to be a common symptom in *C. pyrrhus* envenomations (Bush et al., 2002; Hartnett, 1931; Klauber, 1997b). Both patients were also hemodynamically stable throughout their hospital course. Early hematologic toxicity is well-described and relatively common sequelae of *Crotalinae* envenomation.

Thrombocytopenia, hypofibrinogenemia, and coagulopathy (e.g. prolonged prothrombin time) can develop, although significant bleeding events appear to be surprisingly uncommon (Tanen, Ruha, Graeme, & Curry, 2001). The North American Snakebite Registry (NASBR), a database established by the American College of Medical Toxicology to collect detailed prospective information related to snakebite, has defined thrombocytopenia in the setting of envenomation syndrome as a platelet count of ≤ 120,000 K/mm^3 (Ruha et al., 2017). The patient described in case 1 had an initial platelet count of 120,000 K/mm^3, representing early and mild thrombocytopenia. Unfortunately, a repeat hemogram was inexplicably not recorded until nine hours after initial laboratory assays were obtained. It is unclear in the medical record how many doses of antivenom the patient received before repeat laboratory assays were obtained, but the platelet count had normalized by 12 hours post bite, and remained within normal range throughout hospitalization. Administration of adequate antivenom likely reversed early venom-induced thrombocytopenia in this patient. The unremarkable hematological parameters lab results recorded for case 1 contrasted with details shared informally by hospital staff with the patient, and one of the authors (CC), during course of treatment.

The two bites differed markedly in amount of local tissue injury. Hemorrhagic bullae formation and severe local tissue injury were present only in the envenomation from the Phoenix Mountains and necessitated the eventual amputation of the distal portion of the finger. Formation of hemorrhagic bullae and necrosis of tissue appear to be fairly common following envenomation by other *Crotalus* species (Azevedo-Marques et al., 1985; Hardy, Jeter, & Corrigan Jr, 1982; Russell, 1969; Yoshida-Kanashiro, Navarrete, & Rodríguez-Acosta, 2003). That local tissue injury differed between bites

from these two Arizona localities is not surprising as previous reports on bites from *C. pyrrhus* of unknown origin also differed with Hartnett (1931) reporting the formation of hemorrhagic bullae and Corbit and Hayes (2015) reporting that the species scored relatively high for the local wound component used in determining snakebite severity score while Bush et al. (2002) made no mention of local tissue damage. Visual inspection of both gels and RP-HPLC chromatograms showed quantitative but not qualitative differences between both individuals and populations. The increased tissue damage due to the bite from the Phoenix Mountains Preserve snake may be due to the individual receiving a greater dose of venom or due to the victim receiving a bite from an individual snake that possessed a greater amount of SVMP, a toxin known for causing local tissue injury (Casewell et al., 2015; Fox & Serrano, 2010; Gutiérrez, Rucavado, & Escalante, 2010).

Human envenomations by *C. pyrrhus* are surprisingly rare. Though people sparsely populate much of the area the species occupies, *C. pyrrhus* is very common around the Phoenix metropolitan area in places frequented by hikers (CC pers. observ.). The species appears to avoid detection by humans fairly well, as they accounted for only 7.4% of the rattlesnake removals from 2011 to 2014 for a prominent Phoenix rattlesnake removal company, placing a distant second to *C. atrox* (85.1%) (Pitts, Hughes, & Mali, 2017), possibly due to their proclivity for color matching the local, often granite, substrate (Campbell & Lamar, 2004; Klauber, 1936).

The venom of *C. pyrrhus* has yet to receive a detailed venomic characterization. Current antivenom therapies, CroFab® [Crotalidae Polyvalent Immune Fab (Ovine); Protherics Inc., Nashville, Tennessee] and ANAVIP® [Crotalidae Immune $F(ab')_2$

(Equine); Instituto Bioclon, Mexico City, Mexico] do not include *C. pyrrhus* in their immunization pool. The venom of *C. pyrrhus* is not considerably toxic in comparison to other rattlesnake species. The reported LD_{50} for mice is 2.5 μg/g, and due to its low toxicity and higher metalloprotease activity the species is classified as possessing a type I venom (Mackessy, 2008). Despite this low LD_{50} value, bites from the species should be treated as a medical emergency.

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CHAPTER THREE

GEOGRAPHIC VARIATION IN BODY SIZE, SEXUAL SIZE DIMORPHISM, AND SEXUAL BODY COMPONENT DIMORPHISM IN THE SOUTHWESTERN SPECKLED RATTLESNAKE (*CROTALUS PYRRHUS* (COPE 1867))

Chip Cochran[1], Lance R. Pompe[1], and William K. Hayes[1]

[1]Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, California 92354 USA

Abstract

Body size and shape vary considerably in many animal species, which may be evident within individuals during ontogeny, between the sexes, and among different geographic areas. Sexual differences in morphology can encompass overall body size (sexual size dimorphism, SSD) or the size and/or shape of specific body parts (sexual body component dimorphism, SBCD). While natural selection likely shapes geographic variation in body size, a combination of natural and sexual selection, along with non-adaptive processes, likely shapes sexual dimorphism. Studies of SSD and SBCD ideally use an unbiased character as a measure of overall body size, but many studies rely on a biased measure, including essentially all of those that have examined snakes. Here, we demonstrate that overall body size of *Crotalus pyrrhus* varies significantly among biogeographic regions, with the largest snakes occurring in the Mojave Desert, Transverse Ranges, and Peninsular Ranges of California and Nevada; the smallest snakes restricted to the Lower Colorado River subdivision of the Sonoran Desert; and snakes of intermediate size inhabiting the Colorado Desert (California) and Arizona Upland subdivisions of the Sonoran Desert. We also document SSD, with males attaining a greater length than females; however, taking a novel approach using geometric mean and principle component 1 as unbiased measures of overall body, we further show that SBCD also exists, with females possessing relatively longer trunks and bigger heads. Our finding of female-biased trunk length is consistent with prior counts of trunk vertebrae in rattlesnakes and fecundity selection favoring longer trunks in females. Because essentially all prior studies of snake dimorphism have relied on trunk length or SVL,

which are likely to be female-biased, essentially everything we know of sexual dimorphism in snakes needs reevaluation.

Introduction

Geographic variation in body size is a well-studied phenomenon (Bergmann, 1848; Fairbairn, Blanckenhorn, & Székely, 2007; Stillwell, Morse, & Fox, 2007; Wigginton & Dobson, 1999; Yom-Tov & Geffen, 2006). Various hypotheses have been proposed to explain geographic variation in body within a species, including Allen's rule, which states that volume-to-surface ratio of homeothermic animals varies inversely with mean environmental temperature (Allen, 1877), and Bergmann's rule, which states that populations found in colder climates will attain larger sizes than those found in warmer regions (Bergmann, 1848; Mayr, 1956; Rensch, 1938), although the latter does not appear to apply to the majority of squamates (Ashton & Feldman, 2003). Alternative hypotheses have been proposed to account for intraspecific body size variation in ectotherms (See Valenzuela-Sánchez, Cunningham, & Soto-Azat, 2015 for review) but multiple mechanisms likely interact to explain geographic variation in body size for the group (Ficetola et al., 2010). Geographic variation in body size is well documented in many snakes (Forsman, 1991; Krause, Burghardt, & Gillingham, 2003; Madsen & Shine, 1993; Plummer, 1987; Schwaner & Sarre, 1990), including rattlesnakes (Allsteadt, Savitzky, Petersen, & Naik, 2006; Amarello et al., 2010; Ashton, 2001; Beaupre, 1995; Laurence M Klauber, 1997; Spencer, 2008).

Sexual dimorphism, defined as morphological differentiation between sexually mature females and males (Abouheif & Fairbairn, 1997; Fairbairn, 1997; Geber, 1995;

Ghiselin, 1974; King, 1989; Lloyd & Webb, 1977; Nylin & Wedell, 1994), has drawn the interest of researchers since Darwin (Darwin, 1871), and is common among reptiles (Fitch, 1981). Familiar examples of sexual dimorphism include reproductive organs (Brennan, Clark, & Prum, 2010; Eberhard, 1990; Lloyd & Webb, 1977), structures associated with combat or display (Barrette & Vandal, 1990; Emlen, 1996; Gould, 1974; Møller, 1993; Petrie, Tim, & Carolyn, 1991), and body size (M. Andersson & Norberg, 1981; Berry & Shine, 1980; Fairbairn, 1997; Fairbairn et al., 2007; Fairbairn & Preziosi, 1994; Monnet & Cherry, 2002; Richard Shine, 1978). Body features may respond differently to abiotic and biotic factors, resulting in geographic variation in dimorphism (Beaupre, 2002; Dobson & Wigginton, 1996; Madsen & Shine, 1993; Stillwell et al., 2007). Sexual differences can exist in overall body size (sexual size dimorphism, SSD) or between the size and/or shape of individual body components (sexual body component dimorphism, SBCD, often called shape dimorphism; Fox, Cooper, and Hayes (2015)). Evolution of both SSD and SBCD is believed to be driven by sexual selection (Fairbairn, 1997; Fairbairn & Preziosi, 1994; Lindenfors, Tullberg, & Biuw, 2002; Petrie et al., 1991; Richard Shine, 1978; Wikelski & Trillmich, 1997), though ecological differences between the sexes may also play a role in the evolution of SSD (M. B. Andersson, 1994; Fairbairn et al., 2007; Hedrick & Temeles, 1989; Ralls, 1976; Selander, 1972; Richard Shine, 1989).

A major obstacle for studies of dimorphism is that differential scaling of individual body components can make measuring and interpreting body-shape dimorphism difficult (Fox et al., 2015; Kratochvíl, Fokt, Rehák, & Frynta, 2003). In snakes, reports of sexual differences in proportions of the few body components (head,

trunk, tail) exist for a number of species (Bonnet, Shine, Naulleau, & Vacher-Vallas, 1998; King, 1989; Krause et al., 2003; Pearson, Shine, & How, 2002; Richard Shine, 1986; R Shine, Reed, Shetty, & Cogger, 2002; Vincent, Herrel, & Irschick, 2004). However, these studies reporting sexual differences invariable rely on a single trait, snout-vent length (SVL), to control for overall body size. As Kratochvíl et al. (2003) rightfully pointed out for lizards, SVL may, itself, be a dimorphic character. Snout-vent length includes the length of the abdomen, which determines the size of the abdominal cavity; accordingly, if SVL is affected by selection favoring a larger volume of total clutch, then positive allometric growth of the female abdomen would ensure that all other body components would be found dimorphic in regards to relative body length (Braña, 1996; Kratochvíl et al., 2003; Reyes-Gavilán, Ojanguren, & Braña, 1997). This would be especially problematic for head length, which numerous studies show to be dimorphic in many snake species. It is therefore vital that studies examining sexual dimorphism and character scaling use an unbiased reference character as an overall measure of body size (Braña, 1996; Fox et al., 2015; Kratochvíl et al., 2003; Scharf & Meiri, 2013).

Fortunately, several approaches exist to identify or derive an unbiased measure of overall body size. The most direct uses a single character, such as body mass, that is closely associated with overall size, but nutritional state and reproductive condition can markedly influence this measure, especially in ectotherms. A second approach uses discriminant function analysis to identify the least dimorphic character (Fox et al., 2015; Olsson, Shine, Wapstra, Ujvari, & Madsen, 2002), but the relatively simple body plan of snakes can make it unlikely that any of the few body components are unbiased. The third and most widely applied approach is to use the first eigenvalue of a principle component

analysis (principle component 1, PC1), which often encompasses overall size, with the additional orthogonal components generally representing shape (e.g., Bookstein et al., 1985; Jolicoeur, 1963; Somers, 1986). A fourth approach computes the geometric mean of multiple measurements, which effectively removes shape to express overall size (Mosimann, 1970; Mosimann & James, 1979).

Rattlesnakes (genera *Crotalus* and *Sistrurus*) are New World pitvipers that exhibit indeterminate growth (Andrews, 1982), and the majority of species display male-biased SSD in adult SVL (Laurence M Klauber, 1997), or even age-specific SSD (Dreslik, Shepard, Baker, Jellen, and Phillips (2017). Richard Shine (1978) proposed that male-biased SSD in snakes evolves in response to sexual selection for large male body size in species where males engage in intrasexual competition for access to females. Male fighting ("combat") for females is well documented (Aldridge, 1993; Carpenter, 1979; Carpenter, Gillingham, & Murphy, 1976; Gillingham, Carpenter, & Murphy, 1983; Hersek, Owings, & Hennessy, 1992; Laurence M Klauber, 1997) and likely present in most, if not all, members of *Crotalus* and *Sistrurus*. Larger male vipers typically win in combat against smaller rivals (Schuett, 1997). The high cost of reproduction for females relative to males may also drive SSD (Beaupre, 2002; Beaupre & Duvall, 1998; Beaupre, Duvall, & O'Leile, 1998). This latter hypothesis has received support from studies reporting (1) higher mass-specific metabolic rates in gravid female *C. atrox* than non-reproductive females and males (Beaupre & Duvall, 1998); (2) modeling simulations holding all variables constant while only varying the cost of reproduction for females (Beaupre, 2002); and (3) equal growth rates and body size, even after reaching sexual maturity, in captive-raised male and female *C. atrox* on a controlled diet (Taylor &

Denardo, 2005). In addition to male-biased SSD, several rattlesnake species are also known to vary in various other morphological measurements over their geographic range (Allsteadt et al., 2006; Amarello et al., 2010; Ashton, 2001; Smith & Collyer, 2008; Spencer, 2008) with many recent studies documenting male-biased head size dimorphism in pitvipers (Glaudas, Jezkova, & Rodríguez-Robles, 2008; Meik, Setser, Mociño-Deloya, & Lawing, 2012; Vincent et al., 2004).

The southwestern speckled rattlesnake, *Crotalus pyrrhus* (Cope, 1867), ranges from extreme southeastern Utah and southern Nevada southward through western Arizona, southern California, and the northern panhandle of northwestern Sonora, into Baja California Norte, where it can be found as far south as the Vizcaíno region (Campbell & Lamar, 2004; Grismer, 2002; Laurence M Klauber, 1936). A saxicolous species, it is most commonly associated with the rugged and rocky terrain of the mountains of the Mohave and Sonoran Deserts, where it is only rarely encountered in the adjacent desert flats (Campbell & Lamar, 2004; Laurence M Klauber, 1936; Meik, 2016). The species occurs most broadly in the Lower Colorado River and Arizona Upland subdivisions of Sonoran Desertscrub, Mohave Desertscrub, Californian Chaparral, and Californian Coastal Scrub (Brown (1994). It also occurs in portions of Interior Chaparral and Great Basin Conifer Woodland (Campbell & Lamar, 2004; Meik, 2016), and can even be found at the transition zone between Californian Chaparral and Sierran Montane Conifer Forest near Idyllwild, California (C. Cochran, pers. observ.). However, as the result of Pleistocene and Holocene climate change, many populations have become isolated in desert mountain ranges well-separated by large stretches of uninhabitable desert (Douglas, Douglas, Schuett, & Porras, 2006). Because the fragmented populations

are subject to contrasting environmental conditions and have co-evolved with different prey, this rattlesnake species should be a good model for exploring size and shape variation and its potential causes. Indeed, previous studies suggest that this medium-sized rattlesnake exhibits geographic variation in body size (Glaudas & Rodriguez-Robles, 2011; Laurence M Klauber, 1936; Meik, Streicher, Lawing, Flores-Villela, & Fujita, 2015; R. G. Moore, 1978).

The objectives of this study were to: 1) investigate geographic variation in the body size of *C. pyrrhus* across southern California, western Arizona, and southern Nevada; 2) to determine if SSD and SBCD exist within the species; and 3) to evaluate environmental variables that might be associated with body size variation, SSD, and SBCD. We met the first two objectives in this chapter of the dissertation; the third remains to be completed. Our study provides a novel approach to analyzing snake size and shape by evaluating head, trunk, and tail proportions relative to three alternative measures of overall body size: SVL, geometric mean, and PC1.

Materials and Methods

Morphological Measurements

We obtained body measurements from two groups of adult snakes: 171 live specimens collected opportunistically (via active search or road cruising) or provided by snake removal services, and 174 alcohol-preserved specimens. We measured preserved specimens at the Los Angeles County Museum of Natural History (LACM; $N = 41$), and used measurements from Laurence Klauber's original notes, which were supplied by the San Diego Natural History Museum (SDNHM; $N = 133$). We used only adults to avoid

potential sampling bias (abundance of juveniles varies seasonally) and to reduce allometric effects in measuring dimorphism. We classified snakes as adults if their snout-vent length (SVL) exceeded 430 mm, at which size male snakes would likely be mature, given the smallest individual known to copulate was a 443 mm female from the Tinajas Altas Mountains of Yuma County, Arizona (C. Cochran pers. observ.; male rattlesnakes mature at a smaller size than females: Laurence M Klauber (1997)). Our sample represented six major biogeographic regions across Arizona, California, and Nevada, USA (Brown & Lowe, 1994; Schoenherr, 2017), including the Mojave Desert, Transverse Ranges, Peninsular Ranges, and the Colorado Desert, Lower Colorado River, and Arizona Uplands portions of the Sonoran Desert (Fig. 1). Collection locations ranged from 40 to 1640 m in elevation.

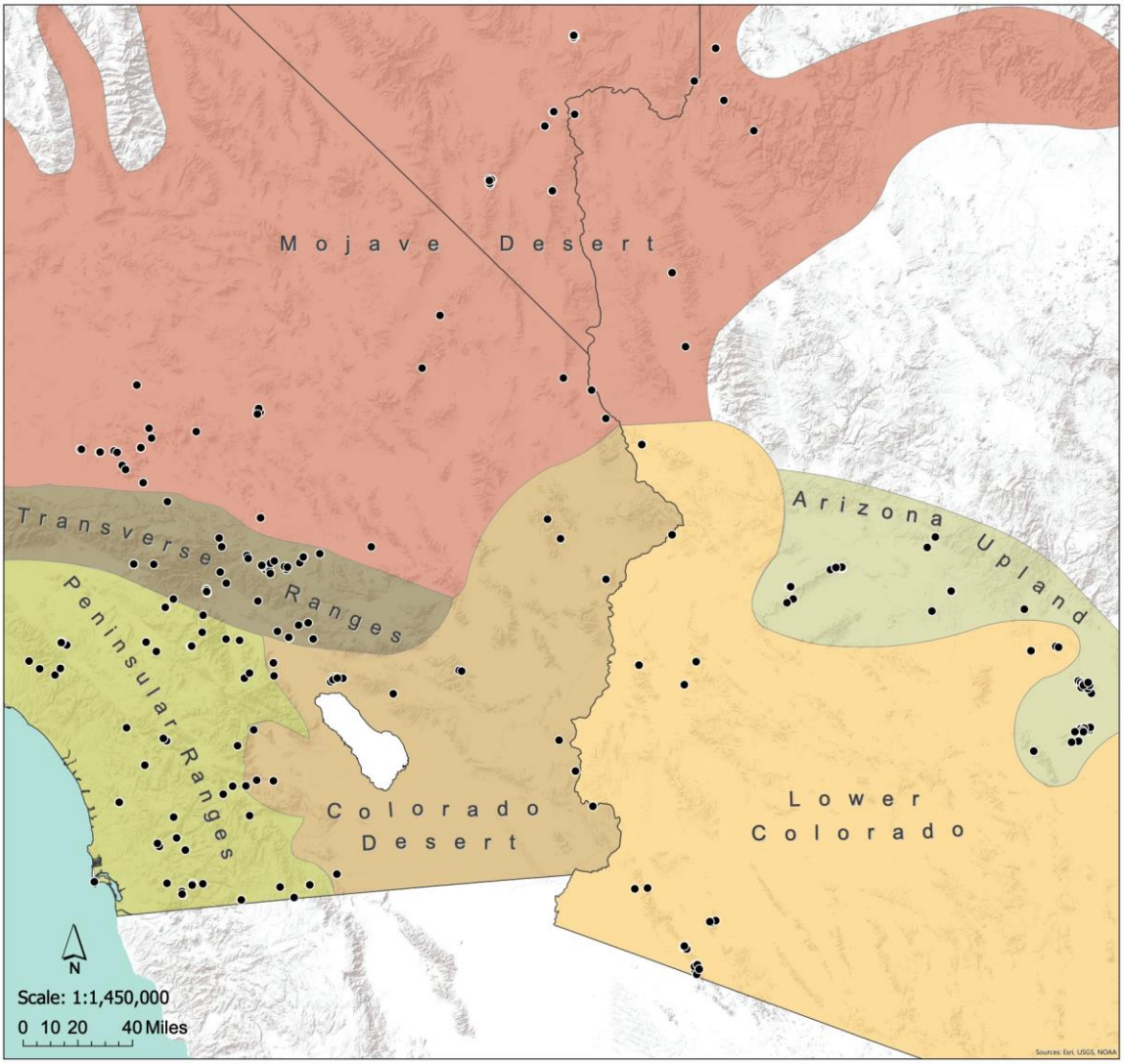

Figure 1. Collection sites for the 345 southwestern speckled rattlesnakes (*Crotalus pyrrhus*). Biogeographic regions adapted from Brown & Lowe, 1994, and Schoenherr, 2017.

