Geographic and Ontogenetic Variation of Venom in the Rattlesnakes Crotalus oreganus oreganus and Crotalus o. helleri

Eric Conrad Kyle Gren

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Geographic and Ontogenetic Variation of Venom in the Rattlesnakes *Crotalus oreganus oreganus* and *Crotalus o. helleri*

by

Eric Conrad Kyle Gren

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

March 2015
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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DEDICATION

To Papa Don, whose love for God, family, and life will always be warmly remembered.

To Mamma Jean, Grandpa K and Grandma K, who shared with me their love of exploring creation.

To Mr. Verlo, who helped me fall in love with biology and encouraged me to pursue my love of science.

To Mom and Dad, who have always loved me unconditionally and encouraged me to do what I love.

To Kandi, whose constant love and support are always there for me.
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I thank Wayne Kelln and Ben Gardner for their dedicated instruction in instrumental methods and theory. Our lab would not be where we are today without their tireless commitment.

I thank Carl Person and Gerad Fox for all their logistical support—from experimental design suggestions, to sample collection, to animal care—and for helping me remember the larger context of our research.

I thank Allen Cooper, David Nelsen, and Chip Cochran who, like Carl and Gerad, were always happy to share their insight and extensive literature knowledge to help improve my projects.
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ABBREVIATIONS

PLA$_2$  Phospholipase A$_2$
SVMP  Snake Venom Metalloproteinase
HPLC  High-pressure Liquid Chromatography
RP  Reversed-phase
CHCA  -Cyano-4-hydroxy Cinnamic Acid
Gln  Glutamine
Glu  Glutamic Acid
ANOVA  Analysis of Variance
$\eta^2$  Eta-squared
CRiSP  Cysteine-rich Secretory Protein
BPP  Bradykinin-potentiating Peptide
BIP  Bradikinin-inhibiting Peptide
MT  Mojave Toxin
3Ftx  Three-fingered Toxin
LC-MS  Liquid Chromatography-Mass Spectrometry
MALDI  Matrix-assisted Laser Desorption/Ionization
ToF  Time-of-flight
NP  Natriuretic Peptide
SBP  Small Basic Peptide
MTA  Mojave-toxin A
GF  Growth Factors
MTB  Mojave-toxin B
<table>
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<th>Description</th>
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<tr>
<td>SVSP</td>
<td>Snake Venom Serine Protease</td>
</tr>
<tr>
<td>LAAO</td>
<td>L-amino Acid Oxidase</td>
</tr>
<tr>
<td>CLR</td>
<td>Centered-log Ratio</td>
</tr>
<tr>
<td>PCA</td>
<td>Principle Component Analysis</td>
</tr>
<tr>
<td>cyt-b</td>
<td>Cytochrome-b</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>NDVI</td>
<td>Normalized Difference Vegetation Index</td>
</tr>
<tr>
<td>$t_{50}$</td>
<td>50% Twitch Strength</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>CCh</td>
<td>Carbachol</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>DFA</td>
<td>Discriminant Function Analysis</td>
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<tr>
<td>PC</td>
<td>Principle Component</td>
</tr>
<tr>
<td>Enviro PC</td>
<td>Environmental Principle Component</td>
</tr>
<tr>
<td>Veg PC</td>
<td>Vegetation Principle Component</td>
</tr>
<tr>
<td>ALA</td>
<td>Alameda County, California</td>
</tr>
<tr>
<td>STA</td>
<td>Stanislaus County, California</td>
</tr>
<tr>
<td>KER</td>
<td>Kern County, California</td>
</tr>
<tr>
<td>LA</td>
<td>Los Angeles County, California</td>
</tr>
<tr>
<td>SBD</td>
<td>San Bernardino County, California</td>
</tr>
<tr>
<td>RIV</td>
<td>Riverside County, California</td>
</tr>
<tr>
<td>SD</td>
<td>San Diego County, California</td>
</tr>
<tr>
<td>GeogDist</td>
<td>Geographic Distance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GenDist</td>
<td>Genetic Distance</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median Lethal Dose</td>
</tr>
<tr>
<td>LACM</td>
<td>Los Angeles County Museum of Natural History</td>
</tr>
<tr>
<td>SVL</td>
<td>Snout-vent Length</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ER</td>
<td>Elution Range</td>
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I investigated the extent of variation of venom protein composition (venome) and neurotoxicity in the Northern Pacific (Crotalus oreganus oreganus) and Southern Pacific (C. o. helleri) rattlesnakes, with special emphasis on geographic variation across the species’ California range. In the first of three empirical studies, I used reversed-phase liquid chromatography and mass spectrometry to examine the venome of four C. o. helleri populations. Substantial geographic variation existed, with small basic peptides (myotoxic β-defensins) expressed in large amounts by all populations, metalloproteinases abundant in two populations but only moderate and trace amounts in the other two populations, and a potent presynaptic neurotoxic phospholipase A2 present in just one population. The second study comprised a more thorough analysis of venome variation and neurotoxicity among C. oreganus individuals at 40 sampling locations across their California range. In this study, I tested for associations between venom composition and three potential factors that contribute to venome variation: geographic distance, genetic relatedness, and environmental variation. Again, venom profiles and neurotoxicity varied substantially across the species’ California distribution. Environmental variables had the
strongest and most consistent association with venom composition among statistical models, but geographic distance and genetic distance were also significant in several models. The third study examined diet and ontogeny as possible sources of venom variation in two populations of *C. o. helleri* having highly divergent venoms. For this study, I analyzed the gut contents and venomes of juvenile and adult snakes from a coastal population, Santa Catalina Island, and a high-elevation montane population, Mt. San Jacinto. I found no significant difference in diet composition (lizard versus mice) between the two populations. Differences in venom profiles between snake age classes were limited to three protein families in the Mt. San Jacinto populations and one in the Santa Catalina Island population, but effect sizes for overall ontogenetic change in venom was similar for the two populations. Collectively, these findings document the considerable extent of variation in venom composition and toxicity among populations within the species and support the emerging consensus that such variation is driven by a combination of environmental, geographic, and phylogenetic factors.
CHAPTER ONE
INTRODUCTION

Venom: Definition, Phylogenetic Distribution, and Functional Roles

Venom has been defined as any biologically-produced substance that causes pathophysiological change in the recipient and is delivered to the tissues (or cells) via mechanical injury (Nelsen et al., 2014). Mode of delivery distinguishes venom from other classes of biological toxins. Poisons, for example, must be introduced through ingestion, inhalation, or absorption. Since venom toxins are injected directly into the recipient’s tissue, their functionality is not constrained by molecular size as are poisons and toxungens, whose delivery depends on passive absorption (Nelsen et al., 2014). Thus, many venoms contain large proteins and enzymes with highly specific mechanisms of action.

Although venom use is widely recognized among many animal groups, organisms that meet the criteria for being venomous are widely represented among diverse life forms. Bacteriophages infect bacteria by attaching to the recipient cell's exterior and inject their genome into the host cytoplasm using a hypodermic needle-like apparatus (González-Huici, 2004; Rossman et al., 2004). Certain bacteria employ a needle-like structure called a type-III secretion system (T3SSs), which spans their inner and outer membranes and projects externally, to deliver their toxins into nematode, insect, plant, or animal targets. The T3SSs docks with target cell surfaces to form a conduit by which toxin may be delivered to the cytosol or cytosol surface of the target (Cornelis, 2010; Erhardt et al., 2010; Chatterjee et al., 2013). The ciliate Dileptus gigas discharges harpoon-like organelles called toxicysts when pursuing prey. These toxin-filled
projectiles rupture the victim’s cell membrane and expel their venom content, resulting in paralysis or death of the target (Visscher, 1923; Miller, 1968). Phytopathogenic fungi enter plants by using appressoria, which are specialized cells that form a minute peg and penetrate the cuticle of the plant via turgor pressure (Möbius and Hertweck, 2009). As the hyphae penetrate, they secrete toxins that destroy the plant’s cells to derive nutrition from the dead cells and to protect against the host’s defense (Möbius and Hertweck, 2009).

Plants from at least four families (Thurston and Lersten, 1969), including the *Urticaceae* (nettles), are equipped with sharply-pointed hair-like structures called trichomes. When an animal brushes against one of these plants, the hollow, hypodermic needle-like, silicified upper end of the trichomes penetrate and break off in the animal’s skin, releasing toxins including acetylcholine, histamine, and serotonin, which cause intense immediate pain (Kulze and Greaves, 1988), and oxalic acid and tartaric acid, which produce longer lasting pain (Fu et al., 2006), itching, tingling, burning, piloerection, arterial dilation, local sweating, rash development, and even neuropathy (Kulze and Greaves, 1988; Hurley, 2000; Hammond-Tooke et al., 2007; Schmitt et al., 2013).

The remarkable variety of delivery mechanisms and specific toxins employed by venomous taxa reflect the specific ecological needs of the various species. Venom typically functions for defense or prey acquisition. However, as Nelsen et al. (2014) discussed, venom may also be used for a range of other purposes. Male Duck-billed Platypuses (*Ornithorhyncus anatinus*), for example, use their toxins and delivery apparatus primarily for mate competition, using it against male conspecifics during mating and territorial disputes (Torres et al., 2000). Certain corals and anemones use venom for predation and defense, but also possess specialized tentacles to attack other
nearby colonies, thereby protecting and expanding their own territory against
intraspecific and interspecific competitors (Williams, 1991). In addition to the use of
venom for self and/or colony defense (generally by injection), some hymenopterans also
spray their "venom" to keep their broods free of parasites in the context of hygiene (Oi
and Pereira, 1993), and some ants spray the same secretion that is used as a venom for
trail marking in the context of communication (Blum, 1966; Mashaly et al. 2010).

**Venom Variation and Its Importance**

Because of the essential utility venom serves for organisms that employ it, its
composition is often finely tuned by selection to meet specific ecological needs. Widow
spider (*Latrodectus* spp.) venoms, for example, contain individual toxins specific to prey
and predator taxa, including several insect-specific "insectotoxins" (Rohou et al., 2007), a
crustacean-specific "crustatoxin" (Bettini, 1971), and a vertebrate-specific neurotoxin
(Frontali and Grasso, 1964). The venom of several species of *Echis* and *Vipera* snakes is
tailored to their arthropod diet, in contrast to congeners who primarily prey on mammals
and whose venom exhibits pronounced mammalian toxicity (Barlow et al., 2009).

Cone snails (genus *Conus*) provide perhaps the most dramatic illustration of the
interplay between venom composition and behavioral and ecological requirements.
Certain *Conus* species prey almost exclusively on fish. Because the snails are much
slower and less agile than their prey, their venom must act rapidly to incapacitate the fish
before their prey can escape. Such swift immobilization is achieved via small, highly
specific neurotoxic peptides which diffuse through the target tissue more efficiently than
the larger toxins common in other venoms. In addition to prey acquisition, cone snails
must also use their venom to defend against a wide variety of predators and competitors. Due to the extreme receptor specificity of conotoxins, individual toxins may vary widely in their effectiveness against various target taxa. Thus, to cope with the complex and shifting allospecific assemblages in their marine habitats, *Conus* species have evolved rapid hypermutation mechanisms to accelerate development of novel venom peptides (Olivera, 1997) and snails that feed on a broader spectrum of prey species exhibit more diverse toxin-encoding genes (Chang et al., 2015).

**Snake Venom Variation**

Snake venom composition varies widely among taxa. The factors that influence venom composition are complex, and exert their impact at various stages of venom production. At its most fundamental level, snake venom protein composition is first dependent on toxin-encoding genes within a snake’s genome. Until recently, these venom genes, often referred to collectively as the snake’s venome (Fry, 2005), were generally understood to have originated as duplicated, relatively non-toxic body genes that were subsequently recruited to the venom gland, whereupon they experienced neofunctionalization under natural selection to alter the toxicity and function of their products (Casewell et al., 2012, 2013; Fry et al., 2005, 2006, 2009, 2012; Fujimi et al., 2003; Ivanov, 1981; Ivanov and Ivanov, 1979; Kwong et al., 2009; Lynch, 2007; Margres et al., 2013; Vonk et al. 2013). This improbable scenario, however, has been challenged, with recent whole-body and comparative transcriptomic and genomic analyses suggesting, instead, that snake venom evolves via the duplication and subfunctionalization of genes encoding existing salivary proteins (Hargreaves et al., 2014; Reyes-Velasco et al., 2014). As a consequence of this process, venom profiles differ among lineages in ways that are
broadly characteristic of snake families and genera (Fry, 2005; Mackessy 2002, 2010a; Mackessy et al. 2006). Venom composition also varies among species, as well as intraspecifically among populations (Boldrini-França et al., 2010; Fry et al., 2002; Fry et al., 2003; Castro et al., 2013; Daltry et al., 1996a,b; Forstner et al., 1997; French et al., 2004; Mackessy, 2010b; Salazar et al., 2009; Wilkinson et al., 1991), between sexes (Daltry et al., 1996a,b; Menezes et al., 2006), and ontogenetically within individuals (Calvete et al., 2009b; Daltry et al., 1996a,b; Lopez-Lozano et al., 2002; Mackessy, 1988). Variation in the regulation of gene transcription and RNA translation, as well as post-translational modifications of gene products, generates considerable variation even among closely related snakes with similar venomes (e.g., Fox and Serrano, 2008; Casewell et al., 2014).

Intraspecific venom variation often correlates to geographic location, with members of a given population exhibiting greater venom similarity than individuals from more distant localities. Jiménez-Porras (1964) reported, for instance, that venom profiles of the Jumping Viper (Atropoides nummifer) from specific geographic localities were so distinct that venom composition could be used to predict where a venom sample was collected. The underlying causes of geographic variation in venom have been the subject of considerable debate. Daltry et al. (1996a,b, 1997, 1998) tested associations of geographic proximity, phylogenetic relatedness, and diet with venom composition in the Malayan Pitviper (Calloselasma rhodostoma), and found only diet to be significantly correlated with venom content. More recent studies support the interpretation that diet and habitat are major drivers of venom evolution (e.g., Chijiwa et al., 2000; Barlow et al., 2009). Mackessy et al. (2003) suggested that local climate may also influence venom
composition, with cool or highly variable climates conferring an adaptive advantage on snakes with venom components capable of accelerating meal digestion at lower temperatures. Others studies implicate phylogeny as the main source of venom variation (e.g., Jones, 1976; Boche et al., 1981; Gregory-Dwyer et al., 1986; Chippaux et al., 1991; Williams et al., 1988). The emerging consensus seems to be that phylogeny, geographic distance, environment, and other factors act in concert to influence venom composition. Even when diet seems to exert only minimal influence on venom, such as in the North American *Agkistrodon* pitviper complex (Lomonte, 2014), venom is increasingly viewed as a labile trophic adaptation shaped by ecological pressures.