For live snakes, we took measurements from specimens anesthetized within a translucent plastic tube with a vial at the distal end containing a cotton ball saturated with 0.5 mL of isoflurane. After removing the snake from the tube, we used digital calipers to measure head width (HW; at widest part of head) and head length (HL; tip of rostral to the plane joining the posterior tips of the mandibular bones, on the left side only) to the nearest 0.1 mm, and a tape measure (Stanley PowerLock® 33-428 8 m/26') was used to

measure SVL (tip of snout to center of anal plate) and tail length (TL; center of anal plate to forward edge of the proximal rattle) to the nearest 0.5 mm. We subsequently calculated trunk length (TrL) as SVL – HL. One of us (CC) obtained all measurements to minimize measurement error. Sex was determined by manually probing for the presence of hemipenes (Schaefer, 1934).

For preserved snakes, CC obtained measurements from LACM specimens using the same methods employed for live snakes, but we opted to use the measurements reported in Klauber's original notes for the remaining preserved snakes, presumably all housed at SDNHM. Because of well-documented shrinkage that occurs during the preservation process, we multiplied the SVL of preserved snakes by 2.0% to make them comparable to measurements from live snakes (Laurence M Klauber, 1938, pg. 3). Because body components (head, trunk, tail) exhibit differential shrinkage, we included preserved snakes only in analyses of overall body size and SSD, and excluded them from analyses of SBCD.

Visual inspection of bivariate scatterplots led us to doubt the sex of Klauber specimen LMK37464, purportedly female, because of its obviously short tail, so we discarded data from it. We also assigned the sex of female to a live specimen with a short tail whose sex was ambiguous during cloacal probing.

Derived Measures of Overall Body Size

We obtained relatively unbiased measures of overall body size via two approaches. First, using the Mosimann method, we computed the geometric mean (*n*th root of the product of *n* variables) of four body measures (head length, head width, trunk

length, tail length), which effectively isolates size from shape (Mosimann, 1970; Mosimann & James, 1979). Second, we used principle components analysis to derive the first eigenvector or principle component (PC1), which likewise is recognized as a measure of overall body size, with the other components extracted incorporating shape (Bookstein et al., 1985; Jolicoeur, 1963; Mosimann, 1970; Somers, 1986).

Statistical Analyses

We conducted three major sets of analyses using SPSS 20.0 for Macintosh (Statistical Package for the Social Sciences, Inc., Chicago, 2011). Prior to doing so, we subjected data to standard tests for univariate normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test), and screened for multivariate outliers using Mahalanobis distances obtained from regression (Tabachnick & Fidell, 2013). We removed two outlier measurements from live snakes: a head length from one specimen, and a tail length from another. Visual inspection of bivariate scatterplots across all variables, including geometric mean and PC1 (derived measures described above), indicated the original measurements met the assumption of linearity. Log-transformed measurements created mild curvilinearity in some bivariate relationships, so we used only untransformed data for all analyses. The principle components analysis that we conducted to generate PC1 used a correlation matrix, unrotated factors, and SPSS defaults.

Our first analysis considered geographic variation in body size. We conducted a 6 × 2 analysis of variance (ANOVA; Tabachnick and Fidell (2013)), with SVL as the measure of body size, and biogeographic region and sex treated as between-subjects

variables. By using SVL, we were able to use the combined data set from live and preserved snakes while accounting for sexual bias in this measure.

Our second set of analyses measured SSD and SBCD, which are properties of populations rather than individuals. We used only measurements from live snakes because, as mentioned previously, preservation disproportionately affects head and tail size relative to the trunk. We also pooled snakes from all biogeographic regions after learning that SVL differences between the sexes were similar among all biogeographic regions (see Results). Measuring SSD was straightforward: we calculated the Lovich and Gibbons (1992) index for SVL and geometric mean separately using the following equations: (1) if females were larger, then SSD = ((mean female geometric mean) / (mean male geometric mean)) - 1; (2) if males were larger, then SSD = - ((mean male geometric mean) / (mean female geometric mean)) + 1. With this index, SSD = 0 means absence of sex differences; positive values indicate female-biased dimorphism; negative values indicate male-biased dimorphism; and values multiplied by 100 provide the percent difference relative to the smaller sex, which we report because doing so is more intuitive and avoids values with excessive decimal places. We could not compute an index value for SSD using PC1 because of the scaling effect associated with extraction. We tested the significance of SSD using independent-samples *t*-tests (Sokol & Rohlf, 1969).

Measuring SBCD for each of the four body components (HL, HW, TrL, TL) required multiple models. We examined HL and HW in three multivariate analysis of covariance (MANCOVA; Tabachnick and Fidell (2013)) models using sex as a predictor and either SVL, geometric mean, or PC1 as the covariate encompassing overall body size. We also examined TrL and TL in separate ANCOVA (Tabachnick & Fidell, 2013)

models (three for each) that likewise included sex as a predictor and one of the three measures of body size as covariates. For all models, we 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. We computed estimated marginal means for each sex to derive the (Lovich & Gibbons, 1992) index of dimorphism for each body component using the three different covariates for overall body size.

Because null-hypothesis tests (*P*-values) are greatly influenced by sample size, we place greater confidence in effect sizes, which are much more meaningful, can be compared more readily across studies, and should always be reported in addition to statistical significance. For the three estimates of SSD based of SVL, geometric mean, and PC1, we calculated Cohen's *d*, with values of ~0.2, ~0.5, and ≥0.8 corresponding loosely to small, medium, and large effects, respectively (Cohen, 1988). For the estimates of SBCD, we computed multivariate eta-squared (η^2) and partial eta-squared as measures of effect size for each variable and covariate in the MANOVA and ANCOVA models, respectfully. Eta-squared can be interpreted as the proportion of variance in a dependent variable explained by an independent variable (Cohen, 1988). We loosely considered values of ~0.01, ~0.06, and ≥0.14 to be small, medium, and large effects, respectively (Cohen, 1988). Because η^2 is upward-biased when multiple variables are included (Pierce, Block, & Aguinis, 2004), we adjusted these when values for main effects (and interaction if present) summed to >1.0 by dividing each value by the sum of all values (Revell & Hayes, 2009). Following Nakagawa (2004), we chose not to adjust alpha for multiple tests because doing so overemphasizes the importance of null hypothesis testing

when effect size is more meaningful, and unacceptably increases the probability of making type II errors (i.e., the hyper-Red Queen phenomenon: the more research one does, the lower the probability that a significant result will be found; Moran (2003).

Results

Geographic Variation in Snout-vent Length

For the combined data sets from live and preserved snakes, mean body size measured as SVL varied significantly among the six biogeographic regions ($F_{5,327}$ = 22.71, p = < 0.001, partial η^2 = 0.26) and differed between the sexes ($F_{1,327}$ = 51.11, p = < 0.001, partial η^2 = 0.14). No interaction existed between biogeographic region and sex ($F_{5,327}$ = 0.90, p = 0.48, partial η^2 = 0.01), suggesting that SSD measured by SVL was similar among biogeographic regions. Tukey's post-hoc multiple comparisons revealed three groupings: snakes in the Mojave Desert, Transverse Ranges, and Peninsula Ranges were larger than snakes in the Colorado Desert and Arizona Upland subdivisions of the Sonoran Desert, which were larger than the diminutive snakes from the Lower Colorado River subdivision of the Sonoran Desert (Fig. 2). A separate model adding data set (live versus preserved snakes) as another variable yielded a negligible effect (not shown) for source of snakes and no interactions, which justified pooling data for live and preserved snakes to increase group sample sizes and statistical power.

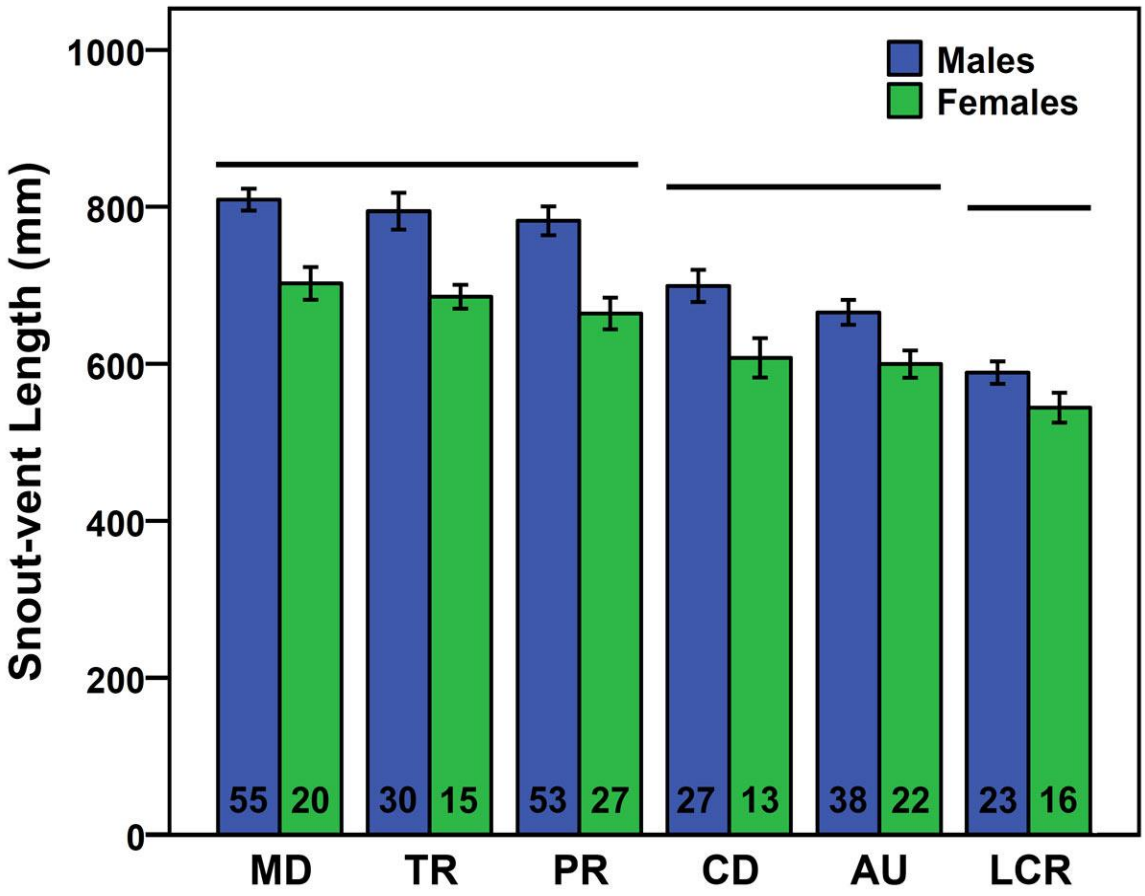

Figure 2. Mean (± 1 S.E.) snout-vent length of male and female southwestern speckled rattlesnakes (*Crotalus pyrrhus*) from six biogeographic regions (see Fig. 1): Mojave Desert = MD; Transverse Ranges = TR; Peninsular Ranges = PR; and subregions of Sonoran Desert designated as Colorado Desert = CD, Arizona Uplands = AU, and Lower Colorado River = LCR. Body size differed significantly between the sexes (ANOVA: $P < 0.001$) and among biogeographic regions (ANOVA: $P < 0.001$), with horizontal lines indicating statistical similarity among biogeographic regions (but not sex) by Tukey's multiple comparisons. Sample size for each mean is indicated at the base of each bar.

Sexual Size Dimorphism and Sexual Body Component Dimorphism

For the four measurements obtained from live snakes, the first component (PC1) extracted from principle components analysis captured 93.0% of the variance in the correlation matrix, and was comprised near-equally of HL (component score 0.988), HW (0.980), and TrL (0.979), with a weaker contribution of TL (0.910). The eigenvalue of 3.72 for PC1 was considerably larger than those of the three remaining components (all ≤

0.22), which collectively explained negligible variance (all ≤ 5.5%). We interpreted PC1 to represent overall body size.

The SSD index differed between the two measures evaluated for overall body size (SVL and geometric mean). Males were larger than females for both SVL (t_{169} = 5.19, P < 0.001, Cohen's d = 0.84) and geometric mean (t_{167} = 7.54, P < 0.001, Cohen's d = 1.22), but SVL provided a smaller SBCD index value (-0.164) than geometric mean (-0.238). In other words, males were 16.4% larger than females for SVL (mean ± 1 SE: 733 ± 12.2 mm and 630 ± 14.2 mm, respectively) and 23.8% larger than females for geometric mean (80.6 ± 1.3 and 65.1 ± 1.4, respectively). The third measure of overall body size, PC1, could not be expressed as a percentage difference (see Methods), but mean values were larger for males than females (0.35 and -0.66, respectively; t_{167} = 7.08, P < 0.001, Cohen's d = 1.55). Percent differences between the sexes and corresponding effect sizes (Cohen's d) demonstrate greater dimorphism when using geometric mean and PC1 compared to SVL.

The SBCD index demonstrated dimorphism for all four body components; however, the interpretation varied dramatically depending on the reference variable used for overall body size (Fig. 3).

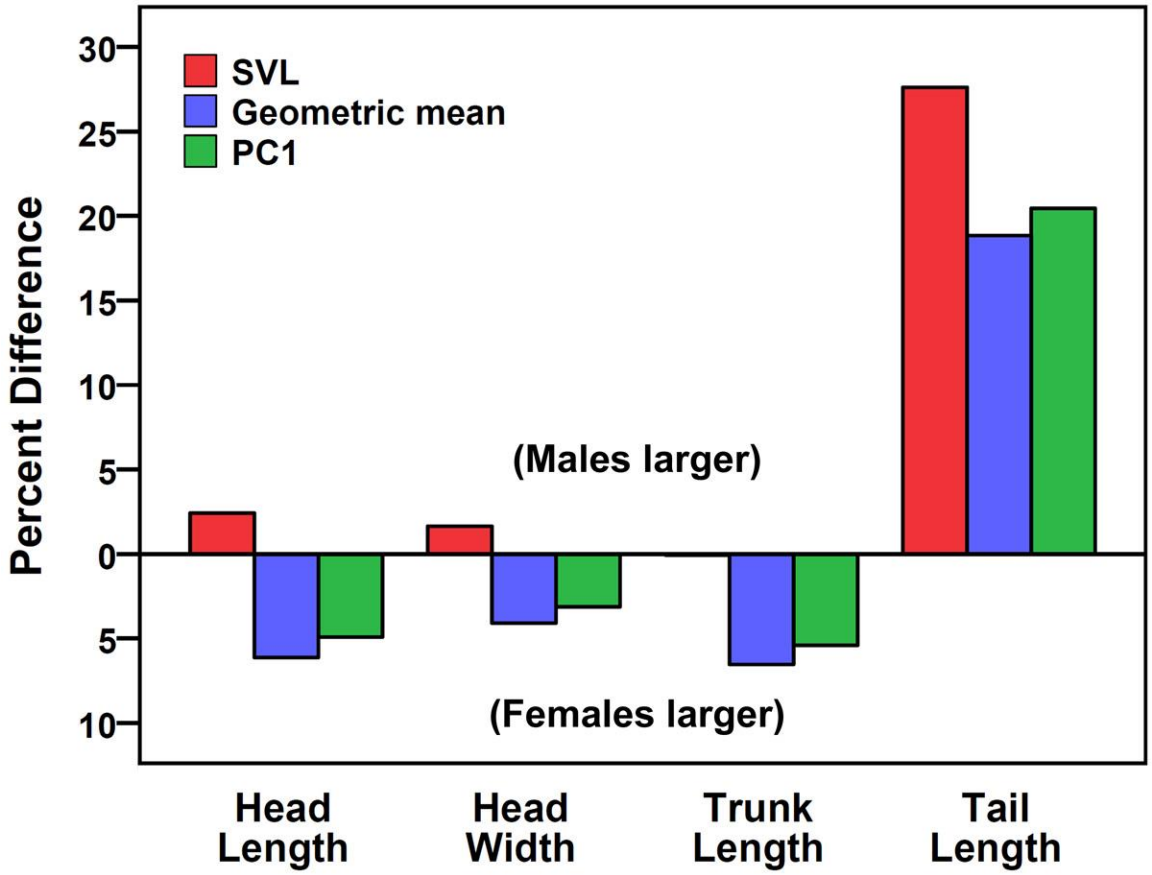

Figure 3. Percent difference between male and female southwestern speckled rattlesnakes (*Crotalus pyrrhus*) relative to the smaller sex for four body components: head length, head width, trunk length, and tail length. Interpretation of sexual body component dimorphism (SBCD) differed markedly depending on which of three measures was used to control for overall body size: snout-vent length (SVL), geometric mean, or principle component 1 (PC1).

Use of SVL as the covariate resulted in males having larger heads than females (2.4% greater HL and 1.6% greater HW), whereas use of geometric mean and PC1 both resulted in females having markedly larger heads (6.5% and 4.4% greater HL and HW, respectively, for geometric mean, and 5.2% and 3.2% greater HL and HW, respectively, for PC1). Trunk length differed negligibly for SVL (0.1% larger for females; ANCOVA), but was markedly greater for females with geometric mean (7.0%) and PC1 (5.7%) used as the covariate. Finally, TL differed most substantially between the sexes, being longer

for males regardless of covariate (27.6%, 18.8%, and 20.5% larger for SVL, geometric mean, and PC1, respectively). Results from the MANCOVA and ANCOVA models are summarized in Table 1. All three ANCOVAs for TL and one ANCOVA for TrL (with geometric mean as the covariate) violated the assumption of homogenous regression slopes because the difference between the sexes increased with increasing body size; however, the effect size was relatively small for the interactions (η^2 = 0.02–0.05), suggesting minimal relevance. Estimated marginal means for the sexes were similar for the models with and without the interaction term, so for consistency we computed index values using models without the interaction. Marginal means were estimated at SVL of 697 mm, geometric mean of 75.3, and PC1 of 0.0.

Table 1. Sexual body component dimorphism. MANCOVA and ANCOVA results for body component comparisons between the sexes of Southwestern Speckled Rattlesnakes (*Crotalus pyrrhus*) using three alternative measures of overall body size: snout-vent length (SVL), geometric mean, and principle component 1 (PC1). Head length = HL; head width = HW; trunk length = TrL; tail length = TL.

		SVL				Geometric Mean				PC1			
Model	**Variables**	***N***	***F***	***P***	**Partial η^2**	***N***	***F***	***P***	**Partial η^2**	***N***	***F***	***P***	**Partial η^2**
HL x HW	Sex	170	8.44	<0.001	0.088	169	57.39	<0.001	0.295	169	44.19	<0.001	0.262
	Covariate		1694.70	<0.001	0.912		3737.61	<0.001	0.705		4878.43	<0.001	0.738
HL	Sex	170	14.57	<0.001	0.077	169	67.54	<0.001	0.229	169	47.12	<0.001	0.184
	Covariate		3407.80	<0.001	0.923		5650.49	<0.001	0.771		7118.88	<0.001	0.816
HW	Sex	170	2.41	0.123	0.014	169	83.80	<0.001	0.257	169	59.70	<0.001	0.215
	Covariate		1579	<0.001	0.904		4180.57	<0.001	0.743		4626.55	<0.001	0.785
TrL	Sex	170	14.57	<0.001	0.926	169	0.00	0.993	0.000	169	96.93	<0.001	0.276
	Covariate		1463429.60	<0.001	0.074		3481.85	<0.001	0.955		5274.17	<0.001	0.724
	Sex x covariate						4.40	0.037	0.026				
TL	Sex	170	0.23	0.623	0.001	169	0.82	0.366	0.005	169	251.11	<0.001	0.417
	Covariate		473.35	0.000	0.740		797.12	<0.001	0.829		702.93	<0.001	0.560
	Sex x covariate		9.15	0.003	0.052		4.35	0.039	0.026		5.62	0.019	0.023

Note: Interaction term is included for models when a significant interaction existed. Partial eta-squared (η^2) effect sizes are adjusted when they sum to >1.0 for a model (see text for method).

Discussion

In this study, we examined geographic variation in body size, and sexual dimorphism in overall body size (SSD) and specific body components (SBCD for head, trunk, and tail). The most important finding is that geometric mean and PC1, both derived from multiple measurements, comprise more appropriate measures of overall body size in snakes than SVL. Here, we compare our findings to those of prior studies, especially of rattlesnakes, and discuss the factors that might lead to variation in body size and sexual dimorphism.

Geographic Variation in Body Size

We found that body size of *C. pyrrhus* sampled from six biogeographic regions of Arizona, California, and Nevada varied significantly, forming three distinct groups (Fig. 2). The largest snakes occurred in the Mojave Desert, Transverse Ranges, and Peninsular Ranges of California; the smallest snakes were restricted to the Lower Colorado River subdivision of the Sonoran Desert; and snakes of intermediate size inhabited the Colorado Desert (California) and Arizona Upland subdivisions of the Sonoran Desert. Klauber (1936) remarked that the largest *C. pyrrhus* were found in the Peninsular Ranges; however, with our substantial data set, snakes from those ranges were similar in size to those from the Mojave Desert and the Transverse Ranges. Although our longest male (991 mm SVL) was collected from the Peninsular Ranges, our longest female (854 mm SVL) was captured in the Mojave Desert. Meik et al. (2015) stated that the smallest specimens occur in western Arizona and northeastern Baja California, Mexico, which is consistent with our results. Our sample size benefitted greatly by combining data

collected from live and preserved snakes, but our general conclusions would have been similar had we analyzed a single data set.

Without completing our intended analyses of environmental variation, we can only speculate on the best explanation(s) for body size variation in *C. pyrrhus*. Valenzuela-Sánchez *et al*. (2015) considered six hypotheses to explain geographic variation in body size of ectotherms (other than amphibians), including two that involve seasonality (starvation resistance and growing season length), three that relate to temperature, and one that invokes primary productivity. We suspect seasonality does not explain variation in *C. pyrrhus* body size because Amarello *et al*. (2010) found no effect of temperature seasonality on body size of 10 populations of *C. atrox* in Arizona, which is broadly sympatric with *C. pyrrhus*. The authors proposed that the relatively short periods of stressful conditions in Arizona were insufficient to influence body size. Two of the three temperature-related hypotheses (heat balance and temperature-size rule) predict larger animals at colder climates. Larger body size has been associated with cooler and wetter sites in both *C. atrox* (Amarello et al., 2010) and *C. lepidus* (Beaupre, 1995), and may explain variation in *C. pyrrhus* as well. The third temperature-related hypothesis (optimal body temperature) predicts the reverse, with snakes a larger size at warmer locations. Our data refute this hypothesis, as some snakes with the longest SVL were collected at high elevation in the San Jacinto Mountains of California, comprising the only population we sampled that regularly experiences snowfall in the winter (CC, pers. observ.). Primary productivity, addressed by the last hypothesis, undoubtedly differs among our six biogeographic regions, and might contribute to variation in *C. pyrrhus* body size.

Sexual Size Dimorphism and Sexual Body Component Dimorphism

We found male-biased SSD among all six biogeographic regions studied, with male *C. pyrrhus* attaining significantly larger body sizes—whether measured by SVL, geometric mean, or PC1—than females. Male-biased SSD is well documented in rattlesnakes (Laurence M Klauber, 1997), including southwestern speckled rattlesnakes (Glaudas & Rodriguez-Robles, 2011). Prior studies suggest that male-biased SSD results from the higher cost of reproduction faced by females compared to males (Beaupre, 2002; Beaupre & Duvall, 1998; Beaupre et al., 1998; Taylor & Denardo, 2005).

The most novel findings relate to SBCD. By using geometric mean and PC1 as alternatives to SVL as a reference variable (covariate) for overall body size, our results provide strong evidence that SVL is a biased measure of overall body size, as it is composed largely of trunk length, which has likely been subject to fecundity selection. We should not be surprised that trunk length is greater in female compared to male rattlesnakes because females in each of the 15 rattlesnake species examined by Laurence Monroe Klauber (1943), including *C. pyrrhus*, had a greater number of ventral scales, which corresponds to number of vertebrae.

Perhaps most surprising was the discovery of female-biased head size dimorphism in *C. pyrrhus*. When controlling for overall body size using geometric mean or PC1, females possessed heads that were 3.2–4.4% wider and 5.2–6.5% longer than males of equivalent body size. Laurence M Klauber (1938) reported the absence of head size dimorphism in six rattlesnake species, and a clear female bias in a seventh species, the sidewinder (*C. cerastes*). More recent studies have documented male-biased head size dimorphism in the Great Basin Rattlesnake (*C. lutosus;* Glaudas et al. (2008)), a species

Klauber studied but failed to detect it in, and in the Mexican Lance-headed Rattlesnake (*C. polystictus*; Meik et al. (2012)) and cottonmouth (*Agkistrodon piscivorus*; Vincent et al. (2004)). Rattlesnakes are gape-limited predators, so an increase in head size would allow consumption of larger prey (Arnold, 1993; Rodríguez- Robles, Bell, & Greene, 1999; Rick Shine, 1991).

Selection for larger head size in females of *C. pyrrhus* would allow them to swallow larger prey items than males, thereby reducing competition for resources (Richard Shine, 1991) and possibly allowing them to recoup energy lost during reproduction in fewer meals. Male *C. polystictus* possessed not only relatively larger heads that differed in shape from those of females, but also consumed a greater number of large mammals (Meik et al., 2012). Male *A. piscivorus* possessed longer quadrate bones and greater lateral surface area of the head than females, and consumed a different diet of taller prey and a higher proportion of fish instead of the reptiles more often captured by females (Vincent et al., 2004). Both of the latter studies used trunk length to control for overall body size, which itself might be a dimorphic character.

An alternative hypothesis for female-biased head size could be selection acting to reduce head size in males in an attempt to minimize mass and corresponding energy expended during locomotion. Male *C. pyrrhus* travel longer distances per unit time in the mating and post-mating season than females and have larger home ranges (Glaudas & Rodriguez-Robles, 2011). This sex difference in movements and space use is consistent with the closely related tiger rattlesnake (*C. tigris*; Goode, Smith, & Amarello, 2008) and in rattlesnakes in general (J. A. Moore & Gillingham, 2006; Parker & Anderson, 2007;

Putman, Lind, & Taylor, 2013; Shipley, Chiszar, Fitzgerald, & Saviola, 2013; Timmerman, 1995; Waldron, Lanham, & Bennett, 2006; Wastell & Mackessy, 2011).

Male snakes possess hemepenes in the base of their tails, and therefore normally possess longer tails than females. Thus, our finding of a finding that males possess tails 18.8–27.6% longer (depending on measure) than similarly-sized females is not surprising.

Conclusions

This study represents a comprehensive effort to identify potential patterns and causes of size and shape variation in a rattlesnake that likely experiences differential selection among geographic regions and between the sexes. We have shown that overall body size varies geographically, presumably due to environmental variation, and that both SSD and SBCD are present in *C. pyrrhus*, with males attaining a greater length, but females possessing relatively longer trunks and bigger heads. More importantly, we show that either of two measures—geometric mean or PC1—should be less-biased and preferred alternatives to SVL or trunk length in representing overall body size. The latter measurements are likely female-biased in most snakes because of fecundity selection favoring a longer trunk in females. Because PC1 has a scaling constraint that prohibits calculating a dimorphism index value, geometric mean may have greater utility.

Our findings turn upside down our understanding of head size dimorphism in rattlesnakes. Because female-biased trunk size appears to be widespread in rattlesnakes, we suspect that future studies with a proper reference character for overall size will eventually show that most rattlesnakes possess female-biased head size. But the implications go further. Numerous studies have documented head size dimorphism in

snakes using SVL or trunk length to control for overall body size, and because we suspect most if not all snakes have female-biased trunk length, and therefore female-biased SVL, everything we have learned about head size dimorphism in snakes needs reevaluation.

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CHAPTER FOUR

ONTOGENETIC DIETARY VARIATION IN THE SOUTHWESTERN SPECKLED RATTLESNAKE (*CROTALUS PYRRHUS* (COPE 1867)) ACROSS THE SOUTHWESTERN UNITED STATES

Chip Cochran[1], Katherine L. Edwards[2], Zachary D. Travis[1], and William K. Hayes[1]

[1]Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, California 92354 USA

[2]Unaffiliated

Abstract

Dietary studies are central to our understanding of organismal biology. We describe the diet of the southwestern speckled rattlesnake (*Crotalus pyrrhus*) using data collected from fecal samples collected among six biogeographic regions across the species United States range of Arizona, California, and Nevada. Examination of 185 specimens yielded 104 prey items from 72 snakes. The diet of *C. pyrrhus* consisted predominantly of mammals (80.8%), in particular terrestrial squirrels (39.4%) and the heteromyid rodent genus *Chaetodipus* (26.9%). Male and female snakes consumed similar diets, but an ontogenetic shift occurred from primarily ectothermic prey (lizards) to endothermic prey (rodents and birds). Diet also varied among biogeographic regions, with snakes of the Lower Colorado River biogeographic region consuming a higher proportion of endotherms than those from the California/Nevada biogeographic regions, in part because the Tinajas Altas Mountains, Yuma County, Arizona population relies on birds to a greater extent than other *C. pyrrhus* populations.

Introduction

Dietary studies provide valuable insights for our understanding of snake biology. Shifts in diet likely played a large role in the evolutionary radiations and success of snakes (Colston, Costa, & Vitt, 2010; Greene, 1983). Information gained from detailed dietary studies can inform us about movements and habitat use (Baxley & Qualls, 2009; Heard, Black, & Robertson, 2004; Hirai, 2004; Sperry & Weatherhead, 2009), interspecific competition (Dugan & Hayes, 2017; Luiselli, 2003, 2006), intraspecific partitioning of resources (Glaudas, Jezkova, & Rodríguez-Robles, 2008; Meik, Setser, Mociño- Deloya, & Lawing, 2012; S. E. Vincent, Herrel, & Irschick, 2004), and

conservation (Holycross, Painter, Barker, & Douglas, 2002). Individual snake species span the dietary spectrum from specialists to generalists (Brischoux, Bonnet, & Shine, 2009; Greene, 1997). Rattlesnakes are generally regarded as ambush predators that opportunistically predate a variety of species across various phyla and classes (Clark, 2016). Detailed dietary analyses, however, are still lacking for many rattlesnake species (Campbell & Lamar, 2004; Klauber, 1997), even though they are the most-studied group of snakes (Beaman & Hayes, 2008).

As gape-limited predators, snakes face functional constraints on prey acquisition due to prey item diameter (Arnold, 1993; Hampton & Moon, 2013; Rodríguez- Robles, Bell, & Greene, 1999; Rick Shine, 1991). Thus, diet may play a key role in the evolution and maintenance of morphological features that take part in food acquisition (Gans, 1961; Greene, 1983; Mushinsky, 1987; Pough & Groves, 1983). Members of Viperidae possess stouter bodies, larger heads, and longer jaws than members of other snake families, and these morphological adaptations allow them to reduce handling costs for prey of all sizes while simultaneously increasing the maximum size of prey they can swallow (Pough & Groves, 1983). Intraspecific associations of skull morphology and diet have been documented among localities (Forsman, 1991; Forsman & Shine, 1997; Margres et al., 2015) and between sexes (Meik, Setser, et al., 2012; S. E. Vincent et al., 2004).

As a consequence of gape limitation, large snakes can consume larger prey items than smaller snakes (Forsman & Lindell, 1993; Mushinsky, 1987; Mushinsky, Hebrard, & Vodopich, 1982; Pough & Groves, 1983; Rick Shine, 1991), so it is not surprising that ontogenetic dietary shifts within vipers are well documented (Andrade & Abe, 1999; Hartmann, Hartmann, Cechin, & Martins, 2005; Santos et al., 2007; R Shine & Sun,

2003), including rattlesnakes (Clark, 2002; Glaudas et al., 2008; Mackessy, 1988; Mackessy, Williams, Ashton, & Lannoo, 2003; Sparks, Lind, & Taylor, 2015; Taylor & Price, 2001). As rattlesnakes grow, diet typically shifts from smaller ectothermic prey to larger endothermic prey, with corresponding shifts in venom composition from more toxic to more digestive (Mackessy, 1988, 2010). However, not all species follow this dietary pattern (Dugan & Hayes, 2012; Salomão, Santos, & Puorto, 1995).

Snake species with large geographic distributions often exhibit geographical variation in diet (Creer, Chou, Malhotra, & Thorpe, 2002; Luiselli, Filippi, & Capula, 2005; Santos et al., 2007). Several rattlesnake species demonstrate this variation (Clark, 2002; Dugan & Hayes, 2012; Glaudas et al., 2008; Holycross & Mackessy, 2002), whereas others maintain a similar diet across their distributional range (Holycross, Painter, Prival, et al., 2002; Sparks et al., 2015; Webber, Jezkova, & Rodríguez-Robles, 2016). Geographic variation in diet appears to be more pronounced in juvenile than adult *C. o. oreganus* (Sparks et al., 2015). Because diet may drive venom evolution (Barlow, Pook, Harrison, & Wüster, 2009; Daltry, Wüster, & Thorpe, 1996; Richards, Barlow, & Wüster, 2012; Wüster, Daltry, & Thorpe, 1999); see Jackson et al. (2016) for a discussion on prey metabolic state and its possible influence on venom evolution, studies of diet variation can inform venom research. A thorough understanding of geographic dietary variation can inform future venom research while also improving our understanding of a species' foraging mode and trophic morphology (Forsman & Shine, 1997; Pleguezuelos, Fernández-Cardenete, Honrubia, Feriche, & Villafranca, 2007; S. Vincent, Brandley, Herrel, & Alfaro, 2009) and how the environment may influence body size and sexual size dimorphism (Amarello et al., 2010; Beaupre, 2002).

The southwestern speckled rattlesnake, *Crotalus pyrrhus* (Cope, 1867), is a medium-sized pitviper typically under a meter in length. The species ranges from extreme southwestern Utah and southern Nevada, south through western Arizona, southern California, and the northern panhandle portion of northwestern Sonora, into Baja California Norte, where it can be found as far south as the Vizcaíno region (Campbell & Lamar, 2004; Grismer, 2002; Klauber, 1936). Populations are also present on various Gulf of California islands, including El Muerto and Smith (Campbell & Lamar, 2004; Grismer, 2002; Meik, Lawing, & Pires-daSilva, 2010; Meik, Schaack, et al., 2012; Meik, Streicher, Lawing, Flores-Villela, & Fujita, 2015). A saxicolous species, it is most commonly associated with the rugged and rocky terrain of the mountains of the Mojave and Sonoran Deserts, where it is rarely encountered in the adjacent desert flats (Campbell & Lamar, 2004; Klauber, 1936; Meik, 2016). *Crotalus pyrrhus* associates with a number of biotic communities defined by Brown and Lowe (1994), including the Lower Colorado River and Arizona Upland subdivisions of Sonoran Desertscrub, Mohave Desertscrub, Californian Chaparral, and Californian Coastal Scrub. The species also occurs locally in Interior Chaparral and Great Basin Conifer Woodland (Campbell & Lamar, 2004; Meik, 2016). It can even be found at the transition zone between Californian Chaparral and Sierran Montane Conifer Forest in the San Jacinto Mountains (C. Cochran, pers. observ.). As an ambush predator and dietary generalist, *C. pyrrhus* predates upon a diverse array of prey types across its distribution (Table 1). Small mammals and lizards appear to make up the majority of its diet (Klauber, 1936, 1997; Lowe, Schwalbe, & Johnson, 1986; Meik, Schaack, et al., 2012), though birds are also recorded with regularity (Cornett, 1987; Klauber, 1936, 1997; Meik, 2016; Meik,

Schaack, et al., 2012; Miller & Stebbins, 1964) and appear to be an important dietary component of the El Muerto island population (Meik, 2016).

Table 1. Prey consumed, in the wild, by *Crotalus pyrrhus*, including locality and source. Matching superscripts indicate multiple prey items from a single snake.