**Biomedical Implications of Snake Venom Variation**

The toxins of snake venoms often pose serious health risks, so an improved understanding of venom composition and extent of variation has important implications for advances in the clinical treatment of envenomation. Perhaps equally important, the incredible spectrum of toxins also represents a treasure trove of biologically active compounds which perform their functions with incredible specificity and efficiency, and therefore offer enormous potential for novel pharmaceutical applications.

Venomous snakes possess several unique characteristics that make them excellent models for venom investigation by toxinologists. In contrast to many venomous animals, snakes generally secrete high volumes of venom. Although snake venom toxins vary widely in their toxicity—some being lethal even in tiny volumes while others are less so—the amount of venom typically injected, together with snakes’ frequent encounters with people, ensure that snake venom research has direct medical applications. Generous
venom yield has proven valuable in the laboratory, allowing convenient sample collection in sufficient quantities for multiple experiments, eliminating the need to pool samples from multiple extractions. The wide distribution of venomous snake species across a multitude of habitats and ecological niches has resulted in a diverse assortment of venoms, each varying both in their protein toxin components and in the relative abundance of individual toxins. The venoms produced by different snakes have a long and unique history of interacting with the physiology of other organisms, and therefore possess properties that can be tapped for biotechnology and pharmaceutical applications (Teichert and Olivera, 2010; Zhu et al., 2011, 2012; Vonk, 2012; Alves and Albuquerque, 2013). Thus, snake venoms can provide not only critical insights into the biological and ecological role of animal toxins and the factors that shape their evolution, but also can benefit humanity.

**Venom Variation among Rattlesnakes**

Rattlesnakes (genera *Crotalus* and *Sistrurus*) comprise the most studied group of all snakes (Beaman and Hayes, 2008). All of the 80 recognized taxa (Beaman and Hayes, 2008) possess relatively complex venom, with numerous toxins identified (Table 1). Many of these toxins exhibit pronounced geographic variation. Mojave toxin and its homologs, for example, are phospholipase A_2 (PLA_2) β-neurotoxins comprised of non-covalently linked heterodimers of acidic and basic subunits, which presynaptically block neuromuscular activity by inhibiting release of acetylcholine neurotransmitter. To date, this toxin has been described in a number of rattlesnakes species (Table 2). However, in a
Table 1. Common components of rattlesnake venoms and general characteristics (from Mackessy, 2008).

<table>
<thead>
<tr>
<th>Component Name</th>
<th>Approximate Mass (kDa)</th>
<th>Function</th>
<th>Biological Activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphodiesterase</td>
<td>94-140</td>
<td>Hydrolysis of nucleic acids and nucleotides</td>
<td>Depletion of cyclic, di- and tri-nucleotides; hypotension/shock (?)</td>
<td>Mackessy, 1998; Aird, 2002</td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>53-82</td>
<td>Hydrolysis of 5'-nucleotides</td>
<td>Nucleoside liberation</td>
<td>Rael, 1998; Aird, 2002</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>73</td>
<td>Hydrolysis of interstitial hyaluronan</td>
<td>Decreased interstitial viscosity – diffusion of venom components</td>
<td>Tu and Kudo, 2001</td>
</tr>
<tr>
<td>L-amino acid oxidase (homodimer)</td>
<td>85-150</td>
<td>Oxidative deamination of L-amino acids</td>
<td>Induction of apoptosis, cell damage</td>
<td>Tan, 1998</td>
</tr>
<tr>
<td><strong>Snake venom metalloproteases:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M12 reprolysins P-IV</td>
<td>48-85</td>
<td>Hydrolysis of many structural proteins, including basal lamina components, fibrinogen, etc.</td>
<td>Hemorrhage, myonecrosis, prey digestion</td>
<td>Fox and Serrano, 2005</td>
</tr>
<tr>
<td>P-III</td>
<td>43-60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-II</td>
<td>25-30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-I</td>
<td>20-24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serine proteases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin-like</td>
<td>31-36</td>
<td>Catalysis of fibrinogen hydrolysis</td>
<td>Rapid depletion of fibrinogen; hemostasis disruption</td>
<td>Markland, 1998; Swensen and Markland, 2005</td>
</tr>
<tr>
<td><strong>Kallikrein-like</strong></td>
<td>27-34</td>
<td>Release of bradykinin from HMW kininogen; hydrolysis of angiotensin Peptidase and esterase activity</td>
<td>Induces rapid fall in blood pressure; prey immobilization</td>
<td>Nikai and Komori, 1998</td>
</tr>
<tr>
<td><strong>“Arginine esterase”</strong></td>
<td>25-36</td>
<td></td>
<td></td>
<td>Schwartz and Bieber, 1985</td>
</tr>
<tr>
<td><strong>Non-enzymatic proteins/peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine-rich secretory proteins (CRiSPs)/helveprins</td>
<td>21-29</td>
<td>Possibly block cNTP-gated channels</td>
<td>Induced hypothermia; prey immobilization (?)</td>
<td>Yamazaki and Morita, 2004</td>
</tr>
<tr>
<td>PLA(_2)-based presynaptic neurotoxins (2 subunits, acidic and basic)</td>
<td>24</td>
<td>Blocks release of acetylcholine from axon terminus</td>
<td>Potent neurotoxicity; prey immobilization</td>
<td>Aird et al., 1985; Ducancel et al., 1988; Faure et al., 1994</td>
</tr>
<tr>
<td>C-type lectins</td>
<td>27-29</td>
<td>Binds to platelet and collagen receptor</td>
<td>Anticoagulant, platelet-modulator</td>
<td>Leduc and Bon, 1998</td>
</tr>
<tr>
<td>Category</td>
<td>Mass (kDa)</td>
<td>Function</td>
<td>Reference(s)</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------</td>
<td>--------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Disintegrins</td>
<td>5.2-15</td>
<td>Inhibit binding of integrins to receptors</td>
<td>Calvete et al., 2005</td>
<td></td>
</tr>
<tr>
<td>Myotoxins – non-PLA₂</td>
<td>4-5.3</td>
<td>Modifies voltage-sensitive Na channels; interacts with lipid membranes</td>
<td>Laure, 1975; Fox et al., 1979; Bieber and Nedelhov, 1997</td>
<td></td>
</tr>
<tr>
<td><strong>Smaller peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradykinin-potentiating peptides</td>
<td>1.0-1.5</td>
<td>Increases potency of bradykinin</td>
<td>Wermelinger et al., 2005</td>
<td></td>
</tr>
<tr>
<td>Tripeptide inhibitors</td>
<td>0.43-0.4</td>
<td>Inhibit venom metalloproteases and other enzymes</td>
<td>Francis and Kaiser, 1993; Munekiyo and Mackessy, 2005</td>
<td></td>
</tr>
<tr>
<td><strong>Smaller organic compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purines and pyrimidines (AMP, Hypoxanthine, Inosine)</td>
<td>AMP = 0.347</td>
<td>Broad effects on multiple cell types (?)</td>
<td>Aird, 2002, 2005</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>0.192</td>
<td>Inhibition of venom enzymes</td>
<td>Francis et al., 1992; Freitas et al., 1992</td>
<td></td>
</tr>
</tbody>
</table>

Mass in kilodaltons (kDa). Note that this list is not all-inclusive and that masses, functions, and activities do not apply to all compounds isolated from all rattlesnake venoms. Specific rattlesnake venoms may not contain all components. (?) indicates hypothetical function and/or activity.
Table 2. Taxonomic distribution of heterodimeric presynaptic neurotoxin complexes in venoms among rattlesnakes (from Werman, 2008). Taxonomic names conform to Campbell and Lamar (2004).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Neurotoxic complex</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Crotalus basiliscus</em></td>
<td>Crotoxin-like</td>
<td>Chen et al., 2004</td>
</tr>
<tr>
<td><em>Crotalus durissus cascaella</em></td>
<td>Crotoxin homolog</td>
<td>Santoro et. al. 1999</td>
</tr>
<tr>
<td><em>Crotalus durissus collilineatus</em></td>
<td>Crotoxin homolog</td>
<td>Santoro et al. 1999; Toyama et al., 2005</td>
</tr>
<tr>
<td><em>Crotalus durissus cumanensis</em></td>
<td>Crotoxin homolog</td>
<td>Chen et al. 2004</td>
</tr>
<tr>
<td><em>Crotalus durissus ruruima</em></td>
<td>Crotoxin homolog</td>
<td>Dos-Santos et al. 2005</td>
</tr>
<tr>
<td><em>Crotalus durissus terrificus</em></td>
<td>Crotoxin</td>
<td>Aird S. D. et al. 1985</td>
</tr>
<tr>
<td><em>Crotalus durissus vegrandis</em></td>
<td>Vegrandsis toxin</td>
<td>Kaiser and Aird, 1987</td>
</tr>
<tr>
<td><em>Crotalus horridus atricaudatus</em></td>
<td>Canebrake toxin</td>
<td>Hawgood, 1982</td>
</tr>
<tr>
<td><em>Crotalus lepidus klauberi</em></td>
<td>Mojave toxin</td>
<td>Rael et al., 1992; Powell et al., 2008</td>
</tr>
<tr>
<td><em>Crotalus mitchellii mitchellii</em></td>
<td>Crotoxin-like</td>
<td>Chen et al., 2004</td>
</tr>
<tr>
<td><em>Crotalus oreganus concolor</em></td>
<td>Concolor toxin</td>
<td>Pool and Bieber, 1981</td>
</tr>
<tr>
<td><em>Crotalus oreganus helleri</em></td>
<td>Mojave toxin</td>
<td>French et al., 2004</td>
</tr>
<tr>
<td><em>Crotalus scutulatus scutulatus</em></td>
<td>Mojave toxin</td>
<td>Hagwood, 1982; Wooldridge et al., 2001</td>
</tr>
<tr>
<td><em>Crotalus simus simus</em></td>
<td>Crotoxin homolog</td>
<td>Gutierrez et al. 1991; Chen et al., 2004</td>
</tr>
<tr>
<td><em>Crotalus tigris</em></td>
<td>Mojave toxin</td>
<td>Powell et al., 2004</td>
</tr>
<tr>
<td><em>Crotalus viridis viridis</em></td>
<td>Mojave toxin/Concolor toxin</td>
<td>Glenn and Straight, 1990</td>
</tr>
<tr>
<td><em>Sistrurus catenatus catenatus</em></td>
<td>Sistruxin</td>
<td>Sanz et al. 2006</td>
</tr>
<tr>
<td><em>Sistrurus catenatus tergeminus</em></td>
<td>Sistruxin</td>
<td>Chen et al., 2004; Sanz et al., 2006</td>
</tr>
</tbody>
</table>

number of taxa, expression of the toxin varies geographically. Mojave toxin is common among *C. scutulatus scutulatus* populations, for instance, but is absent in certain localities (Glenn et al., 1983; Glenn and Straight, 1989; Massey et al., 2012; Borja et al., 2014). By contrast, *C. o. helleri* and *C. viridis viridis* typically lack PLA$_2$ neurotoxins but have been documented to express them in localized populations (French et al., 2004; Glenn and Straight, 1990; Sunagar et al., 2014). In snake venoms, potent neurotoxicity and pronounced metalloproteolytic activity appear incompatible with one another. Snake venoms, then, can generally be classified as either predominately neurotoxic or
predominately proteolytic (Mackessy et al., 2003). The distribution of neurotoxic and proteolytic venoms shows no clear phylogenetic pattern in rattlesnakes and has therefore been interpreted as influenced mainly by local adaptation (Mackessy, 2008).