Prey taxon	n	Locality	Source
Aves	17		
Callipepla gambelii	1	South Mtn, Maricopa Co., AZ	Meik (2016, p. 554)
Columbina inca	1	South Mtn, Maricopa Co., AZ	Meik (2016, p. 554)
Dendroica sp.	1	El Muerto Island, MX	Meik et al. (2012, p. 558)
Myiarchus tyrannulus	1	South Mtn, Maricopa Co., AZ	Meik (2016, p. 554)
Pipilo chlorurus	1	South Mtn, Maricopa Co., AZ	Meik (2016, p. 554)
Spinus tristis	8	N of Desert Center, RIV Co., CA	Klauber (1936, p. 168)
Toxostoma curvirostre	1	South Mtn, Maricopa Co., AZ	Meik (2016, p. 554)
Zonotrichia leucophrys	1	South Mtn, Maricopa Co., AZ	Meik (2016, p. 554)
Unidentified bird	1	Joshua Tree NP, SBE Co., CA	Cornett (1987, p. 125)
Unidentified bird	1	Not provided	Klauber (1997, p. 628)
Mammalia	36		
Ammospermophilus leucurus	1	Providence Mtns, SBE Co., CA	Johnson et al. (1948, p. 275)
Ammospermophilus leucurus[a]	1	Providence Mtns, SBE Co., CA	Johnson et al. (1948, p. 275)
Dipodomys agilis	1	Not provided	Klauber (1997, p. 628)
Otospermophilus beecheyi	1	San Pedro Mártir Mtns, BCN, MX	Klauber (1997, p. 628)
Peromyscus crinitus	1	Turtle Mtns, SBE Co., CA	Camp (1916, p. 534)
Peromyscus maniculatus	1	Highland Valley, San Diego Co., CA	Klauber (1997, p. 628)
Peromyscus sp.	1	El Muerto Island, MX	Klauber (1949, p. 103)
Peromyscus sp.	1	El Muerto Island, MX	Klauber (1949, p. 103)
Peromyscus truei[a]	1	Provedince Mtns, SBE Co., CA	Johnson et al. (1948, p. 275)
Sylvilagus auduboni	1	Not provided	Klauber (1997, p. 628)
Unidentified mammal	7	El Muerto Island, MX	Klauber (1949, p. 103)
Unidentified mammal	18	Not provided	Klauber (1997, p. 628)
Unidentified mammal[b]	1	Not provided	Klauber (1997, p. 628)
Reptilia	20		
Eumeces skiltonianus	1	Not provided	Klauber (1997, p. 628)
Sauromalus ater	1	Not provided	Klauber (1997, p. 628)
Sauromalus hispidus	1	Smith Island, MX	Meik et al (2012, p. 558)
Sceloporus sp.	1	Not provided	Klauber (1997, p. 628)
Uta stansburiana	1	Not provided	Klauber (1997, p. 628)
Unidentified lizard	4	El Muerto Island, MX	Klauber (1949, p. 103)
Unidentified lizard	9	Not provided	Klauber (1997, p. 628)
Unidentified lizard	1	Not provided	Klauber (1997, p. 628)
Unidentified lizard[b]	1	Not provided	Klauber (1997, p. 628)
Totals	73		

The main objective of this study was to investigate how body size, sex, and location influence diet among 23 populations of *C. pyrrhus* within six biogeographic regions across southern California, western Arizona, and southern Nevada. Our sample size was sufficient to test three hypotheses regarding consumption of ectothermic (reptile) versus endothermic (bird and mammal) prey: (1) the percentage of endotherms increases ontogenetically, as documented for other rattlesnake species (Clark, 2002; Glaudas et al., 2008; Mackessy, 1988; Mackessy et al., 2003; Sparks et al., 2015; Taylor & Price, 2001); (2) no difference exists between the sexes when controlling for snake size; and (3) no difference exists among the different populations and biogeographic regions when controlling for snake size, as demonstrated in another similarly distributed species (Webber et al., 2016). We also tested a fourth hypothesis, that snakes from the Tinajas Altas Mountains (TAM), Yuma County, Arizona, rely more heavily on birds than those of other populations. We suspected greater bird consumption because snakes from this population tend to exhibit arboreality (i.e., climbing into shrubs/trees; C. Cochran, pers. observ.).

Methods

Sources of Prey Items

We examined 185 *C. pyrrhus* from 23 localities representing six major biogeographic ranges across Arizona, California, and Nevada, USA (Brown & Lowe, 1994; Schoenherr, 2017); Fig. 1). We collected snakes during the period 2012–2017 via active search, road cruising, and specimens provided by snake removal services. Because we sampled no museum specimens, our study assesses contemporary diet during a long-

term regional drought (Cayan et al., 2010; Weiss, Castro, & Overpeck, 2009). Collection locations ranged from 40 to 1640 m in elevation.

Upon collection, we anesthetized snakes within a plastic tube in which a vial containing a cotton ball saturated with 0.5 mL of isoflurane was placed (Hardy Sr & Greene, 1999). We measured the snout-vent length (SVL) of the anesthetized snake to the nearest millimeter, and determined sex by eversion of hemipenes or cloacal probing (Schaefer, 1934). We then firmly palpated the snake's posterior to expel fecal matter into 118-mL Nasco Whirl-Pak® bags (Fort Atkinson, Wisconsin, USA). Fecal material was stored in a -20°C freezer until transfer to a glass vial containing 70% ethanol. We included stomach samples from three snakes of the Tinajas Altas Mountains of Yuma County, Arizona, which were collected for a previous study (Tellez & Cochran, 2017). We also obtained one fecal sample from South Mountain of Maricopa County, Arizona, which was assigned to a snake of unknown sex because two snakes of different sexes were captured by a snake removal company and placed in the same bucket, whereupon one individual defecated. The two snakes measured 669 mm and 700 mm in SVL, respectively, so we arbitrarily assigned the prey item to one of the snakes and an SVL of 700 mm to both snakes, as either measurement placed the snake in the same SVL grouping.

Identification of Prey Items

We classified prey items as lizards, birds, or mammals; no invertebrates or amphibians were identified in the feces. We used diagnostic scale characteristics of sympatric species (Stebbins, 2003) for lizard identification when possible. To identify

mammal prey to genus or species, dorsal guard hairs were cut in half at the widest point and soaked in xylene 24 h to allow maximum penetration of the medulla (T. D. Moore, Spence, & Dugnolle, 1974). We then mounted the hairs on glass slides using Seche Vite™ Dry Fast Top Coat, immediately placed a glass coverslip, and allowed them to set overnight. We attempted to identify samples to the lowest possible taxonomic level by comparing them with known dorsal guard-hair patterns observed under a light microscope (T. D. Moore et al., 1974). When possible, the known distributions of prey species were used to reach species-level identification (Jameson & Peeters, 2004; Reid, 2006). However, we disregarded the possibility of hairs being from bats (order Chiroptera), which are very rarely consumed by rattlesnakes, with only one naturally-occurring record we could find (for *C. horridus*; Uhler, Cottam, and Clarke (1939)) despite numerous detailed studies of diet for many species. In sampling only fecal material, we recognize the limitations of prey detection and identification, including reduced detection of invertebrate and amphibian remains (Glaudas, Kearney, & Alexander, 2017).

Sources of Dietary Variation

We examined the potential influences of body length, sex, and biogeographic region on diet variation. We classified prey items as ectotherms (lizards) and endotherms (birds and mammals). We used SVL in statistical analyses, but to depict ontogenetic variation graphically, we placed snakes into six groups: <430 mm (juvenile), 431–530 mm, 531–630 mm, 631–730 mm 731–830 mm, and >831 mm. To assess geographic variation, we assigned individual snakes to six biogeographic regions (Brown & Lowe,

1994; Schoenherr, 2017): Mojave Desert (MD, $N = 12$), Transverse Ranges (TR, $N = 3$), Peninsular Ranges (PR, $N = 6$), Sonoran Desert Colorado Desert (SDCD, $N = 3$), Sonoran Desert Lower Colorado River (SDLCR, $N = 17$), and Sonoran Desert Arizona Upland (SDAU, $N = 31$; Fig. 3-1; see chapter 3). However, due to relatively sparse samples from the four regions west of the Colorado River (MD, TR, PR, SDCD), we pooled these into a single California-Nevada region (CA/NV), leaving three regions for analyses: CA/NV, SDLCR, and SDAU. We have shown elsewhere that males of this species average larger than females, and that body size varies significantly among biogeographic regions, with snakes from SDLCR averaging smallest (Chapter 3). Supplemental analyses (not provided) confirmed these findings for snakes examined in the present study.

Statistical Analyses

We conducted two sets of analyses using SPSS 20.0 for Mac (Statistical Package for the Social Sciences, Inc., Chicago, 2011), with $\alpha = 0.05$. First, we relied on binary logistic regression (Mertler & Vannatta, 2010; Tabachnick & Fidell, 2013) to determine whether percent endotherms in diet varied ontogenetically (SVL), between the sexes (2 levels), and among biogeographic regions (3 levels). We included all prey items, including multiple items from the same snake (i.e., mild pseudoreplication); prior studies have similarly treated multiple food items from the same snake as independent data (Dugan & Hayes, 2012; Santos et al., 2007; Tuttle & Gregory, 2009), though bias could arise from individual foraging preferences.

For the second analysis, we tested whether snakes of the TAM population rely more heavily on birds compared to snakes of other populations. Due to sparse cells, we

collapsed data to two diet categories (bird vs. other) and two biogeographic regions (TAM vs. other). Because the resultant cells remained too sparse to control for sex and SVL in another logistic regression model, we resorted to univariate tests (Field, 2013). We used a Mann-Whitney *U* test to compare the mean body size of snakes consuming birds versus other prey, and chi-square tests to examine associations of bird consumption with sex and with biogeographic region. The chi-square tests violated the assumption of <20% of cells having an expected frequency <5, but the results for both tests were unambiguous despite the reduction in statistical power. Again, we included all prey items.

We computed effect sizes for each statistical test, as these are independent of sample size (in contrast to statistical significance) and more readily compared among different data sets and different studies (Cohen, 1988; Hojat & Xu, 2004; Nakagawa & Cuthill, 2007). For logistic regression, we computed Nagelkerke R^2 as the effect size for the omnibus model, with 0.01, 0.09, and 0.25 loosely corresponding to small, medium, and large effects, respectively. We also calculated an odds ratio with 95% confidence interval (CI) for each predictor. For the univariate tests, we computed r^2 for the Mann-Whitney test (calculated as $[z/\sqrt{N}]^2$ and interpreted the same as Nagelkerke R^2; Field (2013)), and phi (Φ) for the chi-square test, with values of ~0.1, ~0.3, and ≥0.5 loosely deemed small, moderate, and large effects, respectively (Cohen, 1988).

Results

Prey Items

Of the 185 *C. pyrrhus* collected, 69 (37.3%) snakes yielded 70 fecal samples (Muddy Mountains #2 was caught twice, 44 weeks apart, and provided a fecal sample each time), and three snakes from a previous study provided stomach contents, from which 104 prey items (Table 2) were at least partially identifiable. Twenty-eight fecal samples contained multiple prey items (40.0%); of these, 22 contained two mammals, three contained the remains of both a lizard and a mammal, and three contained two mammals and one lizard. Identification of multiple prey from feces was only possible if at least two different prey species were found, leading to likely underestimation of snakes containing multiple prey items. The 72 snakes containing food items were distributed as follows: CA-NV, 12 ♂♂, 12 ♀♀, 349–868 mm SVL; SDLCR, 7 ♂♂, 6 ♀♀, 3 unknown body size and sex, 1 unknown body size, 296–658 mm SVL; SDAU, 17 ♂♂, 13 ♀♀, 1 unknown sex, 413–793 mm SVL.

Among the 104 prey items, we identified the remains of 13 (12.5%) lizards, seven (6.7%) birds, and 84 (80.8%) mammals. The five lizards identifiable to genus included one *Aspidoscelis* (whiptail lizards), three *Sceloporus* (spiny lizards), and one *Uta* (side-blotched lizards). We were unable to identify any bird remains (feathers) to even the family level. The 84 mammal remains identifiable to family were distributed among three families: Cricetidae (15), Heteromyidae (28), and Sciuridae (41). We were unable to assign any sciurids to the generic level, but the remaining rodents were distributed among three genera: 28 *Chaetodipus* (pocket mice), one *Neotoma* (woodrats), and 14 *Peromyscus* (deer mice). Up to six of the 104 prey items were omitted from statistical

analyses because of missing data for the snake's sex (N = 2), SVL (N = 1), or both sex and SVL (N = 3 TAM snakes).

Table 2. Prey consumed by *Crotalus pyrrhus* in this study, including, individual, locality, and source.

Prey taxon	n	Snake	SVL (mm)	Locality
Aves	7			
Unidentified bird	1	LSB #11	708	Little San Bernardino Mountains, Riverside Co., CA
Unidentified bird	1	SJ #8	765	San Jacinto Mountains, Riverside Co., CA
Unidentified bird	1	TA #?	?	Tinajas Altas Mountains, Yuma County, AZ
Unidentified bird	1	TA #3	?	Tinajas Altas Mountains, Yuma County, AZ
Unidentified bird	1	TA #4	526	Tinajas Altas Mountains, Yuma County, AZ
Unidentified bird	1	TA #15	624	Tinajas Altas Mountains, Yuma County, AZ
Unidentified bird	1	TA #29	500	Tinajas Altas Mountains, Yuma County, AZ
Mammalia	84			
Chaetodipus spp.	1	AV #7	854	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #3	620	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #4	604	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #5	528	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #6	463	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #7	792	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #9	466	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #10	498	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #13	592	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #15	655	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #16	732	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	HAR #17	572	Harcuvar Mountains, La Paz Co., AZ
Chaetodipus spp.	1	LSB #6	551	Little San Bernardino Mountains, Riverside Co., CA
Chaetodipus spp.	1	MUD #6	582	Muddy Mountains, Clark Co., NV
Chaetodipus spp.	1	PMP #1	629	Phoenix Mountains Preserve, Maricopa Co., AZ
Chaetodipus spp.	1	PMP #6	478	Phoenix Mountains Preserve, Maricopa Co., AZ
Chaetodipus spp.	1	PMP #8	750	Phoenix Mountains Preserve, Maricopa Co., AZ
Chaetodipus spp.	1	PIS #5	646	Pisgah Crater, San Bernardino Co., CA
Chaetodipus spp.	1	PLO #1	598	Plomosa Mountains, La Paz Co., AZ
Chaetodipus spp.	1	SM #1	683	South Mountain, Maricopa Co., AZ
Chaetodipus spp.	1	SM #7	746	South Mountain, Maricopa Co., AZ
Chaetodipus spp.	1	SM #8	491	South Mountain, Maricopa Co., AZ
Chaetodipus spp.	1	SM #10	597	South Mountain, Maricopa Co., AZ
Chaetodipus spp.	1	SM #?	700	South Mountain, Maricopa Co., AZ
Chaetodipus spp.	1	TA #6	561	Tinajas Altas Mountains, Yuma Co., AZ
Chaetodipus spp.	1	TA #8	502	Tinajas Altas Mountains, Yuma Co., AZ
Chaetodipus spp.	1	TA #17	296	Tinajas Altas Mountains, Yuma Co., AZ
Chaetodipus spp.	1	TA #23	443	Tinajas Altas Mountains, Yuma Co., AZ
Neotoma spp.	1	AV #6	800	Bell Mountain, San Bernardino Co., CA
Peromyscus spp.	1	AV #13	615	Fairview Mountain, San Bernardino Co., CA
Peromyscus spp.	1	HAR #3	620	Harcuvar Mountains, La Paz Co., AZ
Peromyscus spp.	1	HAR #7	792	Harcuvar Mountains, La Paz Co., AZ
Peromyscus spp.	1	LQ #2	675	La Quinta, Riverside Co., CA
Peromyscus spp.	1	McC #5	868	McCullough Range, Clark Co., NV
Peromyscus spp.	1	MUD #2	867	Muddy Mountains, Clark Co., NV
Peromyscus spp.	1	MUD #5	860	Muddy Mountains, Clark Co., NV
Peromyscus spp.	1	PMP #2	413	Phoenix Mountains Preserve, Maricopa Co., AZ

Peromyscus spp.	1	PMP #3	793	Phoenix Mountains Preserve, Maricopa Co., AZ
Peromyscus spp.	1	SM #3	678	South Mountain, Maricopa Co., AZ
Peromyscus spp.	1	TA #9	572	Tinajas Altas Mountains, Yuma Co., AZ
Peromyscus spp.	1	TA #17	296	Tinajas Altas Mountains, Yuma Co., AZ
Peromyscus spp.	1	TA #21	658	Tinajas Altas Mountains, Yuma Co., AZ
Peromyscus spp.	1	TA #27	477	Tinajas Altas Mountains, Yuma Co., AZ
Ground Squirrel	1	CHK #2	703	Chuckwalla Mountains, Riverside Co., CA
Ground Squirrel	1	HAR #3	620	Harcuvar Mountains, La Paz Co., AZ
Ground Squirrel	1	HAR #7	792	Harcuvar Mountains, La Paz Co., AZ
Ground Squirrel	1	HAR #10	498	Harcuvar Mountains, La Paz Co., AZ
Ground Squirrel	1	HAR #13	592	Harcuvar Mountains, La Paz Co., AZ
Ground Squirrel	1	HAR #14	637	Harcuvar Mountains, La Paz Co., AZ
Ground Squirrel	1	HAR #15	655	Harcuvar Mountains, La Paz Co., AZ
Ground Squirrel	1	HAR #16	732	Harcuvar Mountains, La Paz Co., AZ
Ground Squirrel	1	HAR #17	572	Harcuvar Mountains, La Paz Co., AZ
Ground Squirrel	1	McC #5	868	McCullough Range, Clark Co., NV
Ground Squirrel	1	McC #9	860	McCullough Range, Clark Co., NV
Ground Squirrel	1	MUD #2	867	Muddy Mountains, Clark Co., NV
Ground Squirrel	1	MUD #2	867	Muddy Mountains, Clark Co., NV
Ground Squirrel	1	ORO #1	581	Orocopia Mountains, Riverside Co., CA
Ground Squirrel	1	ORO #2	821	Orocopia Mountains, Riverside Co., CA
Ground Squirrel	1	PMP #1	629	Phoenix Mountains Preserve, Maricopa Co., AZ
Ground Squirrel	1	PMP #3	793	Phoenix Mountains Preserve, Maricopa Co., AZ
Ground Squirrel	1	PMP #5	504	Phoenix Mountains Preserve, Maricopa Co., AZ
Ground Squirrel	1	PMP #6	478	Phoenix Mountains Preserve, Maricopa Co., AZ
Ground Squirrel	1	PMP #8	750	Phoenix Mountains Preserve, Maricopa Co., AZ
Ground Squirrel	1	PMP #10	715	Phoenix Mountains Preserve, Maricopa Co., AZ
Ground Squirrel	1	PMP #12	575	Phoenix Mountains Preserve, Maricopa Co., AZ
Ground Squirrel	1	PLO #1	598	Plomosa Mountains, La Paz Co., AZ
Ground Squirrel	1	SJ #3	757	San Jacinto Mountains, Riverside Co., CA
Ground Squirrel	1	SM #1	683	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	SM #2	570	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	SM #3	678	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	SM #6	737	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	SM #7	746	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	SM #8	491	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	SM #10	597	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	SM #13	685	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	SM #14/15	700	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	SM #18	602	South Mountain, Maricopa Co., AZ
Ground Squirrel	1	TEM #4	668	Temescal Mountains, Riverside Co., CA
Ground Squirrel	1	TEM #6	709	Temescal Mountains, Riverside Co., CA
Ground Squirrel	1	TA #8	502	Tinajas Altas Mountains, Yuma Co., AZ
Ground Squirrel	1	TA #9	572	Tinajas Altas Mountains, Yuma Co., AZ
Ground Squirrel	1	TA #21	658	Tinajas Altas Mountains, Yuma Co., AZ
Ground Squirrel	1	TA #23	443	Tinajas Altas Mountains, Yuma Co., AZ
Ground Squirrel	1	TA #24	567	Tinajas Altas Mountains, Yuma Co., AZ
Reptilia	13			
Aspidoscelis tigris	1	TA #?		Tinajas Altas Mountains, Yuma Co., AZ
Sceloporus magister	1	HAR #10	498	Harcuvar Mountains, La Paz Co., AZ
Sceloporus magister	1	PMP #2	413	Phoenix Mountains Preserve, Maricopa Co., AZ
Uta stansburiana	1	TA #?	?	Tinajas Altas Mountains, Yuma Co., AZ
Unidentified lizard	1	HAR #6	463	Harcuvar Mountains, La Paz Co., AZ
Unidentified lizard	1	LSB #5	843	Little San Bernardino Mountains, RIV Co., CA

Unidentified lizard	1	PMP #9	483	Phoenix Mountains Preserve, Maricopa Co., AZ
Unidentified lizard	1	PIS #2	398	Pisgah Crater, San Bernardino Co., CA
Unidentified lizard	1	PIS #7	349	Pisgah Crater, San Bernardino Co., CA
Unidentified lizard	1	PIS #8	830	Pisgah Crater, San Bernardino Co., CA
Unidentified lizard	1	SJ #7	488	San Jacinto Mountains, Riverside Co., CA
Unidentified lizard	1	SM #13	685	South Mountain, Maricopa Co., AZ
Unidentified lizard	1	TA #20	357	Tinajas Altas Mountains, Yuma Co., AZ
Totals	104			

Effect of Body size, Sex, and Biogeographic Region on Percent Endotherms in Diet

The logistic regression model with SVL, sex, and biogeographic region as predictors of prey class provided a significant fit (-2 Log Likelihood = 54.39, χ^2 = 14.44, df = 4, p = 0.006, Nagelkerke R^2 = 0.271, N = 98). The two predictors that successfully classified ectothermic vs. endothermic prey were SVL (p = 0.004) and biogeographic region (p = 0.039). The odds ratio for SVL (1.010, 95% CI 1.003–1.016) indicated that every 10-mm increase in SVL corresponded to a 10% increase in likelihood of consuming an endotherm (Fig. 1). Three additional models testing for two biogeographic regions at a time (i.e., pairwise comparisons) confirmed that snakes from California/Nevada consumed a lower proportion of endotherms (p = 0.039), though this was most apparent for the two smallest size classes. The original model correctly classified 91.8% of the cases, but was better at predicting endotherm prey (100%) than ectotherm prey (27.3%). The low classification success for ectotherms was likely due to two large individuals that consumed lizards: an 830-mm female from Pisgah lava flow in San Bernardino County, California, and an 843-mm male from Little San Bernardino Mountains in Riverside County, California.

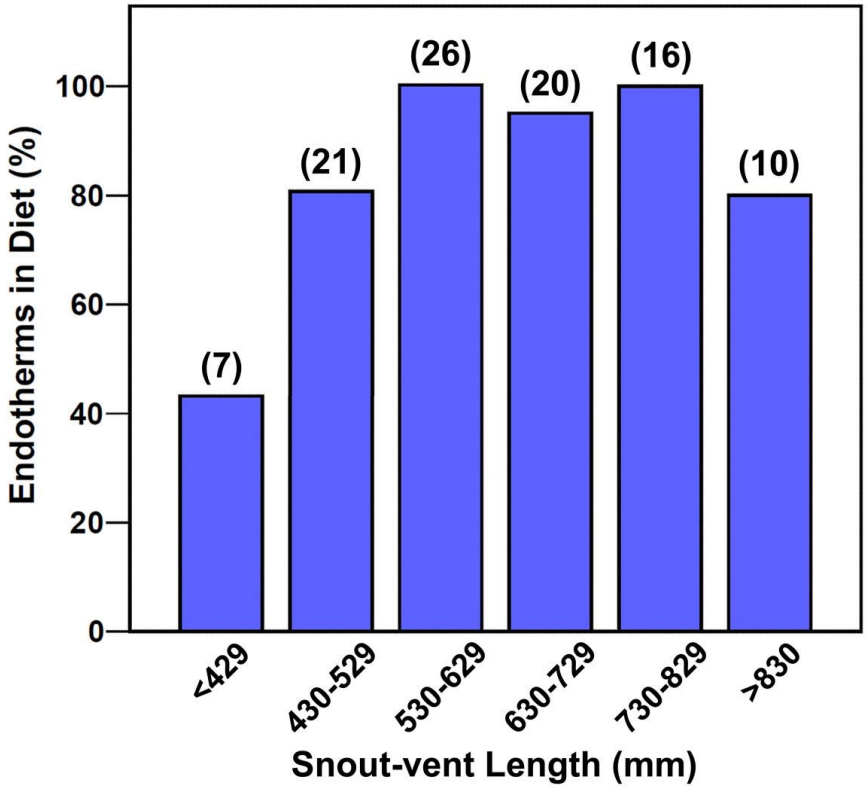

Figure 1. Percentage of Endotherms in the diet of various size classes of southwestern speckled rattlesnake (*Crotalus pyrrhus*). Sample sizes indicated within parentheses.

Frequency of Bird Consumption Among Geographic Regions

Snakes consuming birds (N = 5) vs. other prey (N = 63) were similar in body size (mean ± 1 S.E.: 625 ± 18 mm and 625 ± 51 mm, respectively; z = 0.059, asymptotic p = 0.91, r^2 < 0.0001, N = 5 and 63, respectively), and no difference in proportion of prey types existed between the sexes (males: 5.3% of 38 items were birds; females: 10.8% of 37 items were birds; χ^2 = 0.78, df = 1, asymptotic p = 0.38, Φ = 0.102; 50% of four cells with expected frequency <5). However, snakes from the TAM population consumed a much higher proportion of birds than snakes from other populations (23.8% of 21 prey

items and 2.4% of 83 prey items, respectively; $\chi^2 = 12.23$, df = 1, asymptotic $p < 0.001$, $\Phi = 0.343$, 25% of four cells with expected frequency <5 (Fig. 2)).

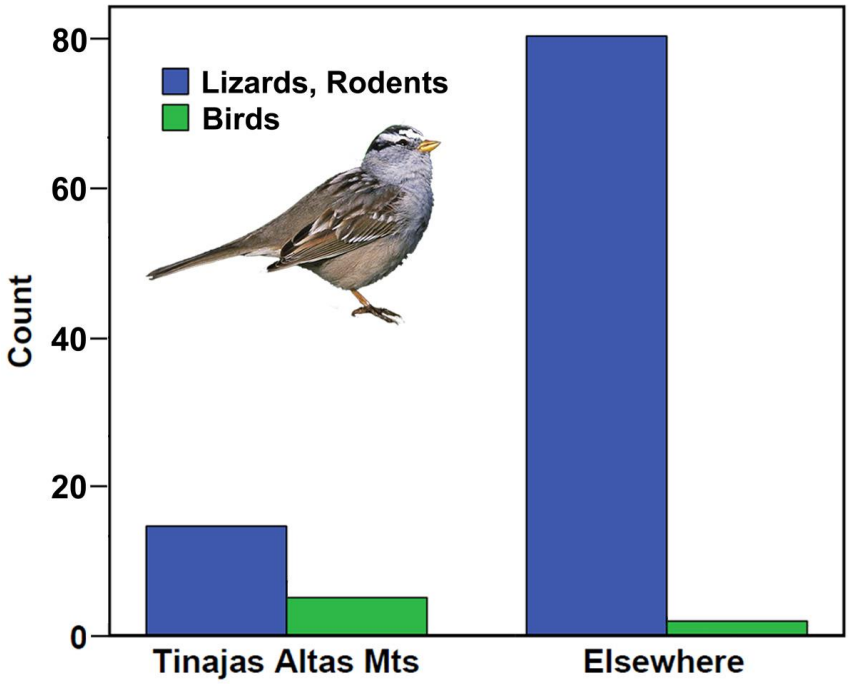

Figure 2. Proportion of birds recovered as dietary items in *Crotalus pyrrhus* from the Tinajas Altas Mountains, Yuma County, Arizona, and all other localities. Sparrow image: pngarts.com, license CC-BY-4.0.

Discussion

Results of this study supported three of our four of our hypotheses. For the first three regarding predation on ectotherms (reptiles) versus endotherms (birds, mammals), *C. pyrrhus* indeed exhibited an ontogenetic shift from ectothermic to endothermic prey and no difference existed between the sexes; however, geographic variation existed, as snakes from CA/NV consumed a lower proportion of endotherms than those from SDLCR. The difference between biogeographic regions was most apparent for the two smallest size classes, for which sample sizes were relatively small. By using logistic regression, we were able to test each of the three hypotheses simultaneously, which

strengthened our conclusions by minimizing potential sampling bias. As anticipated with our fourth hypothesis, the relatively small snakes from TAM (within SDLCR) that tend to exhibit arboreality (shrub/tree use) consumed a higher proportion of birds compared to those from other populations, which likely contributed to the dietary difference between CA/NV and SDLCR. Because of sparse cells resulting from the small number of birds consumed, we could not control for ontogenetic and sex differences simultaneously; however, univariate tests supported our expectation that bird consumption was independent of body size and sex.

Our data revealed that *C. pyrrhus* primarily predates on mammalian prey (80.8% of 104 prey items), supporting previously published anecdotal reports (Glaudas & Rodríguez-Robles, 2011; Klauber, 1936, 1997). Glaudas and Rodríguez-Robles (2011), citing unpublished data from museum specimens, reported that mammals comprised 65% of 77 prey items consumed by adult *C. mitchellii* sensu lato. Consider prey items taken from adults (SVL > 430 mm) in our study, the percentage of mammals rises slightly to 87.1% of 93 items. The high percentage of mammals in our overall sample is likely due to our sampling bias of adult snakes, as we also documented an ontogenetic shift in percent endotherms in the diet. Our finding of an ontogenetic shift from lizards to mammals supports previously published anecdotal reports for *C. pyrrhus* (Klauber, 1936, 1997). Terrestrial squirrels (39.4%) and the heteromyid rodent genus *Chaetodipus* (26.9%) constituted the primary prey items, which contrasts with Glaudas and Rodríguez-Robles (2011), who reported *Peromyscus crinitus*, a cricetid, as the most frequent prey species of *C. mitchellii* sensu lato. Differences between the two studies likely reflect regional and possibly temporal sampling differences. Though we were unable to assign

any of the squirrel remains to species, the majority were likely round-tailed ground squirrels (*Xerospermophilus tereticaudus*) and antelope ground squirrels (*Ammospermophilus* spp.), as suggested by hair length and observed species abundance at collection sites. Prior studies have documented *C. pyrrhus* predation on squirrels, including *A. leucurus* (Johnson, Bryant, and Miller (1948) and adult *Otospermophilus* spp. (Klauber, 1997). One of us (CC) was shown photographs of a large adult *C. pyrrhus* from the Santa Ana Mountains, Riverside County, California, swallowing an adult *O. beecheyi* (R. Carter, pers. comm.).

Mammal-dominated diets are common amongst medium- to large-bodied rattlesnake species, including: *C. abyssus* (Reed & Douglas, 2002), *C. atrox* (Beavers, 1976; Reynolds & Scott Jr, 1982), *C. catalinensis* (Avila-Villegas, Martins, & Arnaud, 2007), *C. durissus* (Salomão et al., 1995; Sant'Anna & Abe, 2007), *C. helleri* (Mackessy, 1988), *C. horridus* (Clark, 2002), *C. lutosus* (Diller & Johnson, 1988; Glaudas et al., 2008), *C. molossus* (Reynolds & Scott Jr, 1982), *C. oreganus* (Fitch & Twining, 1946; MacArtney, 1989; Wallace & Diller, 1990), *C. ruber* (Dugan & Hayes, 2012), and *C. scutulatus* (Reynolds & Scott Jr, 1982). This contrasts with greater reliance on ectothermic prey by smaller species, including *C. cerastes* (Webber et al., 2016), *C. concolor* (Mackessy et al., 2003; Parker & Anderson, 2007), *C. enyo* (Taylor & Price, 2001), *C. lepidus* (Beaupre, 1995; Holycross, Painter, Prival, et al., 2002), *C. pricei* (Prival, Goode, Swann, Schwalbe, & Schroff, 2002), and *C. willardi* (Holycross, Painter, Barker, et al., 2002; Mociño-Deloya, Setser, Heacker, & Peurach, 2015). Season influences dietary composition in *C. cerastes*, with snakes consuming a greater proportion of lizards during moderate temperatures of early spring and autumn, and a

greater percentage of mammals in the higher temperatures of late spring and summer when snakes shift to nocturnal activity (Webber et al., 2016).

Some rattlesnake species exhibit ontogenetic dietary shifts, with juveniles feeding primarily on ectotherms and transitioning to mammalian prey as adults (Glaudas et al., 2008; Holycross & Mackessy, 2002; LaBonte, 2008; Mackessy, 1988; Taylor & Price, 2001). Our data show *C. pyrrhus* follows this pattern, as juveniles (SVL ≤ 429 mm) consumed a higher percentage of ectothermic prey (57.1% of 7 items), but switched to primarily endothermic prey upon reaching reproductive size (SVL > 430 mm; Fig. 2). Ectotherms were not excluded as prey by even the largest size class (SVL ≥ 830 mm). Two individuals, an 830-mm female from the Pisgah lava flow in San Bernardino County, California, and an 843-mm male from the Little San Bernardino Mountains (LSBM) in Riverside County, California, consumed lizards. Based on lizard species observed at each site, we suspect these individuals predated relatively large iguanian lizards, either *D. dorsalis* (can exceed 60 g) (Gleeson & Harrison, 1988; Hazard, 2001) or *S. ater* (can exceed 200 g) (Lappin, Hamilton, & Sullivan, 2006) at Pisgah, and *D. dorsalis* at LSBM; however, scale characteristics do not rule out *Uta stansburiana*, or *Aspidoscelis tigris*, which are considerably smaller in size (<30 g) (Anderson & Karasov, 1988; Tinkle, 1967). *Crotalus pyrrhus* reportedly preys on both *D. dorsalis* (Lowe et al., 1986) and *Sauromalus* spp. (Klauber, 1997; Meik, Schaack, et al., 2012), though it remains unclear whether the former record represents a natural occurrence or captive observation. Adult *D. dorsalis* and *S. ater* possess considerably more mass than many mammalian prey species, confounding any interpretation using prey class as a proxy for prey size. Some *Crotalus* species appear to drop smaller prey species from their diet with

increasing size (Glaudas et al., 2008), whereas others do not (Clark, 2002; Dugan & Hayes, 2012; Mociño-Deloya et al., 2015; Sant'Anna & Abe, 2007; Webber et al., 2016).

Only *C. pyrrhus* from TAM appears to include birds as a substantial dietary component. Our result may be biased due to small samples sizes for many of the mountain ranges sampled, but is surprising for two reasons. The first relates to why bird consumption is most prominent in TAM snakes from SDLCR, the biogeographic region with snakes of the smallest body size. In two rattlesnake species, only the largest individuals consume birds (*C. cerastes*: Webber et al. (2016); *C. willardi obscurus*; Holycross, Painter, Barker, et al. (2002), but see exception in Mociño-Deloya et al. (2015)). The TAM snakes averaged larger (SVL: males = 569 mm, n = 14; females = 516 mm, n = 11; c.f. Meik (2016) than *C. cerastes* (442 and 472 mm, respectively; Reiserer (2001) and *C. w. obscurus* (486 and 456 mm, respectively; A. Holycross, pers. comm.)), with most individuals exceeding the size of *C. cerastes* (46.5 cm; Webber et al. (2016)) and *C. w. obscurus* (<350 mm SVL; Mociño-Deloya et al. (2015)) individuals that preyed on birds. Birds may also be an important part of the diet of the El Muerto Island, Baja California, Mexico, population of *C. pyrrhus* (Meik, 2016; Meik, Schaack, et al., 2012), which averages similar in size (517 mm; Meik et al. (2010)) to the TAM snakes. We found TAM snakes coiled in low bushes and trees more often than those from other localities (C. Cochran, pers. obs.), suggesting a foraging strategy specifically targeting birds. Comparative studies of prey availability could shed light on why TAM snakes rely to a greater extent on birds than snakes of larger size in other populations. The second surprising result is that we failed to document feathers in any fecal samples (N = 13) from the population in South Mountain Park/Preserve, Maricopa County, Arizona, where

numerous photos suggest frequent bird consumption (B. O'Connor, pers. comm.). Sampling at this location and at TAM (16 samples) occurred in both spring and fall, ruling out a temporal bias in explaining the contrasting diets.

The high percentage of diurnal terrestrial squirrels (*Ammosperophilus* spp., *Otospermophilus* spp., *Xerospermophilus tereticaudus*) in the diet of *C. pyrrhus* may reflect the species' ability to remain surface active, even in the summer months, at higher surface temperatures than other sympatric *Crotalus* species (C. Cochran, pers. obs.). When placed in outdoor enclosures, *C. pyrrhus* exhibited a higher preferred body temperature (31.2 C) than *C. cerastes* (25.8 C; R. G. Moore (1978)). Moore hypothesized that greater diurnal activity of *C. pyrrhus* and greater nocturnal activity of *C. cerastes* reduced competition between these two partially syntopic *Crotalus* species. Moore also listed the antelope ground squirrel (*Ammospermophilus leucurus*) as the only diurnal mammalian prey at a nearby site. Our results suggest that *C. pyrrhus* preys upon terrestrial ground squirrels during the entire active season, as we recorded squirrel remains in every month from March to September, including months Moore reported *C. pyrrhus* as being primarily nocturnal. Coupling temperature-sensitive radio-telemetry with increased fecal sampling in areas where *C. pyrrhus* is syntopic and allotopic with other rattlesnake species would inform whether interspecific competition shapes activity patterns, foraging tactics, and/or dietary differences.

In conclusion, our study of fecal material from live specimens captured at 23 locations suggests that diet in *C. pyrrhus* consists largely of rodents, though juveniles, like other rattlesnake species, consume a higher percentage of lizards. As opportunistic predators, species composition of the diet will depend on local prey availability, which

might explain the regional variation that existed in the proportion of ectotherms (lizards) and endotherms (birds, mammals) consumed. Most notably, the proportion of birds in the diet was surprisingly high in the TAM population; further study is needed to assess whether birds are disproportionately more available here, and whether the snakes preferentially target birds. Being based on recently-collected fecal samples, our findings portray a contemporary diet potentially influenced by a long-term regional drought. Diet could shift with changes in precipitation that might affect vegetation and the prey base. Collectively, our results provide baseline data for testing further hypotheses regarding potential interspecific competition, dietary changes associated with climate change, foraging tactics associated with bird predation, venom composition variation, and other factors associated with diet.

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CHAPTER FIVE

GEOGRAPHIC VARIATION OF VENOM COMPOSITION IN THE SOUTHWESTERN SPECKLED RATTLESNAKE (*CROTALUS PYRRHUS* (COPE, 1867)) ACROSS THE SOUTHWESTERN UNITED STATES

Chip Cochran[1], Wayne Kelln[1], Lance R. Pompe[1],
Guangyu Zhang, and William K. Hayes[1]

[1]Department of Earth and Biological Sciences, Loma Linda University, Loma Linda, California 92354 USA

[2]Loma Linda University Mass Spec Core Facility, Loma Linda, California 92354 USA

Abstract

Intraspecific geographic venom variation is well documented in rattlesnakes and snakes in general, and may result from selection, neutral evolutionary processes, or a combination of the two. The broad distribution of the southwestern speckled rattlesnake (*C. pyrrhus*) includes numerous isolated populations in desert mountain ranges, making it an ideal candidate for investigating intraspecific venom variation. We fractionated 151 venom samples from 23 localities within six biogeographic regions across western, Arizona, southern California, and extreme southern Nevada, USA, using reverse-phase high-pressure liquid chromatography. We then defined eight major chromatographic elution regions (ERs) and analyzed their relative protein content for geographic and sexual differences. Venom composition varied geographically in seven of the eight ERs, but most notably with snakes from the Mojave Desert possessing less protein content in ER5 (predominantly serine proteases, snake venom metalloproteinases [SVMPs], and C-type lectins) than other geographic regions. Venom composition also varied between the sexes in three of eight elution regions with the highest level of variation (i.e., the largest effect size), occurring in ER3 (predominately VEGF, PLA2, and SVMP), with males exhibiting higher levels than females. Discriminant analyses revealed a considerable distinction between venoms of eastern (Arizona) and western (California/Nevada) populations. We failed to detect Mojave toxin (or a homolog) in any of our populations. Our findings add to our understanding of venom variation in this widespread species, one that is likely to come into increased human contact as urbanization continues to encroach upon mountain ranges across the American southwest.