**Venom Variation in Pacific Rattlesnakes**

California populations of *C. oreganus* provide an excellent model for investigating environmental influences on intraspecific venom variation. The two subspecies share similar venom profiles that can be readily distinguished from those of other *crotaline* snakes. However, significant intraspecific venomic variation occurs among the various populations (Sunagar et al., 2014). The Northern Pacific Rattlesnake (*C. o. oreganus*) is found from the Canadian province of British Columbia south through Oregon and California to the Tehachappi Mountains of southern California. The Southern Pacific Rattlesnake (*C. o. helleri*) ranges from southern California, USA, southward into Baja California Norte, Mexico, and also occurs on the Pacific island of Santa Catalina (Los Angeles County, California; Klauber, 1997). The two taxa potentially overlap across a fairly narrow region along the northern edge of the Transverse Mountain Range (Klauber, 1997:52). Substantial environmental variation across the species’ range, likely the result of pronounced geologic activity in the region (see Schoenherr, 1992), results in marked habitat variation. Recent urbanization has fragmented the species’ distribution in some areas, potentially diminishing gene flow (c.f. Bolger et al., 1997; Riley et al., 2003). Pacific Rattlesnakes thrive in many of these habitat types, from sea level coastal valleys to grasslands, scrubby foothills, and montane forests up to 3000 m (Klauber, 1997).
As ecological generalists, *C. o. oreganus* and *C. o. helleri* utilize diverse prey types encountered across their distribution (Table 3). Pacific Rattlesnakes are also vulnerable to a wide range of antagonists (e.g., ungulates; Klauber, 1997) and predators (e.g., ophiophagous snakes, Roadrunners [*Geococcyx californianus*] and raptors [hawks and owls], mammalian carnivores; Klauber, 1997). Various animals

*Table 3. Reported prey of adult* *Crotalus oreganus oreganus* *and C. o. helleri.*

<table>
<thead>
<tr>
<th>Prey Type</th>
<th>Region Observed</th>
<th>Reported by</th>
<th>Preferred Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jerusalem Cricket</td>
<td>El Segundo, CA</td>
<td>Von Bloeker, 1942</td>
<td>Damp soils</td>
</tr>
<tr>
<td><strong>Amphibians</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western Spadefoot Toad (<em>Scaphiopus hammondii</em>)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Grassland, scrub, chaparral, oak woodlands</td>
</tr>
<tr>
<td><strong>Reptiles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western Skink (<em>Eumeces skiltonianus</em>)</td>
<td>Northern Idaho</td>
<td>Wallace &amp; Diller, 1990</td>
<td>Pinyon-juniper forest, grassland, desert shrub, rocks</td>
</tr>
<tr>
<td>Checkered Whiptail (<em>Cnemidophorus tesselatus</em>)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Arid woodlands, shrublands, grasslands</td>
</tr>
<tr>
<td>Gilbert's Skink (<em>Plestiodon gilberti</em>, formerly <em>Eumeces gilbert</em>)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Grassland, chaparral, open pine forest</td>
</tr>
<tr>
<td>Western Side-blotched Lizard (<em>Uta stansburiana</em>)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Arid and semi-arid well-drained scrubland</td>
</tr>
<tr>
<td>Western Fence Lizard (<em>Sceloporus occidentalis</em>)</td>
<td>Madera Co., CA; Los Angeles Co., CA</td>
<td>Fitch &amp; Twining, 1946; Cunningham, 1959</td>
<td>Grassland, chaparral, sagebrush, conifer</td>
</tr>
<tr>
<td>Unidentified lizard</td>
<td>Santa Catalina Island, CA; San Jacinto Mts., CA</td>
<td>Gren et al., 2014</td>
<td>------</td>
</tr>
<tr>
<td><strong>Birds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vesper Sparrow (<em>Passerculus sandwichensis</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989</td>
<td>Tundra, grassland, marsh, farmland</td>
</tr>
<tr>
<td>White-crowned Sparrow (<em>Zonotrichia leucophrys</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989</td>
<td>Mixed brush/grassland</td>
</tr>
<tr>
<td>American Robin (<em>Turdus migratorius</em>)</td>
<td>Pateros, Washington</td>
<td>Klauber, 1997</td>
<td>Forests, tundra, urban areas</td>
</tr>
<tr>
<td>Song Sparrow (<em>Melospiza melodia</em>)</td>
<td>Northern Idaho</td>
<td>Wallace &amp; Diller, 1990</td>
<td>Brushland, marsh, general</td>
</tr>
<tr>
<td>Unidentified bird eggs</td>
<td>Napa, California</td>
<td>Klauber, 1997</td>
<td>---</td>
</tr>
<tr>
<td>Doves (<em>Zenaida spp.</em>)</td>
<td>Arbuckle, CA; Pasadena, CA</td>
<td>Klauber, 1997</td>
<td>Open land with scattered cover</td>
</tr>
<tr>
<td>Species</td>
<td>Location</td>
<td>Authors</td>
<td>Habitat</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Downy Woodpecker (<em>Dryobates pubescens</em>)</td>
<td>Mariposa Co., CA</td>
<td>Cunningham, 1959</td>
<td>Forests, especially deciduous</td>
</tr>
<tr>
<td>California Quail (<em>Callipepla californica</em>, formerly <em>Lophortyx californica</em>)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Shrubland, open woodland</td>
</tr>
<tr>
<td>Canyon Towhee (<em>Melozona fusca</em>, formerly <em>Pipilo fuscus</em>)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Brushland, chaparral</td>
</tr>
<tr>
<td>Roadrunner (<em>Geococcyx californicus</em>)</td>
<td>Descanso, CA</td>
<td>Klauber, 1997</td>
<td>Deserts, scrublands</td>
</tr>
<tr>
<td><strong>Mammals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bushy-tailed Woodrat (<em>Neotoma cinerea</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989</td>
<td>Generalist</td>
</tr>
<tr>
<td>Cinereus Shrew (<em>Sorex cinereus</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989</td>
<td>Northern grassland and forests, tundra</td>
</tr>
<tr>
<td>Dusky Grouse (<em>Dendragapus obscurus</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989; Klauber, 1997</td>
<td>Conifer forest</td>
</tr>
<tr>
<td>Meadow Vole (<em>Microtus pennsylvanicus</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989</td>
<td>Northern grasslands and forests</td>
</tr>
<tr>
<td>Muskrat (<em>Ondatra zibethica</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989</td>
<td>Wetlands</td>
</tr>
<tr>
<td>Northwestern Chipmunk (<em>Eutamias amoena</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989</td>
<td>Mountain forests</td>
</tr>
<tr>
<td>Red Squirrel (<em>Tamiasciurus hudsonicus</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989</td>
<td>Conifer forest</td>
</tr>
<tr>
<td>Yellow-bellied Marmot (<em>Marmota flaviventris</em>)</td>
<td>British Columbia</td>
<td>Macartney, 1989</td>
<td>Steppes, meadows, talus, forests above 6,500 ft.</td>
</tr>
<tr>
<td>Northern Pocket Gopher (<em>Thomomys talpoides</em>)</td>
<td>British Columbia; N. Idaho</td>
<td>Macartney, 1989; Wallace &amp; Diller, 1990</td>
<td>Lowland and mountain riparian</td>
</tr>
<tr>
<td>Great Basin Pocket Mouse (<em>Perognathus parvus</em>)</td>
<td>British Columbia; SW Idaho</td>
<td>Macartney, 1989; Diller &amp; Johnson, 1988</td>
<td>Sagebrush, arid and semi-arid shrub and woods</td>
</tr>
<tr>
<td>Montane Vole (<em>Microtus montanus</em>)</td>
<td>British Columbia; SW Idaho</td>
<td>Macartney, 1989; Diller &amp; Johnson, 1988</td>
<td>High-elevation grassland, riparian, cropland</td>
</tr>
<tr>
<td>Deer Mouse (<em>Peromyscus maniculatus</em>)</td>
<td>British Columbia; N Idaho; SW Idaho; Madera Co., CA; Santa Catalina Island, CA</td>
<td>Macartney, 1989; Wallace &amp; Diller, 1990; Diller &amp; Johnson, 1988; Fitch &amp; Twining, 1946; Gren et al., 2014</td>
<td>Generalist</td>
</tr>
<tr>
<td>Mountain Cottontail (<em>Sylvilagus nutalli</em>)</td>
<td>N Idaho; SW Idaho</td>
<td>Diller &amp; Johnson, 1990</td>
<td>High-elevation sagebrush and forest</td>
</tr>
<tr>
<td>Harvest Mouse (<em>Reithrodontomys megalotis</em>)</td>
<td>Northern Idaho</td>
<td>Wallace &amp; Diller, 1990</td>
<td>Grasslands, riparian, marsh</td>
</tr>
<tr>
<td>Vagrant Shrew (<em>Sorex vagrans</em>)</td>
<td>Northern Idaho</td>
<td>Wallace &amp; Diller, 1990</td>
<td>Tundra, wet grassland, swamp, coniferous forest</td>
</tr>
<tr>
<td>Voles (<em>Microtus sp.</em>)</td>
<td>Northern Idaho</td>
<td>Wallace &amp; Diller, 1990</td>
<td>Various grasslands</td>
</tr>
<tr>
<td>Yellow-pine Chipmunk (<em>Tamia amoenus</em>)</td>
<td>Northern Idaho</td>
<td>Wallace &amp; Diller, 1990</td>
<td>Mixed conifer forest, shrub</td>
</tr>
<tr>
<td>House Mouse (<em>Mus musculus</em>)</td>
<td>Southwestern Idaho</td>
<td>Diller &amp; Johnson, 1988</td>
<td>Commensal with humans</td>
</tr>
<tr>
<td>Species</td>
<td>Location</td>
<td>Reference</td>
<td>Habitat</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>---------------------------------------</td>
<td>-----------------------------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>Ord Kangaroo Rat (Dipodomys ordii)</td>
<td>Southwestern Idaho</td>
<td>Diller &amp; Johnson, 1988</td>
<td>Semi-arid open shrubland, grassland, sagebrush</td>
</tr>
<tr>
<td>Townsend Ground Squirrel (Spermophilus townsendii)</td>
<td>Southwestern Idaho</td>
<td>Diller &amp; Johnson, 1988</td>
<td>Arid grassland, open shrubland</td>
</tr>
<tr>
<td>Snowshoe Hare (Lepus americanus)</td>
<td>Ukiah, Oregon</td>
<td>Klauber, 1997</td>
<td>Conifer and mixed forests</td>
</tr>
<tr>
<td>Pika (Ochotona princeps)</td>
<td>Mariposa Co.</td>
<td>Cunningham, 1959</td>
<td>Talus fields, usually high-elevation</td>
</tr>
<tr>
<td>Botta's Pocket Gopher (Thomomys bottae)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Woodland, chaparral, scrubland, agricultural</td>
</tr>
<tr>
<td>California Vole (Microtus californicus)</td>
<td>Madera Co., CA; San Jacinto Mts., CA</td>
<td>Fitch &amp; Twining, 1946; Gren et al., 2014</td>
<td>Various grasslands</td>
</tr>
<tr>
<td>Desert Cottontail (Sylvilagus auduboni)</td>
<td>Madera Co., CA; San Diego Co, CA</td>
<td>Fitch &amp; Twining, 1946; Klauber, 1997</td>
<td>Arid grassland, pinyon-juniper forest</td>
</tr>
<tr>
<td>Dusky-footed Woodrat (Neotoma fuscipes)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Dense chaparral, hardwood, conifer, riparian</td>
</tr>
<tr>
<td>Heermann's Kangaroo Rat (Dipodomys heermanni)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Dry gravel grasslands, open chaparral</td>
</tr>
<tr>
<td>Pocket mice (Perognathus sp.)</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>Arid grasslands</td>
</tr>
<tr>
<td>Unidentified mouse</td>
<td>Madera Co., CA</td>
<td>Fitch &amp; Twining, 1946</td>
<td>-----</td>
</tr>
<tr>
<td>California Ground Squirrel (Otospermophilus beecheyi, formerly Citellus beecheyi)</td>
<td>Madera Co., CA; San Bernardino Co., CA; Santa Catalina Island, CA</td>
<td>Fitch &amp; Twining, 1946; Cunningham, 1959; Gren et al., 2014</td>
<td>Open, well-drained grasslands</td>
</tr>
<tr>
<td>Brush Rabbit (Sylvilagus bachmani)</td>
<td>Los Angeles Co., CA; San Luis Obispo Co., CA</td>
<td>Cunningham, 1959; Klauber, 1997</td>
<td>Dense chaparral, aok, conifer, brush, grassland</td>
</tr>
<tr>
<td>Brown Rat (Rattus norvegicus)</td>
<td>Santa Catalina Island, CA</td>
<td>Gren et al., 2014</td>
<td>Commensal with humans</td>
</tr>
<tr>
<td>Ornate Shrew (Sorex ornatus)</td>
<td>Santa Catalina Island, CA</td>
<td>Gren et al., 2014</td>
<td>Marshes, uplands near water</td>
</tr>
<tr>
<td>Desert Kangaroo rat (Dipodomys deserti)</td>
<td>San Jacinto Mts., CA</td>
<td>Gren et al., 2014</td>
<td>Creosote, desert grasslands, cacti</td>
</tr>
<tr>
<td>Pinyon Mouse (Peromyscus truei)</td>
<td>San Jacinto Mts., CA</td>
<td>Gren et al., 2014</td>
<td>Rocky pinyon-juniper forest</td>
</tr>
<tr>
<td>California Shrew-mole (Neurotrichus gibbsi hyacinthinus)</td>
<td>Terminal Island, Los Angeles Co., CA</td>
<td>Klauber, 1997</td>
<td>Damp forests or brush with deep, loose soil</td>
</tr>
<tr>
<td>Trade Rat (Neotoma albigena)</td>
<td>Riverside Co., CA</td>
<td>Klauber, 1997</td>
<td>Scrubland, forests, and deserts</td>
</tr>
</tbody>
</table>

have been known to prey on rattlesnakes opportunistically, though none feed on rattlesnakes exclusively. Useful predation statistics are elusive but Klauber (1997) mentions rattlesnake predation by badgers, raptors, roadrunners, kingsnakes and racers,
peccaries, coyotes, foxes, raccoons, weasels, minks, bears, turkeys, ravens, and even fish. Klauber also reported rattlesnake mortality caused by deer, antelope, sheep, goats, horses, cattle, ground squirrels, rabbits, and woodpeckers. Taken together, their wide geographic distribution, diverse habitats, fragmented populations, varied prey and predator species encountered make *C. o. oreganus* and *C. o. helleri* excellent models in which to investigate the influences of geographic distance, phylogenetic relatedness, and environmental influences on venom variation.

Medical records along with field, and laboratory observations indicate that, whereas most *C. o. oreganus* and *C. o. helleri* venoms exhibit proteolytic toxicity, certain populations induce severe neurotoxic or myotoxic effects in mammals upon envenomation. French et al. (2004) reported the presence of Mojave toxin, a phospholipase A$_2$ (PLA$_2$) heterodimeric presynaptic β-neurotoxin first discovered in the venom of the Mojave rattlesnake (*C. scutulatus*), in the venom of several *C. o. helleri* using MT-specific antibodies. Neurotoxicity has also been noted after envenomation by snakes from populations shown not to express Mojave toxin, suggesting the presence of other neurotoxins that have yet to be identified (Bush and Siedenburg, 1999; French et al., 2004).

**Study Objectives**

The overall objectives of my research were: 1) to document the extent of geographic variation in venom composition among Pacific Rattlesnakes in California, and 2) to gain insight into factors contributing to the observed venom variation.
The purpose of my first study was to evaluate the extent of variation in venom protein expression among \textit{C. o. helleri} populations in southern California. Venom samples from insular, coastal valley, high desert, and alpine populations were separated by reversed-phase liquid chromatography and the constituent proteins identified by mass spectrometry. Both Mojave toxin subunits were expressed and metalloproteinases were conspicuously absent in venom samples from the mountain population. Conversely, metalloproteinases were secreted in large amounts in all other populations, while PLA$_2$ neurotoxins were not detected. Metalloproteinases were most diverse in the high desert samples while the island population exhibited lower complexity but a much higher relative expression level.

In my second study, I assessed the influence of phylogeny, geographic distance, and habitat on venom composition and neurotoxicity in \textit{C. o. oreganus} and \textit{C. o. helleri} across California. Venom samples from 24 populations were subjected to proteomic analysis as in the previous study and multivariate analyses used to compare venom composition with genetic relatedness, geographic distance, and environmental variables. Habitat showed the strongest associations with venom composition, followed by phylogeny, and geographic distance.

My third study sought to compare the influence of ontogeny and diet on protein composition of neurotoxic and proteolytic venom profiles in \textit{C. o. helleri}. We analyzed juvenile and adult \textit{C. o. helleri} venom from an insular population exhibiting typical \textit{C. o. helleri} venom profiles and an alpine population noted for its pronounced expression of neurotoxins and low metalloproteinase content. We also examined stomach contents and fecal matter from juvenile and adult snakes from each locality to identify consumed prey.
types. Venom composition differed substantially between populations. Snakes in both populations also showed fairly substantial changes in venom composition during ontogeny, with similar effect sizes. Snakes from both populations consumed mainly lizards when young and incorporated more rodents as adults.
References


Glenn JL, Straight RC. Venom characteristics as an indicator of hybridization between Crotalus viridis viridis and Crotalus scutulatus scutulatus in New Mexico. Toxicon 28(7):857–862.


CHAPTER TWO

GEOGRAPHIC VARIATION OF VENOM PROTEIN COMPOSITION IN THE SOUTHERN PACIFIC RATTLESNAKE (CROTALUS OREGANUS HELLERI)

This report is adapted and modified from the following published paper:


This report includes only the portions of the paper that I contributed to, and provides a much expanded Introduction and Discussion

Abstract

Due to substantial toxin variation in the venom of the Southern Pacific Rattlesnake (Crotalus oreganus helleri), management and treatment of envenomation by this species, one of the most medically significant snakes in all of North America, has been a clinical challenge. This taxon has also been the subject of sensational news stories regarding supposed rapid (within the last few decades) evolution of its venom toward supertoxicity. Research reported in this chapter demonstrates for the first time that variable evolutionary selective pressures have sculpted the intraspecific molecular diversity of C. o. helleri venom components. We show that myotoxic β-defensin peptides (i.e., crotamines and small basic myotoxic peptides) are secreted in large amounts by all four examined populations. Hemorrhagic and tissue-destroying snake venom metalloproteinases (SVMPs) were secreted in large amounts by the Catalina Island and Phelan rattlesnake populations, in moderate amounts by snakes in the Loma Linda
population and in only trace levels by the Idyllwild population. Only the Idyllwild population in the San Jacinto Mountains contained a potent presynaptic neurotoxic phospholipase A$_2$ complex characteristic of certain populations of the Mohave Rattlesnake (C. scutulatus) and Neotropical Rattlesnake (C. durissus terrificus).

We not only highlight the tremendous biochemical diversity in the venom-arsenal of C. o. helleri, but we also show that it experiences remarkably variable strengths of evolutionary selective pressures, influencing gene expression within each toxin class among populations and among toxin classes within each population. Mapping of geographical venom variation not only provides additional information regarding venom evolution, but also has direct medical implications by allowing prediction of the clinical effects of rattlesnake bites from different regions. Such information also points to these highly variable venoms as a rich source of novel toxins, which may ultimately prove useful in drug design and development.

**Introduction**

Snake venoms are complex secretions composed of various enzymes, toxins, peptides, small organic molecules, and inorganic compounds that elicit diverse physiological effects upon envenomation (Chippaux et al., 1991; Anaya et al., 1992; Heatwole et al., 1995; Chiszar et al., 1999; Bush et al., 2002; Mackessy and Baxter, 2006; Owings and Coss, 2008; Jansa and Voss, 2011; Casewell et al., 2012; Fry et al., 2012; Massey et al., 2012) facilitating both prey acquisition and snake defense (Anaya et al., 1992; Bush et al., 2002; Casewell et al., 2012; Fry et al., 2012; Mackessy and Baxter, 2006). Recent technological and methodological innovations, particularly in mass
spectrometry (Fry et al., 2002; Fry et al., 2003a,b; Calvete et al., 2007a,b, 2009a,b; Georgieva et al., 2008; Gutierrez et al., 2008; Gutierrez et al., 2009; Boldrini-França et al., 2010; Calvete, 2010; Calvete, 2011; Calvete et al., 2012; Ali et al., 2013) and transcriptomics (Ching et al., 2006; Fry et al., 2006, 2009, 2010, 2012, 2013; Wagstaff et al., 2006, 2009; Casewell et al., 2009; Rokyta et al., 2012, 2013) have significantly accelerated investigation in the field of animal venomics. As our knowledge of venoms has increased, so has our appreciation for their incredible complexity and variation.

Venom variation occurs among snake species within the same genus (van der Weyden et al., 2000; Fry et al., 2002, 2003, 2008; Sanz et al., 2006; Calvete et al., 2007; Gutierrez et al., 2008; Angulo et al., 2008; Lomonte et al., 2008; Tashima et al., 2008; Wagstaff et al., 2009) and among individuals within the same species, with intraspecific differences found among geographic locales (Wilkinson et al., 1991; Daltry et al., 1996a,b; Forstner et al., 1997; Fry et al., 2002, 2003; French et al., 2004; Salazar et al., 2009; Boldrini-França et al., 2010; Mackessy, 2010; Castro et al., 2013), between sexes (Daltry et al., 1996a,b; Menezes et al., 2006), among age groups (Mackessy, 1988; Daltry et al., 1996a,b; Lopez-Lozano et al., 2002; Calvete et al., 2009b), and even between the venom glands of a single individual (Johnson et al., 1987).

Much of the venom variation observed within species or subspecies is associated with geographic location. Members of a given population generally exhibit a higher degree of venom similarity than individuals from distant localities. Jiménez-Porras (1964) reported, for example, that venom profiles of the Jumping Viper (*Atropoides nummifera*) from specific geographic localities were so distinct that venom composition could be used to identify where a venom sample was collected. Studies such as those by
Tsai et al. (2004) and Creer et al. (2003) suggest that major compositional variation of venom among populations may be the result of environmental selection pressures, such as geological isolation and prey ecology, or of discrete founder effects rather than simply through neutral genetic mutations.

Chippaux et al. (1991) argued that geographic variation drives venom evolution based on at least two distinct situations. The first occurs among nearby or sympatric populations and is typified by a divergent population of Mojave Rattlesnakes (Crotalus scutulatus scutulatus) in northeast Arizona. This population’s venom exhibits unexpectedly high LD$_{50}$ values (low toxicity) in the absence of any other discernable morphological differences (Glenn and Straight, 1978; Glenn et al., 1983; Rael et al., 1984). The difference in toxicity was due to the absence of Mojave toxin, a pre-synaptic divalent neurotoxin found through much of the remaining range of the species. Evidence suggests that the neurotoxic and non-neurotoxic populations were once reproductively isolated, but are now free to interbreed and are forming an intergrade zone (Glenn and Straight, 1989). Chippaux mentioned a similar pattern of variability of Mojave toxin expression seen in sympatric Western Diamondback (Crotalus atrox) populations, suggesting a possible common ancestor of C. scutulatus and C. atrox followed by subsequent parallel evolution of the venome under similar evolutionary pressures (Chippaux et al., 1991), though subsequent studies indicated that only the basic subunit of the dimeric neurotoxin is expressed in C. atrox venom (Wooldridge et al., 2001; Werman, 2008).

The second type of geographic variation occurs among populations that are currently isolated. A typical example is the morphologically indistinguishable island
populations of the Japanese Habu Pitviper (*Trimeresurus flavoviridis*). Isolation and shared evolutionary pressures have allowed homogenization of venom within populations, whereas genetic drift causes heterogeneity of minor venom components among localities with only the most biologically crucial components being conserved (Chijiwa et al., 2000). Heterogeneity of venom composition is approximately correlated to time since population divergence (Chippaux et al., 1991). These observations are consistent with the concept suggested by Mebs and Kornalik (1984) that genetic variation influences those venom components with only minor biological roles, whereas other proteins, more vital to the overall functionality of the venom, remain characteristic of the species. Intraspecific variability of components vital to venom toxicity is not unprecedented, though (Glenn and Straight, 1977; Sadahiro and Omori-Satoh, 1980), and provides interesting insights into the processes influencing venom evolution.

The inherent limiting effects of physical separation on the circulation of alleles, either historical or contemporary, and homogenization of venom composition among populations, fail to fully explain all examples of venom variation in widely distributed species. Environmental factors specific to geographic location may also represent an important influence on venom protein expression. Just as ontogenetic shifts in dietary preferences have been shown to correlate with shifts in venom composition, environmental factors such as habitat type, elevation, and annual temperature and precipitation extremes may exert similar influence on venom composition through their effects on prey type availability and exposure to predation (e.g., Mackessy, 2008). In North America, a positive correlation between mammalian-specific venom toxicity and the proportion of mammalian prey in the diet of *Sistrurus* rattlesnake species (Gibbs and
Mackessy, 2009). In South America, populations of Bothrops pitvipers that continue to rely on ectothermic prey into adulthood retain their metalloproteinase-dominated juvenile venoms rather than shifting to the more complex venoms typical of populations where suitable mammalian prey are available (Núñez et al., 2009; Alape-Giron et al., 2008). An increasing number of studies have suggested similar correlations between geographic variation in diet and venom, including several rattlesnake species (e.g., Salazar and Lieb, 2003; Gibbs et al., 2009). Even localized differences in prey behavioral ecology and physiology can apparently influence snake venom composition, as illustrated by the increased mammalian toxicity in the venom of rattlesnakes living near ground squirrel colonies exhibiting increased anti-snake aggression and venom resistance (Poran et al., 1987; Biardi, 2008).

The Southern Pacific Rattlesnake (Crotalus oreganus helleri) is an excellent model for investigation of environmental influences on venom variation. A habitat generalist ranging from Baja California northward through southern California, and the Pacific islands of Santa Catalina (Los Angeles County, California) and Coronado Del Sur (Tijuana, Mexico) (Klauber, 1997), C. o. helleri thrives in sea level coastal valleys, grasslands, scrubby foothills, and montane forests up to 3000 m. Pronounced tectonic activity in the region has produced considerable variation in available habitat (Schoenherr, 1992), and prey species are therefore highly varied. Crotalus o. helleri occurs frequently close proximity to humans and domestic animals, and the species accounts for the majority of reported snake envenomations in southern California (Bush et al. 2002; Wasserberger et al. 2006). Thus, any new information on the venom can potentially have immediate clinical implications.
Material presented in this chapter is adapted from a larger study of the diversity of toxins present in *C. o. helleri*, across its geographic range, using a combined proteomic–transcriptomic approach. The goal was to determine the relative molecular evolution and diversification rates within a given toxin type, and the relative expression levels of particular toxins among populations. This chapter, however, will focus primarily on the proteomic aspects of the project, as they constituted the author’s contribution to the study. The transcriptomic work was performed by our collaborators at the University of Queensland.

**Materials and Methods**

**Sampling**

We sampled four southern California populations of *C. o. helleri* from areas with pronounced geological, elevational, and vegetative differences (Fig. 1). Envenomations by snakes in these regions have exhibited symptoms ranging from hemorrhage, to muscle fasciculations, and paralysis (Bush and Seidenburg, 1999; Richardson et al., 2007; Hoggan et al., 2011; Bush et al., 2014). We chose to study four populations. (1) Catalina Island, dominated by coastal sage scrub and interspersed with chaparral and oak woodland, has never been connected to the mainland (Schoenherr, 1992) and has supported an isolated rattlesnake population since at least the Pleistocene. (2) Idyllwild, in the San Jacinto Mountains, has high altitude pine and cedar montane forests (elevation ~1600 m). (3) Loma Linda, at lower elevation between major mountain ranges consists of low rolling hills covered with grasses and, on north facing slopes, *Salvia mellifera* and other shrubs. (4) Lastly, Phelan comprises a transition zone
between High Desert (Mohave) and coastal mountain scrub. We sampled one snake from each region for transcriptome sequencing. We used the same snake for proteome analysis of the Phelan and Loma Linda populations, and a separate individual of the same sex and size from the exact same locality for the other two locations. To account for potential venom variation among individuals within each population, we subjected venom samples from two additional Catalina Island, Phelan, and Idyllwild snakes to proteomic analysis. Because *C. o. helleri* is scarce in the Loma Linda area, we were only able to collect one

*Figure 1. Crotaulus oreganus helleri* populations investigated in this study. Shading denotes species range.
additional venom sample from that population. We used only adult specimens for venom analysis due to potential ontogenetic shifts in venom composition (Mackessy 2008).

**HPLC**

Lyophilized crude venom was diluted to a concentration of 3 mg/mL in Buffer A (0.065% TFA, 2% acetonitrile in Nanopure water) and centrifuged at 15,000 × g for 10 min. The supernatant (100 μL) was fractionated on an ÄKTAmicro high-pressure liquid chromatography (HPLC) system (GE Healthcare Life Sciences, Piscataway, NJ, USA) fitted with two reversed-phase (RP) columns (SOURCE 5RPC ST polystyrene/divinyl benzene, 4.6 150 mm; GE Healthcare) run in series at a flow rate of 0.5 mL/min, using a linear gradient of 0–100% Buffer B (0.05% TFA, 80% acetonitrile in Nanopure water) over 40 column volumes. Protein elution was monitored at 214 nm using Unicorn 5.0 (GE Healthcare Lifesciences) software, and fractions were collected manually.