Introduction

Snake venoms are complex secretions, produced in a specialized gland, composed of various organic and inorganic compounds including enzymes, peptides, proteins, toxins, polyamines, and salts (Chippaux, Williams, & White, 1991; Fry, Roelants, et al., 2009; Mackessy, 2008). Venom is primarily a trophic adaptation in snakes facilitating prey acquisition (Barlow, Pook, Harrison, & Wüster, 2009; Fry et al., 2008; Li, Fry, & Kini, 2005; Richards, Barlow, & Wüster, 2012) however when forced to use venom in a defensive context, consequences to the offending animal, include morbidity and even mortality (Bush & Siedenburg, 1999; Gutiérrez, Theakston, & Warrell, 2006; Peterson, 2006; Warrell, 2010). Use of mass spectrometry (Calvete et al., 2011; Fry et al., 2002; Fry et al., 2003; Juárez, Sanz, & Calvete, 2004; Núñez et al., 2009) and transcriptomic analysis (Casewell, Harrison, Wüster, & Wagstaff, 2009; Fry et al., 2006; Fry, Vidal, Van der Weerd, Kochva, & Renjifo, 2009; Hargreaves, Swain, Logan, & Mulley, 2014; Reyes-Velasco et al., 2015; Rokyta, Wray, & Margres, 2013; Sunagar et al., 2014), in the field of "venomics" (Calvete, Juárez, & Sanz, 2007), have greatly facilitated and increased our understanding of venom composition and evolution.

Variation in snake venom composition occurs at all taxonomic levels (Chippaux et al., 1991), from broad scale family-level differences (Fry, 2005; Mackessy, 2010b), to interspecific variation within a given genus (Ainsworth et al., 2018; Queiroz, Pessoa, Portaro, Maria de Fátima, & Tambourgi, 2008), intraspecific variation among populations (Calvete et al., 2011; Forstner, Hilsenbeck, & Scudday, 1997; Jayanthi & Gowda, 1988; Saravia et al., 2002; Straight, Glenn, Wolt, & Wolfe, 1991; Sunagar et al., 2014), intrapopulational variation (Anaya, Rael, Lieb, Perez, & Salo, 1992; Margres et al., 2018;

Smiley-Walters, Farrell, & Gibbs, 2019), sexual variation (Daltry, Ponnudurai, et al., 1996; Daltry, Wüster, & Thorpe, 1996; Menezes, Furtado, Travaglia-Cardoso, Camargo, & Serrano, 2006), and even ontogenetic shifts within an individual (Andrade & Abe, 1999; Mackessy, 1988; Mackessy, Sixberry, Heyborne, & Fritts, 2006; Rokyta, Margres, Ward, & Sanchez, 2017; Zelanis, Travaglia-Cardoso, & De Fátima Domingues Furtado, 2008). Molecular mechanisms responsible for variation in venomes of even closely related species include single point mutations, variable regulation of gene transcription, RNA translation, and post-translational modifications (Casewell et al., 2014; Fox & Serrano, 2008; Whittington, Mason, & Rokyta, 2018).

Intraspecific geographic venom variation in snakes is well documented (Forstner et al., 1997; Minton & Weinstein, 1986; Strickland, Mason, Rokyta, & Parkinson, 2018; Sunagar et al., 2014). Some authors have argued venom diversity results from neutral evolutionary processes not subject to natural selection (Mebs, 2001; Sasa, 1999), whereas others have argued that strong natural selection has driven adaptation to particular prey species (Barlow et al., 2009; Daltry, Wüster, et al., 1996; Gangur et al., 2017; Jackson et al., 2016; Richards et al., 2012; Wüster, Daltry, & Thorpe, 1999). Recent work by Margres et al. (2018) provides evidence, in two of three North American pitviper species, for local selection providing a greater effect than migration rate on the spread of toxin alleles.

The southwestern speckled rattlesnake, *Crotalus pyrrhus* (Cope, 1867), is a medium-sized pitviper typically under a meter in length. The species ranges from extreme southwestern Utah and southern Nevada, south through western Arizona, southern California, and the northern panhandle portion of northwestern Sonora, into

Baja California Norte, where it can be found as far south as the Vizcaíno region (Campbell & Lamar, 2004; Grismer, 2002; Klauber, 1936). Populations are also present on various Gulf of California islands, including El Muerto and Smith (Campbell & Lamar, 2004; Grismer, 2002; Meik, Lawing, & Pires-daSilva, 2010; Meik et al., 2012; Meik, Streicher, Lawing, Flores-Villela, & Fujita, 2015). A saxicolous species, it is most commonly associated with the rugged and rocky terrain of the mountains of the Mojave and Sonoran Deserts, where it is rarely encountered in the adjacent desert flats (Campbell & Lamar, 2004; Klauber, 1936; Meik, 2016). *Crotalus pyrrhus* associates with a number of biotic communities defined by Brown and Lowe (1994), including the Lower Colorado River and Arizona Upland subdivisions of Sonoran Desertscrub, Mohave Desertscrub, Californian Chaparral, and Californian Coastal Scrub. The species also occurs locally in Interior Chaparral and Great Basin Conifer Woodland (Campbell & Lamar, 2004; Meik, 2016). It can even be found at the transition zone between Californian Chaparral and Sierran Montane Conifer Forest in the San Jacinto Mountains (C. Cochran, pers. observ.). As an ambush predator and dietary generalist, *C. pyrrhus* predates upon a diverse array of prey types across its distribution. Small mammals and lizards appear to make up the majority of its diet (Klauber, 1936, 1997a; Lowe, Schwalbe, & Johnson, 1986; Meik et al., 2012), though birds are also recorded with regularity (Klauber, 1936, 1997a; Meik, 2016; Meik et al., 2012; Miller & Stebbins, 1964; Chapter 4) and appear to be an important dietary component of the El Muerto island population (Meik, 2016).

The main objective of this study was to analyze geographic venom varation using SDS-PAGE, Multivariate Analysis of Variance (MANOVA) of elution regions, and

Discriminant Functions Analysis (DFA) among 23 populations of *C. pyrrhus* within six biogeographic regions across southern California, western Arizona, and southern Nevada.

Methods

Venom acquisition

We collected venom from 151 *C. pyrrhus* at 23 locations representing six major biogeographic regions (Brown & Lowe, 1994; Schoenherr, 2017) across the species' United States distributional range of Arizona, California, and Nevada. The biogeographic regions included the Mojave Desert, Transverse Ranges, Peninsular Ranges, and the Colorado Desert, Lower Colorado River, and Arizona Upland subdivisions of the Sonoran Desert (Fig 1). Collection locations ranged from 40 to 1640 m in elevation. All samples were from adult snakes (≥430 mm) to avoid potential confounding of geographic variation with ontogenetic variation in venom composition. We procured the samples via voluntary manual venom collection, in which snakes were allowed to bite through a Parafilm® M (Sigma-Aldrich, St. Louis, MO, USA) covered beaker. No massaging of the venom glands occurred. Crude venom was flash frozen in the field in a vapor shipper (Taylor-Wharton CX100, Mechanicsburg, PA, USA), lyophilized upon return to Loma Linda University (Loma Linda, CA, USA), and stored at -80°C until analysis.

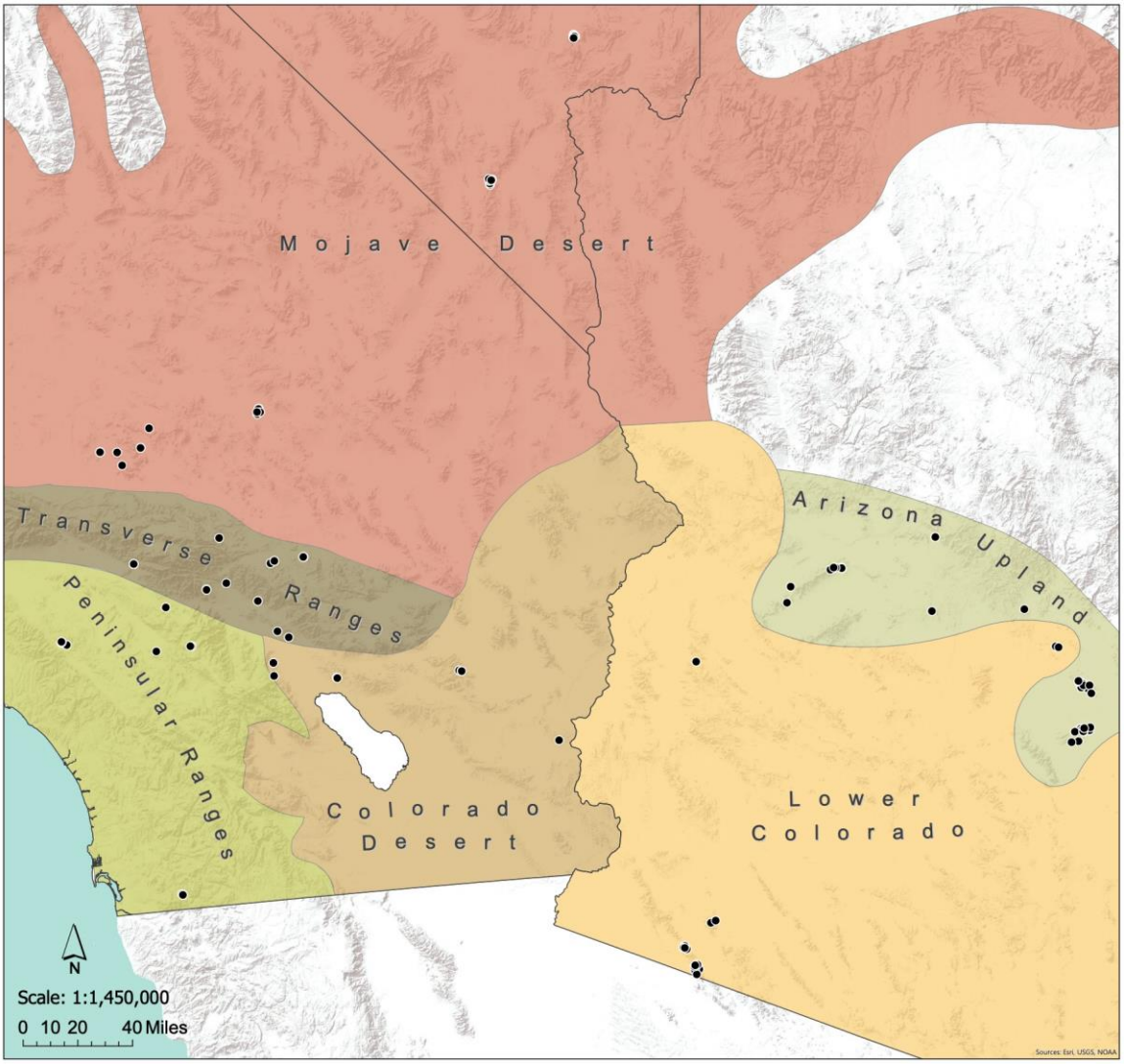

Figure 1. Collection sites for the 151 southwestern speckled rattlesnakes (*Crotalus pyrrhus*) sampled. Some locations had multiple samples. Biogeographic regions adapted from Brown & Lowe (1994) and Schoenherr (2017).

SDS-PAGE

We electrophoresed venom samples on NuPage® Bis-Tris Mini Gels under reduced conditions as suggested by the manufacturer (Invitrogen™, Carlsbad, California, USA), and using their reagents. Briefly, venom samples at a final concentration of 1 µg/µL were prepared in sample buffer containing 2.5 µL NuPage® LDS Sample Buffer (4X), 1 µL NuPage® Reducing Agent (10X) and 6.5 µL of deionized water for a total

volume of 10 µL. Samples were heated at 70°C for 10 min, and 10 µL was then added to each lane. We conducted electrophoresis using 700 mL of 1X SDS Running Buffer with 500 µL NuPage® Antioxidant added to the upper chamber for approximately 50 min, starting at 100–125 V for the first 10 min, and 60–80 V for the final 40 min. Gels were then stained with SimplyBlue™ SafeStain and then de-stained following the manufacturer's protocol with an additional overnight wash in 3.3% NaCl to increase band sensitivity. Gels were photographed using a BioSpectrum™ 500 Imaging System W/LMS-26 Transilluminator, (Analytikjena, Upland, California, USA). Qualitative comparisons were based on band presence/absence and relative band intensity of a given molecular mass class of proteins. We did not digitally quantify individual bands.

RP-HPLC, mass spectrometry, and venom composition differences

Following the methods outlined by Sunagar et al. (2014), we used reversed-phase high-pressure liquid chromatography (RP-HPLC), to fractionate each venom sample and generate a protein profile (chromatogram). We further subjected RP-HPLC fractions from pooled venoms of two populations (Tinajas Altas Mountains [TAM], Yuma County, Arizona, $N = 7$; Apple Valley, San Bernardino County, California, $N = 10$) to proteomic analysis (LC-MS/MS) to identify the toxins present. For mass spectrometry (LC-MS/MS) analysis, an Easy-nLC 1200 liquid chromatography system (Thermo Fisher, Waltham, MA) with an autosampler was attached to an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher, Waltham, MA). Injection of 7 µL of peptide samples in 0.1% FA was then passed through a 15cm home packed C18 column (200 A, 3 micron particle size, Magic). Samples were then eluted and separated on the column with a 2 hours gradient (5% ACN to 30% ACN in 0.1% FA). Collision-induced disassociation

was used to fragment the top 15 most abundant ions and the MS/MS spectra were collected between 350 and 1700 m/z following the parent full scan mass spectrum collected at 60,000 resolution for 2 hours.

Results, were used to establish eight arbitrary RP-HPLC elution regions (ERs, Fig. 2), each of which was primarily composed of proteins identified to 10 major toxins and toxin families (Table 1). For each chromatogram, we integrated the area under the absorbance trace to determine the percent of total protein for each ER (i.e., sum of the area of all peaks in each ER relative to total area). We did this after minor visual alignment of individual chromatograms. Because the resultant data are compositional in nature, we chose to analyze centered logratio-transformed (clr) data (Aitchison, 1986; Pawlowsky-Glahn & Egozcue, 2006). This transformation performs especially well for the multivariate tests we conducted, particularly since our data set was not plagued by outliers (Aitchison, 1986; Reimann, Filzmoser, Garrett, & Dutter, 2011). Isometric logratios (ilr) have certain properties that make them preferable for parametric tests, and would be an excellent alternative to clr, but the dimension of the data set is reduced by one during the transformation and the direct relation to the original variables is completely lost (Egozcue, Pawlowsky-Glahn, Mateu-Figueras, & Barcelo-Vidal, 2003; Reimann et al., 2011). Our data set did not contain zero values, therefore we did not have to replace any zero values using Aitchison's formula (Aitchison, 1986:266-267).

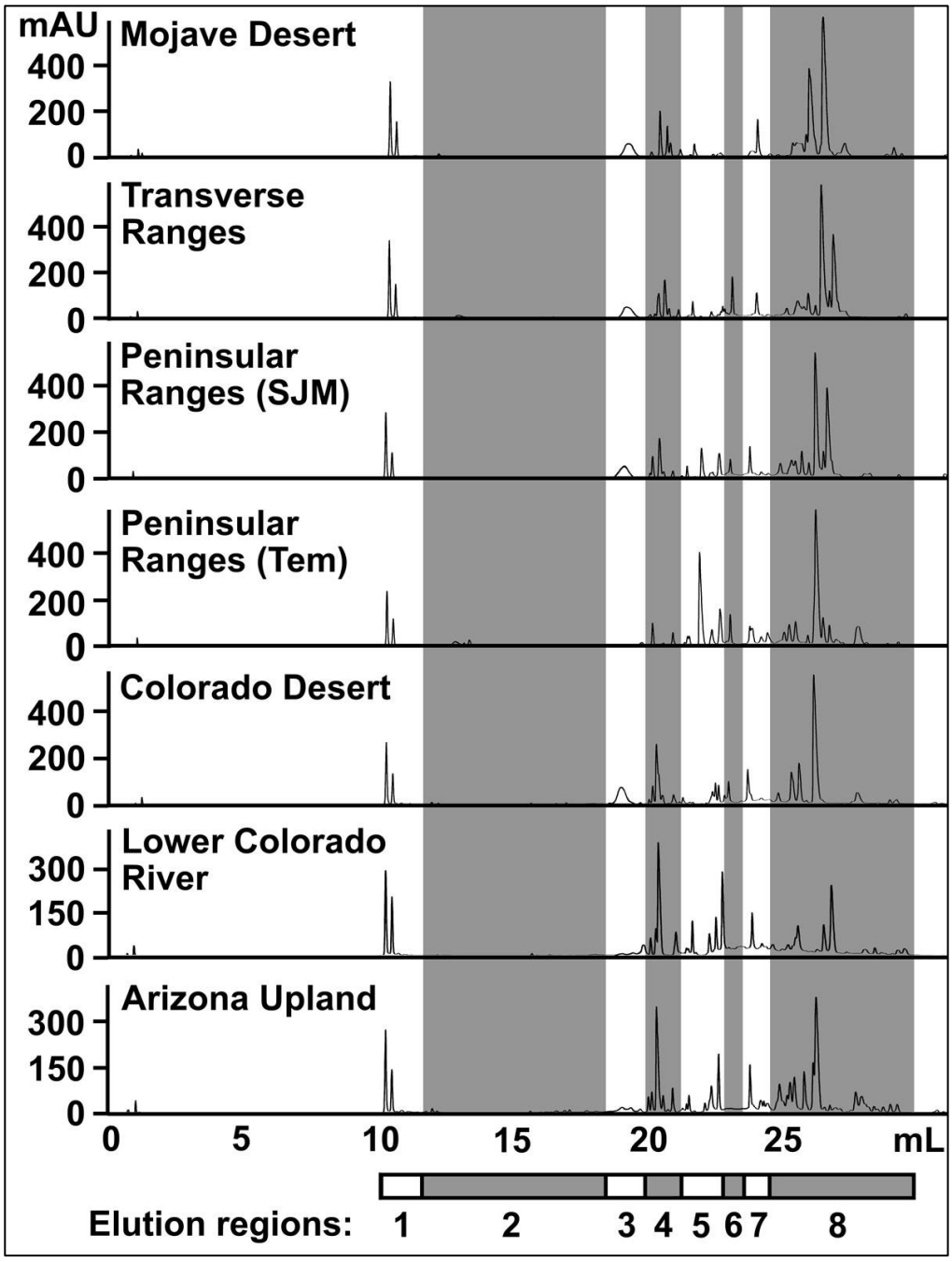

Figure 2. Representative RP-HPLC chromatograms illustrating geographic variation in the crude venom of southwestern speckled rattlesnakes (*Crotalus pyrrhus*). Unshaded and shaded regions illustrate the 8 elution regions (ER) analyzed statistically. Chromatograms are from individual snakes within each biogreographic region. SJM = San Jacinto Mountains, Tem = Temescal Mountains.

Table 1. RP-HPLC elution regions and the proteins and/or protein families[a] identified by mass spectrometry (LC-MS/MS) from peaks within these regions for southwestern speckled rattlesnake (*Crotalus pyrrhus*) pooled venom samples representing two populations.[b]

Elution Region	Primary Content	Tinajas Altas	Apple Valley
1 (9.9-12.0 mL)	Disintegrin	Disintegrin	Disintegrin, SVMP
2 (12.01-18.6 mL)	SVMP	SVMP, CNP	SVMP
3 (18.61-19.9 mL)	VEGF, PLA_2, SVMP	VEGF, PLA_2	PLA_2, SVMP
4 (19.91-20.7 mL)	PLA2, CRiSP	PLA_2, CRiSP	PLA_2
5 (20.71-22.7 mL)	SP, SVMP, CTL	SP	SVMP, CTL, SP
6 (22.71-23.7 mL)	CTL, SP, LAAO	CTL, SP, LAAO	CTL, LAAO
7 (23.71-24.7 mL)	LAAO, PDE	LAAO, CTL, PDE	LAAO, PDE
8 (24.71-32.0 mL)	SVMP, LAAO	SVMP, LAAO, CTL	SVMP, LAAO

[a] Toxin and toxin family abbreviations: CNP = C-type natriuretic peptides; CRiSP = cysteine-rich secretory proteins; CTL = C-type lectins; LAAO = L-amino acid oxidase; PDE = phosphodiesterase; PLA_2 = non-neurotoxic phospholipases A_2; SP = serine proteases; SVMP = snake venom metalloproteinases; VEGF = vascular endothelial growth factor.
[b] Locations in USA: Tinajas Altas Mountains, Yuma County, Arizona; Apple Valley, San Bernardino County, California.