**LC-MS**

Each fraction was subjected to reduction and alkylation prior to enzymatic digestion using dithiothreitol and iodoacetamide, respectively, following the protocol outlined by Matsudaira (1993). Proteins were then digested with proteomics-grade porcine pancreatic trypsin (Sigma-Aldrich, St. Louis, MO, USA). We desalted samples using C18 ZipTips (EMD Millipore, Billerica, MA, USA) according to the manufacturer’s protocol. We analyzed the desalted tryptic peptides with a ThermoFinnigan LCQ Deca XP spectrometer (ThermoFinnigan, Waltham, MA, USA) equipped with a PicoView 500 nanospray apparatus using Xcalibur software (ver. 1.3; ThermoFinnigan, Waltham, MA,
USA) for instrument control and data acquisition. Separation was performed on a 10-cm x 75-m-i.d. C$_{18}$ Biobasic bead column (New Objective, Woburn, MA, USA) by injecting 20-uL samples. Mobile phase B consisted of 98% acetonitrile, 2% water, and 0.1% formic acid. The gradient program was: 0% B at 0.18 mL/min for 7.5 min; 0% B at 0.35 mL/min for 0.5 min; linear gradient to 20% B at 15 min at 0.35 mL/min; linear gradient to 75% B at 55 min at 0.3 mL/min (flow rate constant for remainder of program); linear gradient to 90% B at 60 min; hold at 90% B until 85 min; linear gradient to 0% B at 90 min; hold at 0% B until 120 min. Spectra were acquired in positive ion mode with a scan range of 300–1500 m/z. We converted MS/MS data into peak list files using Extractmsn implemented in Bioworks (version 3.1; ThermoFinnigan) with the following parameters: peptide molecular weight range 300–3,500, threshold 100,000, precursor mass tolerance 1.4, minimum ion count 35. We conducted MS/MS database searches using Mascot (licensed, version 2.2, Matrix Science, Boston, MA, USA) against the National Center for Biotechnology Information non-redundant (NCBI) database within Metazoa. A parent tolerance of 1.20 Da, fragment tolerance of 0.60 Da, and two missed trypsin cleavages were allowed. We specified carbamidomethylation of cysteine and oxidation of methionine in MASCOT as fixed and variable modifications, respectively.

**MALDI ToF MS and MALDI ToF/ToF MS/MS**

RP-HPLC fractions were submitted to the Institute for Integrated Research in Materials, Environments and Society at California State University, Long Beach, to determine whole protein molecular masses and protein identification/similarity. For MALDI ToF/ToF MS/MS analysis, tryptic peptides were mixed with -cyano-4-hydroxy
cinnamic acid (CHCA) matrix and directly spotted onto MALDI plates. MS spectra were collected using 1000 laser shots/spectrum, and MS/MS spectra from 3000 shots/spectrum. Peptides with signal-to-noise ratio above 15 in MS mode were selected for MS/MS analysis, with a maximum of 15 MS/MS spectra allowed per spot. Internal calibration was achieved using ToF/ToF Calibration Mixture (AB SCIEX). We searched MS/MS data against the NCBInr database within Metazoa using GPS Explorer, running Mascot (version 2.1) search engine with a peptide tolerance of 300 ppm, MS/MS tolerance of 0.8 Da, and one missed cleavage allowed. We specified carbamidomethylation of cysteine as a fixed modification, and the following as variable modifications: carbamyl, Gln‡pyro-Glu (Nterm Q), and Glu‡ pyro-Glu (N-term E).

**Statistical Analyses**

To confirm that population differences existed among the 11 snakes with the quantitative RP-HPLC data, we subjected the percent protein present in each of the 11 toxin families (area under the peaks) to a 4 × 11 (population × toxin family) analysis of variance (ANOVA [Green and Salkind, 2005]), treating population as a between-subjects factor and toxin family as a within-subjects factor. We rank-transformed the data to avoid analysis of percentage data that summed to 100 for each individual. Although our samples were small and data were somewhat non-normally distributed and heteroscedastic, general linear models generally handle data well that fail to meet parametric assumptions and the results were extremely robust. We also ran a non-parametric Kruskal-Wallis ANOVA for each toxin family to compare the populations, allowing us to confirm results from the parametric ANOVA; this latter test requires no
assumptions about data distribution (Green and Salkind, 2005). We computed effect sizes (approximate variance explained) as adjusted partial eta-squared ($\eta^2$) for the parametric ANOVA and as $\eta^2$ (computed as $\chi^2 / [\text{total } N - 1]$) for the Kruskal-Wallis ANOVAs (Green and Salkind, 2005; Cohen, 1988). Eta-squared values $\geq 0.14$ are generally deemed large (Nakagawa, 2004). We conducted these analyses using SPSS 13.0 for Windows, with alpha $= 0.05$. Following Nakagawa (2004), we did not apply Bonferroni adjustments to multiple tests.

**Results**

Our proteomic analyses revealed significant differences in the venoms of the four populations (Fig. 2), with venom RP-HPLC profiles within a population largely congruent among individuals (Fig. 3; note: only two Loma Linda specimens were able to be analyzed due to the rarity of *C. o. helleri* in this locality). Proteomic and transcriptomic data for individual toxin constituents are summarized in Table 4. The parametric ANOVA yielded a highly significant interaction between population and toxin family ($F_{9,8,22,9} = 13.15, p < 0.001$, adjusted partial $\eta^2 = 0.31$; Greenhouse-Geisser adjustment of degrees-of-freedom applied), indicating that the distribution of toxins among the protein families differed significantly among the populations. The Kruskal-Wallis ANOVAs confirmed that toxin quantity varied significantly among populations for some (nerve growth factor, cysteine-rich secretory protein [CRiSP], lectin; all $p = 0.21–0.35, \eta^2 = 0.86–0.97$) but not all toxin families. Five additional toxins (BPP, $\beta$-defensin, kallikrein, PLA$_2$, SVMP) approached significance ($p < 0.10$) with large effect sizes ($\eta^2 > 0.63$). Thus, the ANOVAs confirmed population differences despite the small
Figure 2. LC-MS/MS-annotated RP-HPLC chromatograms of the four *Crotalus o. helleri* populations analyzed. Colored regions represent dominant protein families only. β-Defensins include crotamine and other small basic peptides; CRiSPs = cysteine-rich secretory proteins; kallikreins are a subset of serine proteases).
Figure 3. RP-HPLC chromatograms of crude venom from individuals analyzed in each population.
Table 4. *Crotalus o. helleri* intraspecific proteomic and transcriptomic toxin presence (✔) or absence (✖). Adapted from Sunagar et al. (2014).

<table>
<thead>
<tr>
<th>Toxin molecular scaffold type</th>
<th>Catalina Island</th>
<th>Idyllwild</th>
<th>Loma Linda</th>
<th>Phelan</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Defensin</td>
<td>Large amounts, medium complexity</td>
<td>✔</td>
<td>Large amounts, medium complexity</td>
<td>✔</td>
</tr>
<tr>
<td>Bradykinin-potentiating peptide/ Natriuretic peptide (BPP-NP)</td>
<td>Medium amounts, low complexity</td>
<td>✔</td>
<td>Small amounts, low complexity</td>
<td>✔</td>
</tr>
<tr>
<td>Cysteine-rich secretory protein (CRiSP)</td>
<td>Large amounts, medium complexity</td>
<td>✔</td>
<td>✔</td>
<td>Medium amounts, low complexity</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>✖</td>
<td>✔</td>
<td>✖</td>
<td>✔</td>
</tr>
<tr>
<td>Kallikrein (SVSP)</td>
<td>Medium amounts, low complexity</td>
<td>✔</td>
<td>Medium amounts, high complexity</td>
<td>✔</td>
</tr>
<tr>
<td>Kunitz (serine protease inhibitor)</td>
<td>✖</td>
<td>✔</td>
<td>✖</td>
<td>✔</td>
</tr>
<tr>
<td>L-Amino acid oxidase (LAAO)</td>
<td>Medium amounts, low complexity</td>
<td>✔</td>
<td>Medium amounts, low complexity</td>
<td>✔</td>
</tr>
<tr>
<td>Lectin</td>
<td>Large amounts, low complexity</td>
<td>✔</td>
<td>✖</td>
<td>✖</td>
</tr>
<tr>
<td>Nerve growth factor (NGF)</td>
<td>Low amounts, low complexity</td>
<td>✔</td>
<td>✖</td>
<td>✔</td>
</tr>
<tr>
<td>Phospholipase A2 (PLA₂)</td>
<td>Medium amounts, medium complexity</td>
<td>✔</td>
<td>Large amounts, high complexity</td>
<td>✔</td>
</tr>
<tr>
<td>Snake venom metalloprotease (SVMP)</td>
<td>Large amounts, medium complexity</td>
<td>✔</td>
<td>Not detected</td>
<td>✔</td>
</tr>
<tr>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>✖</td>
<td>✔</td>
<td>✔</td>
<td>Low amounts, low complexity</td>
</tr>
<tr>
<td>Vespryn</td>
<td>✖</td>
<td>✔</td>
<td>✖</td>
<td>✔</td>
</tr>
</tbody>
</table>

P = Proteome  
T = Transcriptome
sample sizes.

Consistent with the proteomic results of this study, and a previously published study of San Jacinto Mountain specimens (French et al., 2004), only the Idyllwild population contained both the acidic and basic subunits of the neurotoxic PLA\textsubscript{2} complex type, with both chains showing virtually identical coding sequences to the well-characterized potent presynaptic neurotoxins from \textit{C. d. terrificus} and \textit{C. s. scutulatus}. The Idyllwild population also secreted the lowest amount of SVMPs (Fig. 2), with only a single isoform obtained in the transcriptome and expression only being detectable at trace levels in the proteome. In contrast, the other populations secreted SVMPs in large amounts, with the Phelan samples showing the greatest complexity while the Catalina Island population exhibited lower complexity but a much higher relative expression level. Proteomic profiles were similar among individuals within each locality ($N = 3$; except Loma Linda population, where these animals are scarce) compared to among populations differences. Thus, we are confident that the venom-gland transcriptomics of randomly selected animals closely reflect the overall venomics of the representative population.

**Discussion**

**Sources of Observed Venom Variation**

While the present study confirms the general patterns of geographic variation in venom composition previously described in \textit{C. o. helleri} (e.g., French et al., 2004; Salazar, 2009), our findings provide unprecedented insight into variation of whole venom composition in the species, and which toxin families appear most variable. The most
striking disparity among the sampled populations is the reduced SVMP expression in venoms containing functional neurotoxin (Idyllwild population). This pattern is consistent with the inverse relationship between the relative composition of neurotoxic PLA$_2$ and hemorrhagic SVMP observed in *C. s. scutulatus* venom (Massey et al., 2012; Glenn and Straight, 1978; Glenn and Straight, 1983). Fragmentation due to anthropogenic development and habitat alteration is widespread throughout the range of *C. o. helleri*. However, geographic barriers alone fail to fully explain observed venom variation in the species as even relatively nearby populations with no known geographic barriers between them can exhibit strikingly different venom profiles. It seems likely that local environmental factors are exerting directional selective pressures on the snakes, thereby shaping venom profiles.

Diet is only one of the various factors shaping snake venom composition and its influence apparently varies. Chippaux et al. (1991) reported in their influential review that no relationship between snake venom composition and diet or habitat had been demonstrated, despite investigations of geographic variation in *Agkistrodon* spp. (Jones, 1976), *Bitis* and *Naja* spp. (Boche et al., 1981), or *Crotalus* spp. (Gregory-Dwyer et al., 1986). These and other early studies largely relied on gel electrophoresis and enzymatic activity to assess venom profiles, while diet analysis was limited. However, even detailed chromatographic analysis by Williams et al. (1988), specifically investigating the influence of natural prey type availability, failed to produce evidence of a dietary effect on venom produced by tiger snakes (*Notechis ater niger* and *Notechis scutatus*).

Daltry et al. (1996a,b), however, described a significant association between geographic location and venom composition. She and her colleagues subjected venom
samples from 36 distinct populations of the Malayan Pitviper to isoelectric focusing. They then used partial Mantel tests to assess the degree of correlation between gel band profiles and geographic proximity, phylogenetic relatedness, and diet. The statistical analyses rejected both contemporary gene flow and patristic relatedness as major influences on geographic variation of venom profiles, and only diet was significantly correlated with venom composition. They proposed that selection pressures related to local prey type availability, such as the need for more rapid lethality or digestion of larger prey items, may constitute the primary drivers of venom variability among localities.

Various subsequent studies have lent further credence to dietary affects on venom. Chijiwa et al. (2000) presented an especially compelling case in their investigation of the Habu pitviper (*Trimeresurus flavoviridis*) on several islands off the coast of Japan. Ancestral *T. flavoviridis* on the former Okinawa land mass expressed genes coding for two highly homologous myotoxic PLA$_2$ isoenzymes (BPI and BPII) prior to rises in sea level which split Okinawa into separate islands. Following this eustatic isolation event, alteration of the tandem BPI and BPII genes produced a new pseudogene in *T. flavoviridis* on the new Okinawa island. The myotoxin genes were apparently unaffected in populations on the islands of Amami-Oshima and Tokunoshima. Chijiwa and colleagues noted that such genetic disruptions are not uncommon among highly homologous gene families and the fact that inactivation of the BPI and BPII genes only occurred in Okinawan *T. flavoviridis* suggests the myotoxins serve a more essential ecological role in the other populations.

The toxicity of *T. flavoviridis* venom on Okinawa is greatly reduced due to the loss of BPI, BPII, and a defective hemorrhagic metalloprotease, HR1b. Deficiency of
these three highly toxic venom constituents, the researchers suggest, would represent a significant adaptive disadvantage to Okinawan *T. flavoviridis* unless local ecological pressures there differ from those on Amami-Oshima and Tokunoshima. They further suggested that Okinawan *T. flavoviridis*, which relies heavily on amphibian prey (90% in some areas), may no longer need the level of venom toxicity utilized by populations on Amami-Oshima and Tokunoshima, which feed largely on mammals (86% rats, 14% birds/reptiles/amphibians). This loss of gene function seems to fit a theme seen among island populations in which specific traits are lost when selective pressure to maintain them is removed (McNab, 1994; Blumstein and Daniel, 2005; Zuk et al., 2006).

The emerging consensus seems to be that phylogeny, geographic distance, and ecological factors act in concert to influence venom composition. Even when diet seems to exert only minimal influence on venom, such as in the North American *Agkistrodon* pitviper complex (Lomonte, 2014), venom is increasingly viewed as a labile trophic adaptation shaped by ecological pressures. The relative homogenization of venom profiles among *Agkistrodon* species, for example, has been proposed to reflect shared generalist diets across the complex rather than a lack of dietary influence on venom. The notable variation in venom profiles even among *C. o. helleri* populations separated by minimal patristic distance (Chapter 3 and unpublished data) or geographic distance suggest diet is an important determinate of venom composition in this species. The relationship between venom composition and habitat (a correlate of prey availability) presented in this study is largely descriptive. As Sasa (1999b) pointed out in his critique of Daltry et al. (1996), interpretation of the effect of diet on venom is most appropriate within the context of a toxin’s ecological function. Subsequent investigation, using more
integrative, multi-faceted methods are needed, such as those employed by Barlow et al. (2009) to experimentally test relationships among venom composition, phylogeny, diet, and venom effectiveness in various prey types. Barlow et al. (2009) compared prey type consumed between Echis clades and found that species that preyed more heavily on arthropod prey expressed venom more toxic to scorpions. These results were mapped onto an Echis phylogeny and revealed coevolution of venom toxicity and diet in two independent episodes.

**Clinical and Pharmaceutical Implications**

The observed variability of neurotoxic, hemotoxic, and myotoxic venom-components in *C. o. helleri* greatly complicates clinical treatment of bite victims, not only by eliciting highly variable clinical effects, but also as a consequence the reciprocal variability in the efficacy of antivenom binding. We hope that improved documentation of region-specific toxicity profiles will improve clinical treatment of envenomation by enabling physicians to anticipate symptoms and utilize more tailored treatment strategies based on where the snake was encountered. Improved understanding of the correlation between specific venom protein profiles and clinical symptoms ineffectively ameliorated by current antivenoms should also facilitate development of more effective antivenom.

Naturally-occurring molecules that perform desired biological effects are often used as lead structures from which powerful pharmaceutical applications are developed (Klebe, 2013). However, the process of optimization for therapeutic use is often tedious and costly. Animal venoms represent tremendously diverse sources of bioactive natural products with myriad activities of clinical interest. Selective pressures have already
sculpted the evolution of structure and function in venom components to the point that incredibly precise pharmaceutical selectivity and potency can be achieved with less optimization than other leads. Pharmaceutically-productive species tend to cluster phylogenetically, and the majority of novel drugs are derived from known drug-productive families (Zhu, 2012). Snakes feature prominently among such drug-productive families, with Viperidae representing the second most productive family within Metazoa, behind Hominidae (Zhu, 2011). Proteomic investigation of snake venoms, then, offers significant potential for discovery of novel pharmaceutical applications, particularly in species such as C. o. helleri, which display especially complex and variable protein profiles.

The present study demonstrates dramatic variation in venom protein content and presumed toxicity among populations within a relatively small geographic area. Although the populations investigated here were selected for their unique habitat types and may therefore exaggerate the degree of venom heterogeneity within the species, a more comprehensive range-wide investigation of populations is warranted. Toxicity assays to assess the physiological implications of the observed geographic venome variation are warranted.
References


CHAPTER THREE

GEOGRAPHIC VARIATION OF VENOM COMPOSITION AND NEUROTOXICITY IN THE PACIFIC RATTLESNAKES (CROTALUS OREGANUS OREGANUS AND C. O. HELLERI): EXAMINING THE ROLES OF PHYLOGENY, GEOGRAPHIC DISTANCE, AND HABITAT

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Abstract

Snake venoms comprise complex secretions composed of numerous enzymes, peptides, small organic molecules, and inorganic components which, upon injection, induce a broad range of physiological effects determined by their toxin constituents. Snake venom composition varies dramatically among genera and species, and substantial intraspecific variation also occurs in many species. Clinicians report variability in symptomology and in efficacy of antivenom treatment following envenomation by the Northern Pacific and Southern Pacific Rattlesnakes (*Crotalus oreganus oreganus* and *C. o. helleri*). This study documents substantial geographic variation in venom protein composition and presence of neurotoxicity among *C. oreganus* specimens sampled across California, USA. We used multivariate statistics to assess associations of venom composition with phylogenetic distance, geographic distance, and environmental variables (vegetation index, precipitation, and temperature). Environmental factors showed the strongest correlation with venom composition, followed by genetic relatedness, and geographic distance. The findings have direct implications for improved clinical treatment of snakebite, and, potentially, for novel pharmaceutical applications of toxins.

Introduction

*Factors Influencing Venom Composition*

Snake venoms are complex secretions of numerous peptides, enzymes, and inorganic compounds that cumulatively produce a variety of toxic physiological effects upon envenomation (Mackessy and Baxter, 2006; Mackessy, 2009; Casewell et al., 2012;
Venom is primarily used by snakes to facilitate prey acquisition and defense against predators and antagonists (Chippaux et al., 1991; Heatwole et al., 1995; Chiszar et al., 1999; Owings and Coss, 2008; Casewell et al., 2012). The factors that influence venom composition are complex and varied, and exert their impact at various stages of venom production. At its most fundamental level, snake venom protein composition is first dependent on toxin-encoding genes within a snake’s genome. These venom genes, often referred to collectively as the snake’s venome (Fry, 2005), probably originated via the duplication and subfunctionalization of genes encoding existing salivary proteins (Hargreaves et al., 2014; Reyes-Velasco et al., 2014). As a consequence of genetic drift and/or selection, venom profiles differ among snake lineages in ways that are broadly characteristic of snake families and genera (Fry, 2005). Further variation of venom protein composition occurs among species and intraspecifically among populations (Boldrini-França et al., 2010; Fry et al., 2002, 2003; Castro et al., 2013; Daltry et al., 1996a,b; Forstner et al., 1997; French et al., 2004; Mackessy, 2010; Salazar et al., 2009; Wilkinson et al., 1991), between the sexes (Daltry et al., 1996a,b; Menezes et al., 2006), and ontogenetically within individuals (Calvete et al., 2009b; Daltry et al., 1996a,b; Lopez-Lozano et al., 2002; Mackessy, 1988). Variable regulation of gene transcription, RNA translation, and post-translational modifications of gene products, also exert significant influences on venom composition, producing considerable variation even among closely related snakes with similar venomes (e.g., Fox and Serrano, 2008; Casewell et al., 2014).

Much of the venom variation observed within snake species or subspecies correlates to geographic location, with members of a given population generally exhibiting a higher degree of venom similarity than individuals from distant localities.
Jiménez-Porras (1964) reported, for instance, that venom profiles from specific geographic localities were so similar that venom composition could be used to predict where a venom sample was collected. The underlying causes of geographic variation in venom have been the subject of considerable debate, and the emerging consensus is that genetic drift and environmental factors act in concert to shape venom profiles. Until Daltry et al. (1996a,b) demonstrated significant associations between venom composition and diet, prevailing thought was that geographic variation in adult snake venom profiles was driven mainly by phylogeny (e.g., Jones, 1976; Boche et al., 1981; Gregory-Dwyer et al., 1986; Williams et al., 1988; Chippaux et al. 1991). Subsequent studies, such as those by Creer et al. (2003), Tsai et al. (2004), and Barlow et al. (2009), provided additional evidence that variation of essential venom constituents between populations may be the result of natural selection acting through environmental factors, or of discrete founder effects rather than genetic drift. Given the primary ecological functions of venom in facilitating prey acquisition and defense, selection pressures exerted by environmental factors, such as local prey and predator types, seem plausible. However, this idea has received opposition from some who claim the role of local selection has been exaggerated (e.g., Sasa, 1999ab). The emerging view, nonetheless, suggests that venom composition is, indeed, a fairly labile evolutionary trait and that natural selection acting through factors such as diet variation shape snake venom composition, though this influence varies among taxa (Lomonte et al., 2013; Gibbs et al., 2013).

Multivariate analyses are ideally suited for investigating the potential factors that influence venom variation, as they can control for multiple variables simultaneously. Studies that examine intraspecific variation in venom have considered phylogenetic
relationships, geographic distance, and diet (Thorpe et al., 1995; Daltry et al., 1996ab; Gibbs and Chiu, 2011). The individual influences of each of these variables can be examined using Mantel and partial Mantel tests. Other than the well-established link between diet and venom, analyses of other potential environmental influences on venom composition have been conspicuously absent. Variables such as temperature, precipitation, and altitude affect the morphology, physiology, and ecology of snakes in myriad ways (e.g., Huey, 1991; Ricklefs, 1994; Beaupre, 1995; Filippi et al., 2005; Pizzatto et al., 2007; Gartner et al., 2010; Clusella-Trullas et al., 2011), and potentially contribute to venom variation as well. Cool climate, for example, has been hypothesized to influence venom composition in rattlesnakes by conferring an adaptive advantage to snakes with venom components capable of speeding meal digestion at lower temperatures (Mackessy, 2003). Many snakes, including viperids (e.g., Macartney, 1989; Brown, 1990; Wallace and Diller, 1990), are opportunistic predators with fairly broad diets, and their diets, therefore, reflect to a large extent the prey items available in their particular prey community. Prey community composition, in turn, is shaped to a large extent by environmental features, including temperature, precipitation, elevation, and vegetation. Clearly, disentangling the independent and interacting effects of multiple variables poses a substantial challenge for understanding the factors that shape venom variation. Investigating associations of fundamental habitat characteristics such as temperature, precipitation, elevation, and vegetation with venom variation therefore seems an appropriate place to begin to elucidate the complex details of the interplay between venom and environment.
Various studies (including our own prior work; Sunagar et al. 2014 and Chapter 2) have shown that the strength and direction of selective pressures acting on venom proteins often vary dramatically among protein families within a population, as well as among populations within protein families (e.g., Brust et al., 2013; Pineda et al., 2014). We suspect that generalist species, especially, are likely subject to rugged fitness landscapes with multiple fitness optima (adaptive peaks) and fitness minima (adaptive valleys; Wright, 1932; Whitlock et al., 1995; McCandish, 2011) reflecting the varied applications of their venoms, so that no one adaptive peak represents maximum overall utility to the species. It has been suggested that retention of complex venom phenotypes may be more selectively advantageous than optimization toward a simpler venom profile (Casewell et al., 2011).

**Pacific Rattlesnakes**

The Northern Pacific Rattlesnake (*Crotalus oreganus oreganus*) and Southern Pacific Rattlesnake (*C. o. helleri*) are well-suited for investigation of environmental influences on intraspecific venom variation. These taxa share similar venom protein profiles which are fairly distinct from those of other *Crotaline* snakes (Mackessy, 2008; Hayes and Gren, unpublished data). However, significant intraspecific venomic variation occurs among the various populations (French et al., 2004; Jurado et al., 2007; Sunagar et al., 2014), with higher levels of variation between populations than within populations (Sunagar et al., 2014). In these and other rattlesnakes, nearby populations may possess dramatically different venom in the presence or absence of apparent barriers to gene flow (e.g., Forstner, 2007; Massey et al., 2012), surpassing differences among populations.
separated by much greater distances (Glenn and Straight, 1990; French et al., 2004; Gren et al., this study).

The Southern Pacific Rattlesnake ranges from southern California southward into Baja California Norte, and also occurs on the Pacific island of Santa Catalina (Los Angeles County, California; Klauber, 1997). The Northern Pacific Rattlesnake is found from the Canadian province of British Columbia south through Oregon and California to the Tehachappi Mountains of southern California. The two taxa potentially overlap across a fairly narrow region along the northern edge of the Transverse Mountain Range (Klauber, 1997). Pronounced geologic activity has produced considerable variation in habitat, prey availability, and predator species across the landscape (Schoenherr, 1992). As ecological generalists, *C. o. helleri* and *C. o. oreganus* thrive in many of the habitat types encountered across their range, from sea level beaches to valley grasslands, scrubby foothills, and montane forests up to 3000 m (Klauber, 1997). Recent urbanization has likely fragmented the species’ distribution in some areas, diminishing gene flow and resulting in isolated "island" populations (c.f. Bolger et al., 1997; Riley et al., 2003). Taken together, the wide geographic distribution, diverse habitats occupied, varied prey and predator species encountered, and fragmented populations provide an excellent opportunity to investigate the influences of geographic distance, phylogenetic relatedness, and environmental variation on venom composition.

**Clinical and Pharmaceutical Implications**

Their wide distribution and use of diverse habitat types places *C. o. oreganus* and *C. o. helleri* in frequent close proximity to humans and domestic animals. These taxa
account for the majority of human envenomations in California (Bush et al., 2002; Wasserberger et al., 2006). Moreover, our recent findings suggest that this range of utilized habitat types is associated with pronounced variation in venom profiles, and, presumably, a corresponding range of toxicities (Sunagar et al., 2014). Indeed, physicians report variable symptomology in envenomated patients and variable responses to antivenom administration, resulting in serious clinical challenges to treatment (e.g., Bush and Siedenberg, 1999; Richardson et al., 2007).

*Crotalus oreganus* venom has generally been considered proteolytic in nature, inducing severe, though often localized, tissue damage upon envenomation (Russell, 1983; Mackessy et al., 2003; Norris, 2004; Hoggan et al., 2011; Sprenger and Bailey, 1986). In 1999, Bush and Siedenburg reported the presentation of severe neurotoxic symptoms in a patient following *C. o. helleri* envenomation. Subsequent investigation by French et al. (2004) identified a Mojave toxin (MT) homolog in the venom of several *C. o. helleri* individuals using MT-specific antibodies. Mojave toxin is a well-known phospholipase A$_2$ (PLA$_2$) heterodimeric presynaptic β-neurotoxin first described in the venom of the Mojave rattlesnake (*C. scutulatus*; Cate and Bieber, 1978). However, all MT-expressing individuals in their study came from a single population some 70 miles southeast of where the neurotoxic bite occurred, suggesting the presence of non-MT neurotoxins in the venom of some *C. o. helleri*. Additional sampling could potentially reveal a broader distribution of MT in *C. oreganus*.