Analyses

Prior to analyses, we screened the clr-transformed data to see whether they met univariate and multivariate assumptions of normality and homozygosity. Based on standard criteria for Mahalanobis distances (Mertler & Reinhart, 2010), we removed seven multivariate outliers (Mojave Desert, N = 3; Peninsular Ranges, N = 1; Arizona Upland, N = 3) after several iterations. The final dataset failed to fully meet parametric assumptions, in large part because some populations had one or several heterodox venom samples. The models we used are robust to departures from the assumptions (Tabachnick & Fidell, 2013).

We explored regional differences in venom composition at two levels via SPSS 20.0 for Macintosh (Statistical Package for the Social Sciences, Inc., Chicago, 2011), with

alpha set to 0.05. First, we used multivariate analysis of variance (MANOVA) and post-hoc univariate ANOVA models (Mertler & Reinhart, 2010) to compare the clr-transformed protein representation of each of the eight RP-HPLC ERs among the six biogeographic regions (Fig. 1) and between the sexes. Second, we used discriminant function analysis (DFA; Tabachnick & Fidell, 2013) to examine the extent to which overall venom composition varied in canonical space among the six biogeographic regions. This approach included seven of the eight ERs (ER 8 failed the tolerance test of multicollinearity) in a single analysis that allowed us to assess similarity among the biogeographic regions and to determine which elution regions provided the best discrimination. We conducted the DFA using SPSS program defaults and equal group sizes for prior probabilities. We also used leave-one-out classification, a jackknife procedure, to better accommodate classification bias arising from small samples (Lance, Kennedy, & Leberg, 2000) and to cross-validate accuracy of group assignments. Sample sizes for each geographic region ranged from 144 = 8—39.

To express effect sizes, we computed multivariate eta-squared (η^2) for MANOVAs, partial η^2 for post-hoc ANOVAs, and η^2 for the DFA (as 1 - Wilks' Λ), with values of ~0.01, ~0.06, and ≥ 0.14 loosely considered small, medium, and large effects, respectively (Cohen, 1988). These measures correspond to the approximate percentage of variance in the dependent variable that is explained by an independent variable or interaction. Following Nakagawa (2004), we chose not to adjust alpha for multiple tests because doing so overemphasizes the importance of null hypothesis testing when effect size is more meaningful, and unacceptably increases the probability of making type II errors (i.e., the hyper-Red Queen phenomenon: the more

research one does, the lower the probability that a significant result will be found; Moran (2003).

Results

Venom composition variation among geographic regions

Gel electrophoresis revealed differences in band intensity among various populations (Fig. 3). Mass spectrometry analyses of pooled samples from the TAM and AV populations showed that many elution peaks included several proteins from different toxin families, and toxins from the same toxin family sometimes appeared in multiple elution regions (Table 1). Thus, for most venom components there was no reliable correspondence between peak position and protein identity. We therefore analyzed of venom variation by elution region instead (c.f. Cooper, Fox, Nelsen, & Hayes, 2014; Gren et al., 2017).

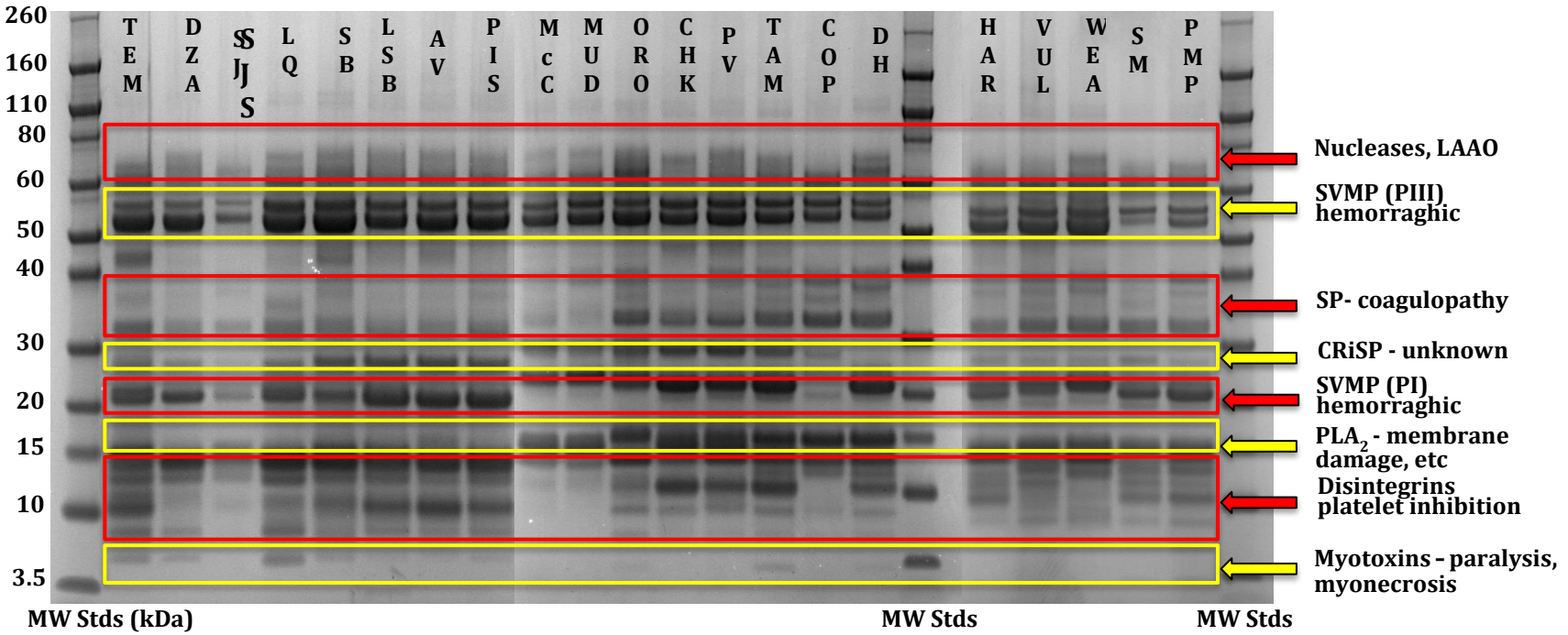

Figure 3. SDS-PAGE analysis (composite gel) of venoms from 23 populations of *Crotalus pyrrhus*. Relevant portions of individual gels were aligned using protein standards run on each gel. Approximate molecular masses (in kiloDaltons) are given on the left, and protein classes of major constituents, and their major actions, are given on the right. It is apparent that many proteins are shared between each population.

The MANOVA results (Table 2) indicated significant variation amongst biogeographic regions Fig. 4. Post-hoc univariate ANOVAs (Table 2) confirmed significant geographic variation for seven of the eight ERs, with protein representation of ER1 (primarily disintegrin) being similar across the range. Protein content of ER5 (predominantly serine proteases [SPs], snake venom metalloproteinases [SVMPs], and C-type lectins [CTLs]) showed the greatest variation (i.e., largest effect size), with snakes from Arizona having the highest levels and those from the Mojave Desert having the least. Protein content of ER2 (primarily SVMPs) was similar among most biogeographic regions, but California (and Nevada) snakes had higher levels than Arizona snakes, with those from the Peninsular Ranges having the highest. Protein content of ER3 (primarily vascular endothelial growth factor [VEGF], phospholipases A_2 [PLA_2], and SVMPs) exhibited a more complicated pattern: Mojave Desert snakes possessed the greatest quantity, followed by those of the Colorado Desert and Transverse Ranges, those of the Arizona Upland and Lower Colorado River, and finally those of the Peninsular Ranges, which had the lowest quantity. Protein content of ER4 (primarily PLA_2 and cysteine-rich secretory proteins (CRiSPs) showed a westward–eastward increase in protein content, with snakes of the Peninsular Ranges possessing the least and those of the Arizona Upland and Lower Colorado Desert having the most. Protein content of ER6 (primarily CTLs, SPs, L-amino acid oxidases [LAAOs]) was greatest in snakes from the Colorado Desert, followed by those of the Transverse Ranges, Mojave Desert, and Arizona regions, and Peninsular Ranges. Protein content of ER7 (primarily LAAOs and phosphodiesterases [PDEs]) was greatest in Arizona snakes, least in those of the Colorado Desert and intermediate for the remaining regions.

Table 2. Results from 6 × 2 (biogeographic region × sex) MANOVA (Wilks' Λ) and univariate ANOVAs (*F*) for protein content in RP-HPLC elution regions (ERs, defined in Table 1) of *Crotalus pyrrhus* venom. *N* = 151.

	Biogeographic Region			Sex			Interaction		
Model	**Λ or *F***	***P***	**η^2**	**Λ or *F***	***P***	**η^2**	**Λ or *F***	***P***	**η^2**
MANOVA	6.619	<0.001	0.262	4.220	<0.001	0.190	1.220	0.184	0.063
ER1	0.727	0.604	0.207	0.821	0.367	0.006	2.199	0.058	0.077
ER2	2.635	0.026	0.091	0.022	0.883	0.000	1.041	0.396	0.038
ER3	11.190	<0.001	0.298	9.841	0.002	0.069	1.591	0.167	0.057
ER4	13.221	<0.001	0.334	2.322	0.130	0.017	1.422	0.220	0.051
ER5	20.775	<0.001	0.440	14.899	0.000	0.101	0.976	0.435	0.036
ER6	5.308	<0.001	0.167	0.031	0.860	0.000	1.002	0.419	0.037
ER7	6.985	<0.001	0.209	2.944	0.089	0.022	0.362	0.873	0.014
ER8	3.033	0.013	0.103	4.504	0.036	0.033	1.783	0.121	0.063

Mean ± SE values portrayed in Fig. 3.
Effect sizes indicated as multivariate eta-squared (η^2) for MANOVA and partial η^2 for ANOVAs.

Protein content of ER8 (primarily SVMPs and LAAOs) varied significantly, with California snakes possessing greater quantities than California and Nevada specimens.

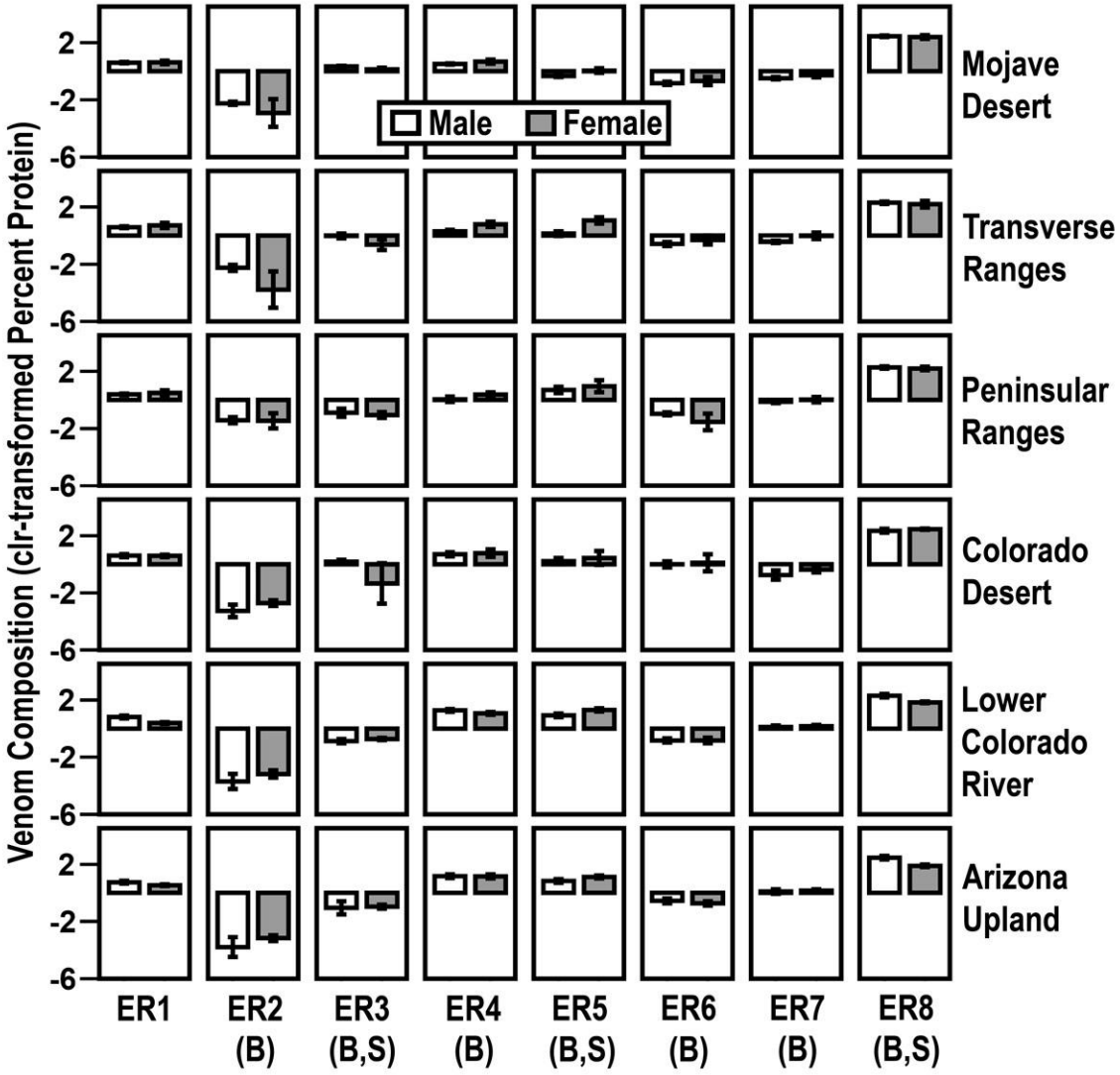

Figure 4. Geographic and sexual comparisons of clr-transformed percent protein for eight elution regions from RP-HPLC chromatograms of individual southwestern speckled rattlesnakes (*Crotalus pyrrhus*). Significant ANOVA effects are indicated parenthetically for biogeographic region (B) and sex (S); there were no interactions.

The DFA used to determine whether overall venom composition differed by biogeographic region generated a significant model (Wilks' $\Lambda = 0.216$, $X^2 = 209.281$, df = 35, N = 144, $P < 0.001$, $\eta^2 = 0.784$) with five discriminant functions. Separation of the populations on the first two functions are depicted in Fig. 5. The first function (63.0% of

variance) consisted largely of ER2 (standardized coefficient 1.29; predominately SVMP), ER4 (0.95; predominately PLA_2, and CRiSPs), and ER5 (0.84; predominantly SPs, SVMPs, and CTLs) all with positive loadings (i.e., positively related to the function), and separated snakes from the California and Nevada biogeographic regions (mostly negative scores) and Arizona biogeographic regions (mostly positive scores). The second function (24.3% of variance) was comprised largely of ER2 (2.11; predominantly SVMPs), ER4 (1.45; predominantely PLA_2 and CRiSPs), ER3 (1.17; predominately VEGF, PLA_2, and SVMPs) with positive loadings, and separated snakes of the Peninsular Ranges (with lower levels) from snakes of the other biogeographic regions. The difference in-group centroid for the Peninsular Ranges can be attributed largely to snakes from the Temescal Mountains area as the five lowest values were from this locality. Snakes from other bioregions clustered quite closely.

The DFA classification results indicated that 56.9% of the venom samples were assigned correctly to the original geographic region. Leave-one-out classification was less successful but still in good agreement at 45.8%, but still far greater than that expected from random for the six groups (16.7%). Classification results accuracy for each geographic region (with leave-one-out results in parentheses) was Mojave Desert 75.0% (75.0%), Transverse Ranges 50% (27.8%), Peninsular Ranges 56.3% (50%), Sonoran Desert Colorado Desert 50% (37.5%), Sonoran Desert Lower Colorado River 63% (48.1%), and Sonoran Desert Arizona Upland 41.0% (25.6%).

Venom composition variation between sexes

The MANOVA results (Table 2) confirmed venom variation between the sexes (Figure 4). Effect sizes indicated that sex explained almost as much variation as biogeographic region (multivariate $\eta^2 = 0.19$ and 0.23, respectively; Table 2). Absence of an interaction between biogeographic region and sex ($P = 0.19$, multivariate $\eta^2 = 0.06$) suggested that differences between the sexes were consistent across the species' range. Post-hoc univariate ANOVAs (Table 2) revealed significant differences for three of the eight ERs. Elution region three (predominately VEGF, PLA2, and SVMP) showed the highest level of variation (i.e., the largest effect size), with males exhibiting higher levels than females. Males also showed higher levels of protein content in ER8 (predominately SVMP, and LAAO) whereas females possessed more protein content in ER5 (predominately SP, SVMP, and CTL).

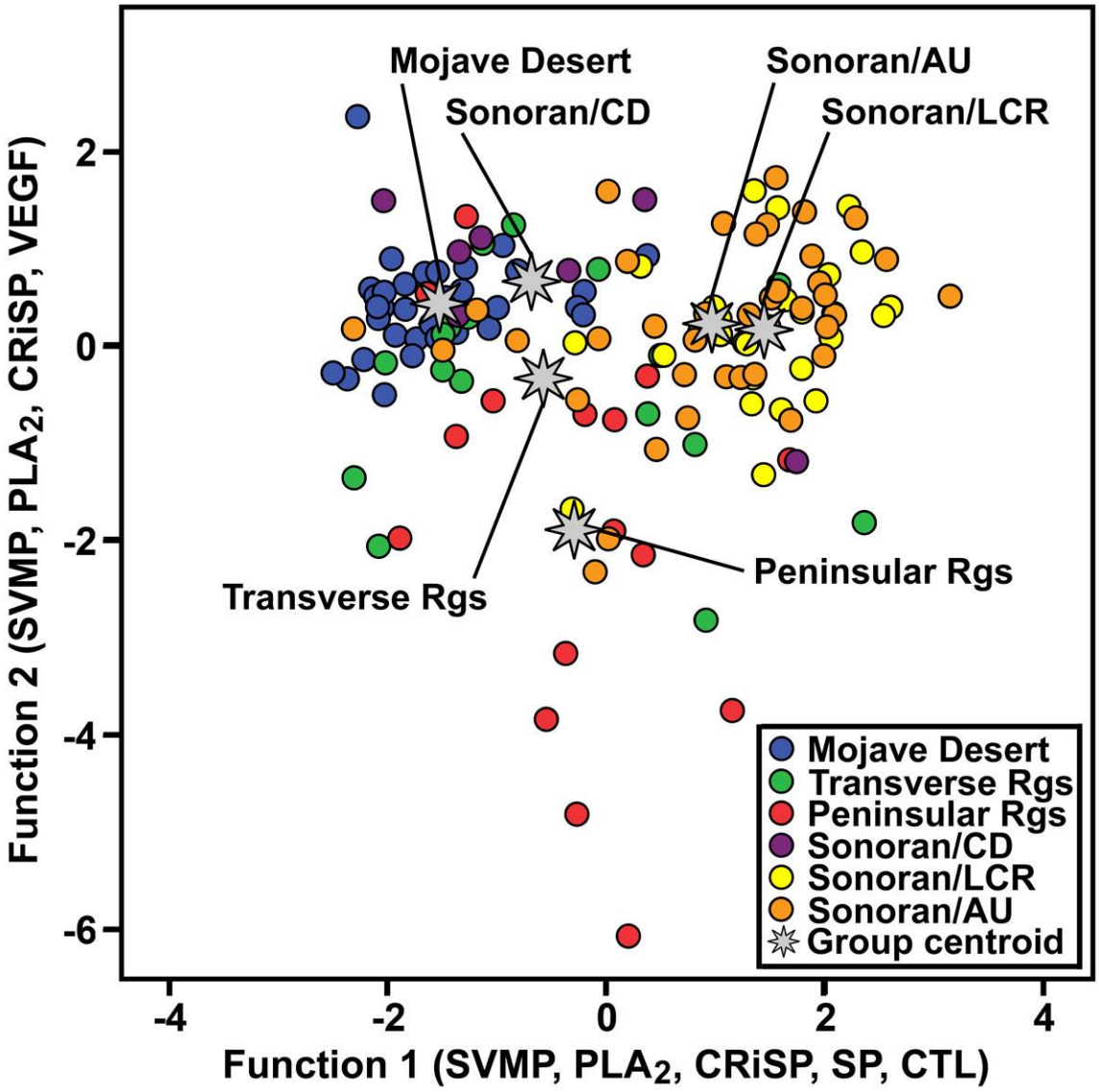

Figure 5. Canonical plot of discriminant scores for venom composition (clr-transformed area under the curve of 8 RP-HPLC elution regions) of individual southwestern speckled rattlesnakes (*Crotalus pyrrhus*) from six geographic regions depicted in Fig 1. Group centroids are shown as stars and labeled. The first function (63.0% of variance) consisted largely of ER2 (predominately SVMP), ER4 (predominately PLA_2, and CRiSPs), and ER5 (predominantly SPs, SVMPs, and CTLs) all with positive loadings (i.e., positively related to the function), and separated snakes from the California and Nevada biogeographic regions (mostly negative scores) and Arizona biogeographic regions (mostly positive scores). The second function (24.3% of variance) was comprised largely of ER2 (predominantly SVMPs), ER4 (predominantely PLA_2 and CRiSPs), ER3 (predominately VEGF, PLA_2, and SVMPs) with positive loadings, and separated snakes of the Peninsular Ranges (with lower levels) from snakes of the other biogeographic regions. The difference in-group centroid for the Peninsular Ranges can be attributed largely to snakes from the Temescal Mountains area as the five lowest values were from this locality. Snakes from other bioregions clustered quite closely.

Discussion

We used protein fractionation to examine intraspecific variation in the venom of a geographically widespread rattlesnake species characterized by numerous, well-isolated populations. We studied only adult snakes to avoid potential confounding of ontogenetic variation (Andrade & Abe, 1999; Mackessy, 1988; Mackessy et al., 2006; Rokyta et al., 2017; Zelanis et al., 2008), but we included both sexes to determine the extent to which differences in venom composition exist. Our results revealed geographic variation, as expected, but we found a surprising level of sexual variation as well in the relative abundance of numerous protein components in the venom of *C. pyrrhus*. Seven of the eight ERs varied geographically, whereas only three ERs differed between the sexes, but effect sizes (approximate proportion of variance explained) were fairly similar.

Our finding of geographically variable venom composition adds *C. pyrrhus* to the list of numerous other rattlesnake taxa studied to date for which geographic venom variation has been documented, including: *C. adamanteus* (Margres et al., 2014; Margres et al., 2015), *C. atrox* (Minton & Weinstein, 1986), *C. durissus* (Boldrini-Franca et al., 2010; Francischetti, Gombarovits, Valenzuela, Carlini, & Guimarães, 2000; Saravia et al., 2002), *C. helleri* (Gren et al., 2017; Sunagar et al., 2014), *C. helleri* and *C. oreganus* (Gren et al., 2017; Sunagar et al., 2014), *C. horridus* (J. Glenn, Straight, & Wolt, 1994), *C. lepidus* (Forstner et al., 1997), *C. scutulatus* (J. L. Glenn & Straight, 1978; J. L. Glenn & Straight, 1989; Massey et al., 2012; Strickland et al., 2018; Wilkinson, Glenn, Straight, & Sites Jr, 1991; Zancolli et al., 2019) and *C. simus* (Castro, Lomonte, del Carmen Gutiérrez, Alagón, & Gutiérrez, 2013). *Crotalus pyrrhus* overlaps in range with *C. atrox*, *C. helleri*, and *C. scutulatus*, but easily has the most disjunct population among them

because of its predilection for rocky montane regions separated by large areas of uninhabitable desert (Klauber, 1997a). In spite of this, *C. helleri* among this group clearly exhibits the highest levels of venom variation for reasons that remain to be understood (Gren et al., 2017; Sunagar et al., 2014). We initially thought *C. pyrrhus* would be a good candidate for high levels of venom composition variation, but that has proven not to be the case.

Discriminant analysis indicated a substantial distinction between venoms of eastern (Arizona) and western (California/Nevada) populations. Arizona snakes had consistently higher expression of proteins in ER2 (predominately SVMPs and CNP for TAM samples; SVMPs for AV samples), ER3 (predominately VEGF and PLA_2 for TAM; PLA_2 and SVMPs for AV), ER4 (predominately VEGF and PLA_2 for TAM; PLA_2 and SVMPs for AV), and ER5 (predominantly SP and PLA_2 for TAM; SVMPs, CTL, and SP for AV) compared to California and Nevada snakes. Considering what we know of genetic structure in *C. pyrrhus*: Douglas, Douglas, Schuett, and Porras (2006), using ATPase 8 and 6 mtDNA markers, recovered two clades: one from Arizona and southern Nevada, and another from southwestern California. Meik et al. (2015), again using ATPase 8 and 6, recovered three clades: one containing snakes from Mexico and a few southwestern California individuals, a second containing individuals from coastal southern California, and a third that included animals from Arizona, interior southern California, and Nevada. Our results are therefore interesting because they show that phylogenetic relatedness does not necessarily determine venom profile.

Discriminant analysis also separated snakes of the Peninsular Ranges from those of other groups at function 2, which corresponded to ER2 (2.05), ER4 (1.445), and ER3

(1.173). Difference in group centroid for the Peninsular Ranges can be attributed largely to snakes from the Temescal Mountains as the five lowest values were from this locality. Snakes from other Peninsular Range localities were very similar to those from other California/Nevada biogeographic regions.

Only ER1 (predominately disintegrins) exhibited an absence of geographic variation. Males and females likewise possessed similar levels of protein content in ER1. Disintegrins are important for prey relocation subsequent to envenomation and release, as they are the venom component that "chemically tags" the envenomated animal (Saviola, Chiszar, Busch, & Mackessy, 2013). Disintegrin content may be at levels optimized by selection, which could account for its lack of variation amongst the populations we studied.

We were surprised by the extent to which venom varied between the sexes of C. pyrrhus. Prior studies have reported small differences between the sexes in other species (Daltry, Ponnudurai, et al., 1996; Daltry, Wüster, et al., 1996; Menezes et al., 2006). However, no species studied to date ["that we are aware of"] exhibits intersexual variation at a level which nearly matches that of geographic variation in this species. We doubt the sexual differences in venom composition relate to diet because we found no sexual differences in prey species consumed (Chapter 4). We omitted body size from our analyses due to the few number of smaller individuals sampled and our desire to avoid a potentially confounding variable, but because diet of the species shifts from lizards to rodents as the snakes grow (Chapter 4), the venom differences observed might simply reflect ontogenetic differences in body size, as females average smaller in length than males (Chapter 3)

Geographic variation in protein content of ER8 (predominately SVMP, LAAO, and CTL) was positively correlated with geographic variation in body size (Chapter 3). This ER included SVMP as one of the dominant toxins, and this toxin family has previously been hypothesized as helping aide digestion of larger mammalian prey items with lower surface area-to-volume ratios (Mackessy, 2008, 2010a; Mackessy, Williams, Ashton, & Lannoo, 2003). Larger *C. pyrrhus* consume a greater proportion of mammalian prey (Chapter 2); thus, if SVMPs are indeed utilized in prey digestion, selection may be acting on gene regulation for increased expression in larger snakes.

We failed to find Mojave toxin in any of the 23 populations among the six biogeographic regions under study. Mojave toxin (and its various homologs) occurs in numerous rattlesnake taxa (French et al., 2004; Weinstein, Minton, & Wilde, 1985), and *C. mitchellii*, a close relative of *C. pyrrhus*, has tested positive against anti-Mojave toxin antibodies and possesses a very low LD_{50} (Arnaud-Franco et al., 2018; J. L. Glenn & Straight, 1985). Our inability to document neurotoxicity in *C. pyrrhus* does not imply that no population possesses the trait. A previous study, utilizing MTX-A and MTX-B primers, found 14 of 20 (70%) *C. pyrrhus* from Arizona and California, including some from areas we sampled, tested positive for the B subunit. Both subunits, however, are necessary for the production of Mojave toxin. Our inability to document Mojave toxin (or a homolog) in *C. pyrrhus* could be due to extreme localization of the trait (Sunagar et al., 2014). While the limited published information on *C. pyrrhus* envenomation does not provide evidence for neurotoxicity (Bush, Green, Moynihan, Hayes, & Cardwell, 2002; Hartnett, 1931; Klauber, 1997b), numerous locals around the town of Wickenburg, Maricopa County, Arizona, claim that *C. pyrrhus*, which they refer to as "Mojave Pinks"

is incredibly toxic and produces neurotoxic symptoms (C. Cochran, pers. obs.). While it is very likely that these are tall tales, the possibility of neurotoxic *C. pyrrhus* in the vicinity of Wickenburg cannot be completely dismissed. Our sampling from this locality was extremely limited (N = 3) and increased sampling from this area would prove valuable in confirming or refuting local's claims.

Our results add to the growing body of evidence that intraspecific geographic venom variation is the norm and not the exception. Whether or not the witnessed variation in *C. pyrrhus* is the result of selection, neutral evolutionary processes, or both we cannot currently say and is in need of future investigation.

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CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, I examined variation in envenomation outcomes, morphology, diet, and venom protein composition of the southwestern speckled rattlesnake (*Crotalus pyrrhus*) from 23 populations within six biogeographic regions across the species' United States range of Arizona, California, and Nevada. Together, these studies represent the most thorough investigation to date of these topics in the species. In this concluding chapter, I begin by revisiting the rationale by which my studies progressed; I will briefly review the principal conclusions drawn from each study; and I will suggest directions for future research.

Concept Development and Progression

My work originated with two friends who suffered envenomation by *C. pyrrhus* at different Arizona localities. The disparity in their outcomes, in regards to tissue destruction, led me to want to understand better the symptoms each suffered, which required an intimate look at venom composition. To better understand why venom varies, I needed to look at diet to see if the challenges of getting food, which can vary from one location to another as well as between the sexes and within an individual's lifetime, might somehow influence venom composition. To better understand how body size and sex influence diet and venom composition, I came to the realization that prior efforts to characterize size and shape in snakes have been flawed, and found a novel approach to better quantify morphological variation in these limbless creatures.

Conclusions from Individual Studies

In Chapter 2, I described two cases of envenoming by *C. pyrrhus* from two Arizona localities (Tinajas Altas Mountains, Yuma County, and Squaw Peak, Maricopa County). Both patients experienced swelling, but neither demonstrated coagulopathy, thrombocytopenia, or hypofibrinogenemia. The patient bitten on Squaw Peak developed hemorrhagic bullae and tissue damage in his bitten extremity, necessitating the amputation of the distal portion of his middle finger. The venome of the two populations, upon visual inspection of 1D-gels and RP-HPLC chromatograms, appeared to differ quantitatively between populations and among individuals within a population, but not qualitatively (i.e., similar toxins were present, but the proportions of some varied).

In Chapter 3, I examined geographic variation in the body size of *C. pyrrhus* across southern California, western Arizona, and southern Nevada to determine if sexual size dimorphism (SSD) and sexual body component dimorphism (SBCD) exist within the species. I demonstrated that overall body size varied significantly among six defined biogeographic regions, with the largest snakes occurring in the Mojave Desert, Transverse Ranges, and Peninsular Ranges of California and Nevada; the smallest snakes restricted to the Lower Colorado River subdivision of the Sonoran Desert; and snakes of intermediate size inhabiting the Colorado Desert (California) and Arizona Uplands subdivisions of the Sonoran Desert. Consistent with the findings of others, we also documented SSD, with males attaining a greater length than females; however, our novel approach using geometric mean and principle component 1 as unbiased measures of overall body size confirmed that SBCD also exists, with females possessing relatively longer trunks and bigger heads. Our finding of female-biased trunk length is consistent

with prior counts of trunk vertebrae in rattlesnakes and the hypothesis of fecundity selection favoring longer trunks in females.

In Chapter 4, I assessed the diet and possible influence of biogeographic region and ontogeny on prey class (ectotherms vs. endotherms) consumed. The diet of *C. pyrrhus* consisted predominantly of mammals (80.8%), in particular terrestrial squirrels (39.4%) and the heteromyid rodent genus *Chaetodipus* (26.9%). An ontogenetic shift occurred from primarily lizards to rodents, but neither biogeographic region nor sex had a significant impact on prey class consumed. However, the Tinajas Altas Mountains, Yuma County, Arizona population appears to rely on birds to a greater extent than other *C. pyrrhus* populations.

In Chapter 5, I examined the influence of biogeographic region and sex on protein composition in eight arbitrarily defined chromatographic elution regions. Venom samples from 151 adult snakes representing 23 populations across six biogeographic regions were subjected to proteomic and multivariate analyses to compare venom composition. Venom composition varied biogeographically, with snakes from the Mojave Desert possessing less protein content in elution region five (predominantly Serine Proteases, PLA2, SVMP, and C-type lectins) than other biogeographic regions. Discriminant analyses indicated a considerable distinction between venoms of eastern (Arizona) and western (California/Nevada) portions of the species' range. We failed to detect Mojave Toxin (or a homolog) in any of our populations.

Collectively, these studies provide meaningful insights on the sources of variation that potentially influence venom composition and clinical symptoms of envenomation in a model rattlesnake species.

Future Directions

My initial study reported on envenomations from two Arizona populations of *C. pyrrhus*. Unfortunately, both medical records lacked pertinent follow-up lab reports that would have proved informative in better understanding venom effects and platelet counts, as well as antivenom efficacy in stabilizing platelet counts. Neither case report had records for total vials of antivenom administered; however, I was able to obtain total vial number used from one patient (Squaw Peak). These shortcomings highlight the importance of much-needed standardization of record keeping in the event of snake envenomation so we can better understand symptoms and antivenom efficacy, while also allowing for future comparative studies among and within species.

My second study on sexual dimorphism invites reevaluation of the way researchers examine sexual dimorphism in various body components of snakes. To my knowledge, essentially all prior studies of snake dimorphism have relied on trunk length or snout-vent length (SVL), which are likely to be female-biased. In order to derive an unbiased measure of overall body size, I utilized the first eigenvalue of a principle component analysis (PC1), which often encompasses overall size, with the additional orthogonal components generally representing shape (e.g., Bookstein et al., 1985; Jolicoeur, 1963; Somers, 1986), and geometric mean of multiple measurements, which effectively removes shape to express overall size (Mosimann, 1970; Mosimann & James, 1979). The use of SVL (female-biased) as a covariate, in place of PC1 or geometric mean (unbiased), drastically changes the results of which sex possesses larger body components when it comes to head size and trunk length, and therefore changes our interpretation of how selection acts on these traits. Future research is also needed that

addresses the impact of environmental variables on *C. pyrrhus* body size, as the largely sympatric *C. atrox* is documented as being larger in cooler and wetter areas (Amarello et al., 2010). Future research on rattlesnake body size should also consider the effect of the presence of syntopic species. The Colorado Desert subdivision of the Sonoran Desert possessed *C. pyrrhus* larger than those of the neighboring Lower Colorado River subdivision of the Sonoran Desert. Environmental variables may differ between the two subdivisions and affect body size, but the syntopic and larger *C. molossus* is noticeably absent from the Colorado Desert (Campbell & Lamar, 2004; Stebbins, 2003). Other syntopic rattlesnake species do not appear to be associated with SVL in *C. pyrrhus*, as the longest specimens were found in the Peninsular Ranges (*C. helleri* and *C. ruber* co-occuring), Transverse Ranges (*C. helleri* and *C. ruber* co-occuring), and Mojave Desert (no truly syntopic rattlesnakes present), and *C. pyrrhus* attains a similar length in all three regions. Increased dietary sampling and telemetry studies comparing *C. pyrrhus* with syntopic rattlesnake species at sites within multiple biogeographic regions would prove useful for determining ecological overlap and potential interspecific competition (eg., Dugan, Figueroa, & Hayes, 2008; Nowak & Schuett, 2016). I hypothesize that *C. molossus* may have a more similar niche to *C. pyrrhus* than either *C. helleri* or *C. ruber*, and that part of the increase in *C. pyrrhus* body length may be due to ecological release when it is freed from competition with *C. molossus.* The montane rattlesnakes of Arizona's sky islands (*C. lepidus klauberi, C. pricei*, and *C. willardi*) would be an incredibly interesting system to investigate the effects of syntopic congeneric species on body size and venom profiles.

In my third study examining dietary variation, I found that *C. pyrrhus* incorporates diurnal ground squirrels as a major component of their diet. Future research should investigate whether this reliance on a diurnal rodent species is associated with behavioral shifts toward increased diurnal activity in comparison to other syntopic *Crotalus* species. Increased diurnal activity may have increased selection pressures for color/pattern matching of local substrate and driven the incredible color diversity seen in *C. pyrrhus* (Klauber, 1936), as camoflouge may benefit avoiding detection from diurnal predators and prey. Future research utilizing DNA barcoding of fecal samples and more intensive sampling of individual populations would prove highly informative for teasing apart variation at a finer scale than I was able to accomplish with my methods, and would be valuable for investigating the role in diet on venom evolution. I also found that the Tinajas Altas Mountains population relies more heavily on birds than other populations. Future research should investigate whether bird predation has led to other trophic adaptations in the population, including the evolution of bird specific toxins and longer fangs and other teeth to help penetrate feathers and hold onto volant prey that would surely be lost if released (Hayes, 1992; Knox & Jackson, 2010; Mackessy, Sixberry, Heyborne, & Fritts, 2006; Pawlak et al., 2006; Pawlak et al., 2009).

In my fourth study, examining geographic variation in venom composition, I found variation amongst the biogeographic regions. This variation, closely related to that previously reported in Chapter 3 on body size variation, was greatest between the Arizona populations syntopic with *C. molossus* and the California and Nevada populations which are not syntopic with *C. molossus (Campbell & Lamar, 2004; Stebbins, 2003)*. Future work should investigate whether interspecific competition among

syntopic congeneric species leads to character displacement in diet and venom profiles. In this system, Nevada population venom profiles resembled those of California animals (Chapter 5) even though the snakes are more closely related to Arizona animals (Douglas, Douglas, Schuett, & Porras, 2006; Meik, Streicher, Lawing, Flores-Villela, & Fujita, 2015). Once again, I believe the syntopic montane sky island species of *C. l. klauberi*, *C. pricei*, and *C. willardi* would be an ideal study system to examine potential outcomes of interspecific competition and character displacement.

My work with *C. pyrrhus* invites numerous follow-up studies involving both it and various closely related species. What selection pressures have led to remarkable color variation among *C. pyrrhus* populations? Are differences in body size and venom composition between *C. pyrrhus* of California and Nevada and those of Arizona a result of ecological release from competition with *C. molossus*? How do other syntopic rattlesnake species change in body size and venom profile when released from competition with congeners? Clearly more work needs to be done, and I propose that the montane sky island species of *C. l. klauberi*, *C. pricei*, and *C. willardi* may be the ideal study system for teasing apart the effects of syntopy on body size, body component size, diet, and venom profiles.

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