Currently, two snake antivenoms are approved for human use in the United States. Antivenin Crotalidae Polyvalent (Wyeth Labs) is a polyvalent equine serum containing whole immunoglobulin raised against the venom of Eastern Diamondback (*Crotalus*
adamanteus), Eastern Diamondback (C. atrox), Tropical Rattlesnake (C. durissus
terrificus), and Lancehead Pitviper (Bothrops atrox). CroFab (Protherics) is a monoclonal
polyvalent bovine serum composed of antibodies raised against the venoms of C.
adamanteus, C. atrox, Mohave rattlesnake (C. scutulatus), and cottonmouth (Agkistrodon
piscivorus). The observed complexity and variability of C. o. helleri and C. o. oreganus
venom profiles and toxicities challenge the assumption that the venoms used in current
antivenom production collectively serve as sufficient representatives to be used in
treatment of all North American pitviper bites. Antibodies against the specific toxins in
C. oreganus venom may be absent or present at insufficient concentrations for effective
venom neutralization. Mapping of the geographic distribution of venom composition and
toxicity may one day facilitate movement away from the current generalized "shotgun"
approach toward development of more effective region-specific treatment.

An equally important implication of venom research is the potential for
biodiscovery. Countless pharmaceuticals have been inspired by naturally-occurring
toxins, of which more than 20 have been developed from snake venoms (Zhu, 2011).
Occasional reports have documented muscle fasciculation following C. oreganus bites
Bush and Seidenburg, 1999; Richardson et al., 2007). Although the protein responsible
has yet to be identified, these muscle tremors resemble those induced by fasciculins,
small neurotoxic members of the three-fingered toxins (3Ftx) protein family, found in the
venom of certain elapid species (Kini and Doley, 1996). Three-fingered toxins are of high
pharmaceutical value because their small size and structural simplicity allow for ease of
synthesis and delivery to target tissues, while their natural extreme binding-site
specificity drastically reduces drug side effects. Crotamine has been implicated in causing
similar muscle tremors (Chang et al., 1983; Ranawaka et al., 2013), and it may be that some of the other myotoxins common in rattlesnake venoms may elicit similar activity.

**Purpose of the Study**

In the present study, we document the extent of variation of venom protein composition and neurotoxicity among Californian *C. o. oreganus* and *C. o. helleri* populations. We also evaluate the associations of geographic distance, phylogenetic relatedness, temperature, precipitation, elevation, and vegetation density with venom composition. Our dataset included venom samples from 69 individuals collected from 40 locations across the range of these taxa in California.

**Materials and Methods**

**Venom Acquisition**

We collected venom from 34 *C. o. oreganus* from 16 locations and 35 *C. o. helleri* from 24 locations representing eight major biogeographical regions of California, USA (Fig. 4). All samples were from adult snakes (≥60 cm) to avoid potential confounding of geographic variation with ontogenetic variation. We procured the samples via manual venom collection, in which snakes were allowed to bite through a membrane-covered container. To allow for adequate regeneration of venom components, samples were collected a minimum of 14 days after snake feeding. Crude venom was lyophilized and stored on Drierite at -20– -80° C until analysis.
Figure 4. Collection sites for the 69 Pacific rattlesnakes (*Crotalus oreganus* ssp.) sampled. Biogeographic regions adapted from Schoenherr, 1992.
**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). The RP-HPLC fractions from the venoms of four individuals from four geographic locations were subjected to proteomic analyses (LC-MS and MALDI-ToF/ToF MS/MS) to identify the toxins present. The results, reported elsewhere (Chapter 2 and Sunagar et al., 2014), were used to establish 11 arbitrary RP-HPLC elution regions, each of which included one or more proteins identified to 13 major proteins and protein families (Fig. 5; Table 5). For each

![Figure 5. Representative composite chromatogram for Pacific rattlesnake (Crotalus oreganus ssp.) venom illustrating the toxin families associated with the 11 elution regions.](image-url)
Table 5. RP-HPLC elution regions (Fig. 5) and the proteins and/or protein families identified by mass spectrometry (LC-MS and MALDI) from peaks within these regions for Southern Pacific Rattlesnake (*Crotalus oreganus helleri*) venom samples from four representative populations. Protein identifications documented in Sunagar et al. (2014).

<table>
<thead>
<tr>
<th>Elution Region</th>
<th>Primary Content</th>
<th>Idyllwild (ID-2)</th>
<th>Phelan (PH-1)</th>
<th>Loma Linda (LL-4)</th>
<th>Catalina Island (CI-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (35–56 mL)</td>
<td>BIP/NP/Disintegrin</td>
<td>BIP Disintegrin</td>
<td>BIP Disintegrin</td>
<td>BIP Disintegrin Myotoxin (SBP) Unknown</td>
<td>BIP Disintegrin</td>
</tr>
<tr>
<td>2 (56–61 mL)</td>
<td>Crotamine/SBP</td>
<td>Crotamine (SBP)</td>
<td>Crotamine (SBP)</td>
<td>Crotamine (SBP) BPP</td>
<td>Crotamine (SBP) BPP Myotoxin (SBP)</td>
</tr>
<tr>
<td>3 (61–78 mL)</td>
<td>Other SBP/Disintegrin</td>
<td>Myotoxin (SBP) Complement C3 homolog</td>
<td>Myotoxin (SBP) Disintegrin</td>
<td>Myotoxin (SBP) Disintegrin</td>
<td>Myotoxin (SBP)</td>
</tr>
<tr>
<td>4 (78–87 mL)</td>
<td>MTA/Growth Factors/SVMP Disintegrins</td>
<td>MTA</td>
<td>NGF</td>
<td>NGF</td>
<td>NGF</td>
</tr>
<tr>
<td>5 (87–95 mL)</td>
<td>MTA/Growth Factors/SVMP Disintegrins</td>
<td>PLA₂ (MTB)</td>
<td>EGF</td>
<td>SVMP-disintegrin SVMP-disintegrin</td>
<td>SVMP-disintegrin</td>
</tr>
<tr>
<td>6 (95–97 mL)</td>
<td>CRISP/PLA₂</td>
<td>CRISP PLA₂</td>
<td>CRISP</td>
<td>VEGF CRISP</td>
<td>CRISP</td>
</tr>
<tr>
<td>7 (97–105 mL)</td>
<td>SVSP/PLA₂</td>
<td>SVSP</td>
<td>SVSP PLA₂</td>
<td>SVSP</td>
<td>SVSP</td>
</tr>
<tr>
<td>8 (105–109 mL)</td>
<td>PLA₂/SVSP</td>
<td>PLA₂</td>
<td>PLA₂</td>
<td>PLA₂</td>
<td>PLA₂</td>
</tr>
<tr>
<td>9 (109–11 mL)</td>
<td>Lectin/PLA₂/SVSP/CRI SP</td>
<td>PLA₂</td>
<td>Lectin SVSP</td>
<td>PLA₂</td>
<td>Lectin SVSP</td>
</tr>
<tr>
<td>10 (111–123 mL)</td>
<td>LAAO/PLA₂/SVMP-Disintegrin/Lectin/SVSP/CRI SP/NP</td>
<td>LAAO PLA₂</td>
<td>LAAO PLA₂</td>
<td>LAAO CRISP</td>
<td>LAAO CRISP</td>
</tr>
<tr>
<td>11 (123–end mL)</td>
<td>SVMP-disintegrin</td>
<td>SVMP-disintegrin VEGF</td>
<td>SVMP-disintegrin Unknown</td>
<td>SVMP-disintegrin PLÀ</td>
<td>SVMP-disintegrin Lectin</td>
</tr>
</tbody>
</table>
chromatogram, we integrated the area under the absorbance trace to determine the percent of total protein for each elution region (i.e., sum of the area of all peaks in each region relative to total area). We did this after minor visual alignment of individual chromatograms.

Compositional data sets comprised of relative abundance data are subject to the constant-sum constraint when all variables sum to 100%. Individual variables cannot vary independently of one another since variation in the proportion of one component adjusts the abundance of the other components proportionally. This induced association violates the assumptions of independence of most multivariate tests (Ranganathan and Borges, 2011) and often hides meaningful relationships among variables (Kucera and Malmgren, 1998). To address this, we performed a centered-log ratio (CLR) transformation of our data following Aitchison (1986). Because CLR transformation requires that the data set contain only non-zero values, we first replaced all zero values using Aitchison’s formula (pp. 266–267). Once transformed, our data satisfied standard assumptions for multivariate analyses.

For analyses, we used both the full data set of 11 elution regions and a reduced data set from principle component analysis (PCA; Mertler and Vannatta, 2010). This procedure, accomplished via SPSS 20.0 for Mac (Statistical Package for the Social Sciences, Inc., Chicago, 2011), recombines variables while taking into account shared variance such that the first few variables (i.e., principle components) of the reconstructed data set account for the majority of variance in the data set. We also created matrices of pairwise differences (or "distances") among all 69 snakes for each of the 11 elution ranges and for each of the principle components.
**Geographic Distances**

We constructed a matrix of pairwise geographic distances (nearest 0.1 km) among all 69 snakes. Linear distances between GPS coordinates of snake capture locations were entered into the matrix.

**Genetic Distances**

To compile matrices of pairwise genetic distances among all snakes, we collected DNA samples from either blood or shed skins following the extraction protocol described by Fetzner (1999). We amplified mitochondrial cytochrome-b (cyt-b) using conventional DNA primers (Carl Person to supply details). Polymerase chain reaction (PCR) amplification was performed using Fermentas HOT START Master mix (Thermo Fisher Scientific, Pittsburg, PA, USA) following the manufacturer’s protocol. Amplified samples were sequenced by Macrogen USA (Rockville, MD, USA). We downloaded the DNA sequence for *C. viridis cerberus* cyt-b (Af147859) from the NCBI database to serve as an outgroup for the Pacific Rattlesnake clade (Pook et al., 2000; Douglass et al., 2002). We obtained 48 useable cyt-b sequences. These were imported into Geneious R6 (Biomatters Ltd. 2006-2012) and aligned with ClustalW (Larkin et al., 2007). Phylogenetic trees and Bayesian distances were generated using Mr. Bayes (Huelsenbeck and Ronquist, 2001). Bayesian distances were entered into separate matrices for each of the mtDNA data sets.
Environmental Variation

We created additional distance matrices among snakes for two environmental variables. These variables were based on principle components extracted by PCA from four environmental variables: elevation, temperature, precipitation, and normalized difference vegetation index (NDVI). Differences between PC scores of individual snakes were entered as distance values in the matrices.

We extracted elevation data for each snake location from the Gtopo digital elevation (United States Geological Survey, 1996) We obtained temperature and precipitation from normalized 30-year (1981–2010) temperature and precipitation data through the PRISM Climate Group (University of Oregon, http://prism.oregonstate.edu). Raster data from PRISM (800-m pixels projected in WGS 84) were re-projected into NAD 83 UTM zone 11 using Arc Map 10.2 before extracting point values.

To create a high resolution representation of vegetative “greenness,” we obtained five high-resolution (30-m pixel size) images of the southern portion of our study area from the OLI/TIRS sensors on the LANDSAT 8 satellite. The images were captured on three separate dates (April 11, 18, and 20, 2013), with time of day varying by only 13 minutes among the images. All images contained similar sun azimuth and sun elevation values, indicating that irradiance would vary little due to solar declination. Cloud cover was prominent in the image of Catalina Island, but most of the cloud cover was located above the ocean, and none intruded upon any of the buffer zones for snake locations (see below). The five images were joined to form a mosaic, and were geometrically rectified using ERDAS Imagine 2013 software. We then created a normalized difference vegetative index (NDVI) image using the near-infrared and visible light red bands. Each
pixel in the resulting gray-scale image represents a value between -1 and +1, where values <0 indicate areas with no vegetation and values >0 indicate vegetative “greenness” of increasing density. Using Arc Map 10.2, we created buffer zones with 500-m radii around each snake collection point to extract NDVI values (see Erbas-White, 2014, for detailed methodology). We were unable to create a matching NDVI for the northern portion of our study because the available images of central and northern California were not orthorectified, so were much less geospatially accurate than the southern California image mosaic. Vegetation values associated with the northern images were also stretched so that they did not align with those from the southern California locations.

To reduce attribute space, we subjected the environmental variables (elevation, temperature, precipitation, NDVI) to PCA. Because NDVI was available only for the southern snakes, we ran two PCAs, one for all snakes (N = 69) that omitted NDVI, and another including NDVI for only the southern snakes (N = 38).

**Chick Biventer Cervicis Assay for Neurotoxicity**

We tested a representative subset of the venom samples (N = 16) for neurotoxic activity using the chick biventer cervicis nerve-muscle assay. Our methods followed those of Fernandez et al. (2014). Each venom sample was tested with 3–4 replicates. Results from the replicates were generalized for three measures: excitatory activity (initial contraction, followed by a gradual decrease in twitch strength), β-neurotoxic activity (substantial decrease in twitch response that can be reversed with addition of exogenous acetylcholine and cholinesterase), and myotoxic activity (inhibited twitch response to exogenous potassium chloride). Relative strength of these measures was
recorded as mild, moderate, or strong toxicity, or no toxicity. We also recorded the time (nearest minute) until there was a 50% reduction of original twitch intensity (t_{50}), though this was achieved for only a portion of venom samples.

**Analyses**

We conducted four major sets of analyses. First, we sought to identify major venom composition differences among populations at a broad geographic scale. Second, we examined how geographic distance, genetic relatedness, and habitat variation potentially influence venom composition, as assessed by associations among these variables. Third, we evaluated how venom composition related to excitatory activity, β-neurotoxic activity, and myotoxic activity. Finally, we tested for correlations of relative abundance for elution regions representing several major toxin constituents.

**Venom Composition Variation Among Geographic Regions**

We explored regional differences in venom composition at two levels. First, we used one-way analysis of variance (ANOVA; Mertler and Vannatta, 2010) models using SPSS to compare the percentage representation (after CLR-transformation) of each of the 11 venom components (elution regions) in the whole venom sample among seven geographic regions (Fig. 4). We omitted the single specimen from the Mojave Desert region from this analysis. We computed eta-squared (\(\eta^2\)) as a measure of effect size, with values of \(~0.01, ~0.06\) and \(\geq0.14\) loosely considered small, medium and large, respectively (Cohen, 1988). Second, we used discriminant function analysis (DFA; Mertler and Vannatta, 2010) to examine the extent to which overall venom composition
varied among the seven geographic regions. This approach included all venom components in a single analysis that allowed us to assess similarity among the geographic regions and to determine which venom components provided the best discrimination. We conducted the DFA using SPSS, with 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 et al., 2000) and to cross-validate accuracy of group assignments. Sample sizes for each region ranged from $N = 4–17$.

**Associations among Venom Composition, Geographic Distance, Genetic Relatedness, and Environmental Variables**

We used Mantel and partial Mantel tests (Mantel, 1967; Manly, 1986) to identify associations among venom composition and three major factors that potentially influence venom composition at the population level (geographic distance, genetic relatedness, and environmental variation). Unlike the simple Mantel test, which can only compare two matrices at a time, the partial Mantel test compares two matrices while simultaneously holding up to eight other matrices constant as controls. Partial Mantel analysis has proven to be a powerful tool in elucidating the complex interplay of factors influencing venom composition (Daltry et al., 1996a,b; Gibbs and Chiucchi, 2011), yet remains under-utilized in venom research. We subjected the aforementioned matrices containing venom composition, geographic, genetic, and environmental distances to both simple and partial Mantel analyses using PASSaGE v2 software (Rosenberg and Anderson, 2011). Each test used one of the venom matrices (either an elution region or PC) as the dependent variable.
and one of the other matrix sets (geographic distances, genetic distances, environmental variables) as the independent variable. For the partial Mantel tests, all remaining variables were held constant as controls.

Given the complexity of our data set, we incorporated multiple permutations in our analyses to gauge congruence among the different approaches and to give us confidence in our conclusions. The permutations included two groups of snakes (all 69 snakes and a subset of only 38 southern snakes), two levels of association (Mantel tests for bivariate matrix associations and partial Mantel tests for bivariate matrix associations while controlling additional variables), and two data sets for venom components (all 11 elution regions and four PCs for the reduced set of toxins). Although strict reliance on principle components might be preferred to reduce the overall number of tests and experimentwise error, we elected to present both sets of results for comparative purposes. These analyses can only identify associations among variables, but this approach comprises a reasonable first step in identifying potential causal relationships.

**Venom Components Associated with Excitatory, Neurotoxic, and Myotoxic Activities**

Because of the small sample size for the chick biventer cervicis assays, we used stepwise binomial logistic regression (Mertler and Vannatta, 2010) to explore potential associations between venom principle components (preferred due to the smaller number of variables) and the presence or absence of each of three measures from the assays: excitatory activity, neurotoxicity, and muscular activity. These analyses were conducted
Correlations Among Venom Components

Previous studies have shown significant negative correlations between metalloprotease and neurotoxin venom components in rattlesnake venoms (e.g., Mackessy, 2008; Calvete, 2012). To assess the extent of this association in Pacific rattlesnake venom, we subjected ER11 (predominantly SVMPs) and ER4 (predominantly MTA/GF) abundance data for all 69 snakes to bivariate correlation analyses in SPSS 20.0 for Macintosh. To see whether a similar negative association exists between metalloprotease and myotoxin expression, we also tested for correlation between ER11 and ER2 (predominantly crotamine), and between ER11 and ER3 (predominantly non-crotamine small basic peptides).

Results

Venom Composition Variation Among Geographic Regions

The ANOVA results (Table 6) revealed significant variation among geographic regions for seven of the 11 elution regions. Elution region 1 (predominantly bradykinin-inhibitor peptide) showed the highest level of variation (i.e., the largest effect size), with snakes from the adjacent Penninsular Ranges and San Jacinto Mountains exhibiting much lower levels than snakes from other regions. Although protein concentration in ER2 (predominantly crotamine) was similar among the geographic regions, ER3 (predominantly non-crotamine SBPs) occurred at much higher levels in snakes from southern regions compared to those from northern regions. Protein content within ER4
Table 6. Percent composition (mean ± 1 S.E., and range in parentheses) of reversed-phase HPLC elution regions (ER) from Pacific Rattlesnake (*Crotalus oreganus* ssp.) venom by region. Analysis of Variance (ANOVA) results compare all populations except for the Mojave Desert.

<table>
<thead>
<tr>
<th>Region</th>
<th>N</th>
<th>ER1</th>
<th>ER2</th>
<th>ER3</th>
<th>ER4</th>
<th>ER5</th>
<th>ER6</th>
<th>ER7</th>
<th>ER8</th>
<th>ER9</th>
<th>ER10</th>
<th>ER11</th>
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<td></td>
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<tr>
<td>Coastal Ranges</td>
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<td>10.5±1.1</td>
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<td>9.2±3.5</td>
<td>11.7±1.6</td>
<td>5.9±1.9</td>
<td>2.4±0.6</td>
<td>14.0±0.7</td>
<td>20.0±7.0</td>
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<td>(7.0-13.5)</td>
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<td>(3.9-31.2)</td>
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<td>(12.7-16.5)</td>
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<td>(5.9-28.8)</td>
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<td>0.2</td>
<td>3.0</td>
<td>11.8</td>
<td>12.7</td>
<td>0.3</td>
<td>12.2</td>
<td>11.8</td>
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<td>2.5±0.4</td>
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<td>(13.8-27.8)</td>
<td>(0.0-0.4)</td>
<td>(0.0-0.7)</td>
<td>(0.0-2.4)</td>
<td>(10.0-16.3)</td>
<td>(8.5-15.8)</td>
<td>(0.0-10.6)</td>
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<td>(15.7-27.9)</td>
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<td>San Jacinto Mountains</td>
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<td>7.1±1.6</td>
<td>37.0±5.8</td>
<td>6.3±0.9</td>
<td>12.2±1.4</td>
<td>2.0±0.5</td>
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<td>4.0±0.5</td>
<td>8.8±1.8</td>
<td>0.6±0.4</td>
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<td></td>
<td>(2.0-18.6)</td>
<td>(10.3-57.3)</td>
<td>(3.3-20.5)</td>
<td>(7.7-10.4)</td>
<td>(0.0-13.1)</td>
<td>(9.9-17.1)</td>
<td>(9.0-13.6)</td>
<td>(0.0-5.6)</td>
<td>(2.9-17.2)</td>
<td>(0.0-3.5)</td>
<td></td>
</tr>
</tbody>
</table>

| F<sub>6,61</sub> | 4.17  | 1.22  | 12.68  | 6.71  | 5.71  | 11.37  | 1.42  | 1.90  | 4.27  | 1.60  | 7.76  | 40.57 |
| P               | < 0.001 | 0.311 | < 0.001 | < 0.001 | < 0.001 | 0.220  | 0.096  | 0.001 | 0.161  | < 0.001 | < 0.001 |
| η<sup>2</sup>   | 0.29  | 0.11  | 0.56  | 0.36  | 0.53  | 0.12  | 0.16  | 0.30  | 0.14  | 0.43  | 0.80  |

Protein contents of individual elution regions are provided in Table 5.
and ER5 (predominantly MTA/GF, and MTB/GF, respectively) was substantially higher in the San Jacinto Mountains than elsewhere. Two elution regions, ER1 (predominantly bradykinin-inhibitor peptide) and ER10 (predominantly LAAOs), were substantially reduced in the San Jacinto Mountains compared to other regions. Elution region 8 (predominantly phospholipases A\textsubscript{2} and SVSPs) showed a complex trend, with relatively low levels in the disjunct Coastal Ranges and San Jacinto Mountains.

The DFA used to determine whether overall venom composition differed by geographic region generated a significant model (Wilks’ Λ = 0.023, \( \chi^2_{60} = 220.95, N = 68, p < 0.001 \)) with six functions. Separation of the populations on the first two functions is depicted in Fig. 6. Classification results indicated that 73.5% of the venom samples were assigned correctly to the original geographic region. Leave-one-out classification was less successful at 51.5%, but still far greater than that expected from random for the seven groups (14.3%). Accuracy for each geographic region (with leave-one-out results in parentheses) was Catalina Island 100% (25.0%), Central Valley 64.3% (42.9%), Coast Ranges 100% (40.0%), Penninsular Ranges 100% (50.0%), San Jacinto Mountains 77.8% (77.8%), Sierra Nevada 80.0% (66.7%), and Transverse Ranges 52.9% (41.2%).

**Associations Among Venom Composition, Geographic Distances, Genetic Distances, and Environmental Variation**

The PCA of venom composition (for the 11 elution regions) yielded four components, which collectively accounted for 73.4% of the variance. Venom PC1 (32.2%) was comprised of (in order of loading) ER11, ER1, ER8, and ER10 with positive
Figure 6. Canonical plots of discriminant scores for venom components (area under the curve of 11 RP-HPLC elution regions) of individual Pacific rattlesnakes (*Crotalus oreganus* ssp.) from seven geographic regions depicted in Fig. 4 (the single Mojave Desert sample is excluded). Group centroids are also shown. The first function (57.3% of variance) consisted largely of ER11 and ER1 with negative loadings, which separated snakes of the San Jacinto Mountains with minute quantities of these proteins from those of other regions having greater quantities. The second function (33.2% of variance) consisted largely of ER3 with positive loadings, which separated the northern snakes with smaller quantities of proteins eluting in this region (Coast Ranges, Great Central Valley, and Sierra Nevada) from the southern snakes with higher quantities (Catalina Island, Penninsular Ranges, San Jacinto Mountains, Transverse Ranges). An additional DFA (results not provided) that included the Mojave Desert specimen placed it within the canonical space of specimens from the Transverse Ranges.
loadings, and ER4 and ER5 with negative loadings. Venom PC2 (17.3%) consisted of ER7 and ER3 with positive loadings. Venom PC3 (13.5%) was composed of ER6 with a positive loading and ER9 with a negative loading. Venom PC4 (10.5%) consisted of ER2 with a positive loading.

The PCA of environmental data for all snakes omitted NDVI, and produced a single component that explained 68.1% of the variance. This component, PC1 (hereafter Environmental PC), consisted of elevation and precipitation with positive loadings, and temperature with a negative loading. The PCA for the southern snakes included NDVI, and yielded two components. PC1, essentially identical to that of the PCA for all snakes, and therefore also labeled Environmental PC, explained 68.1% of the variance and was similarly composed of elevation and precipitation with positive loadings, and temperature with a negative loading. PC2 (hereafter Vegetation PC) explained 22.4% of the variance and consisted of NDVI with a positive loading.

Consensus results for partial Mantel tests of the 11 components (Table 7) found some support for associations between venom components and geographic distance. Of the five venom components with significant associations, three associations were relatively small and two were moderate, though minor differences existed between the tests for all snakes and the tests for just southern snakes. Two associations were negative and three were positive. In some cases, the direction of association differed between the Mantel tests and the partial Mantel tests, suggesting likely covariation between geographic distance and other variables.
Consensus results for partial Mantel tests of the 11 components (Table 7) yielded stronger support for associations between venom components and genetic distances. Of the six venom components with significant associations, one association was relatively small, two were moderate, and three were large. Two associations were negative and four

Table 7. Consensus from partial Mantel tests of associations between relative protein content of toxins and toxin families of Pacific rattlesnake (*Crotalus oreganus* ssp., *N* = 69) venom and other factors. Associations are shown as negative (-) or positive (+), and relative strength as small (-/+), moderate (- -/++), or large (- - -/+++).

<table>
<thead>
<tr>
<th>Elution Region</th>
<th>Geographic Distance Associations</th>
<th>Genetic Distance Associations</th>
<th>Environmental Associations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>++</td>
<td>-</td>
<td>Enviro PC: +</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Veg PC: +</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>- -</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>- -</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>6</td>
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<td>- +</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>11</td>
<td>++</td>
<td>+++</td>
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</tr>
</tbody>
</table>

Significant associations 5 6 9 8

Associations were tested among matrices of pairwise distances for venom components and matrices of pairwise distances or differences for three other major factors that potentially influence venom composition: geographic distance, genetic distance (for the mtDNA markers cyt-b), and environmental variation (see below).

Two environmental variables consisting of principle components (PC) from a principle component analysis were tested, including (with negative or positive association with PC : 1) environmental PC (Enviro PC), comprised largely of elevation(+), precipitation (+), and temperature (-); and 2) Vegetation PC (Veg PC), comprised largely of a normalized difference vegetative index (NDVI). Detailed results from the tests are provided in Appendices 1 and 2.

Protein contents of individual elution regions are provided in Table 5.
were positive. Associations were consistent between the full sample of all snakes and the subset of southern snakes with one exception: a relatively small association for ER10 existed for the southern snakes, but was absent from the test of all snakes (Appendix 1). Mantel and partial Mantel tests were largely congruent, except that the Mantel tests for ER5 showed a significant negative association that was absent from the partial Mantel tests (Appendix 1), suggesting likely covariation between genetic distance and other variables.

Consensus results for partial Mantel tests of the 11 components (Table 7) provided the strongest support for associations between venom components and environmental variables. Of the nine venom components significantly associated with Environmental PC (elevation, precipitation, temperature), three associations were relatively small, three were moderate, and three were large. Six associations were negative and three were positive. Associations were largely consistent between the full sample of all snakes and the subset of southern snakes (with two minor exceptions; Appendix 1) and between the Mantel and partial Mantel tests (with one minor exception; Appendix 1). Of the eight venom components significantly associated with Vegetation PC, three were relatively small, one was moderate, and four were strong. Four were negative and four were positive. These tests were conducted only for southern snakes, but results were identical for Mantel and partial Mantel tests.

Partial Mantel tests using Venom PCs (Appendix 2) were generally congruent with those using the 11 venom components individually (Appendix 1), but some notable differences existed. Venom PC1 was positively associated with geographic distance, which was consistent for most but not all of the six elution regions analyzed individually;
it was positively associated with genetic distance, which was consistent for all but one elution region analyzed individually; and it was negatively associated with both Environmental PC and Vegetation PC, which was consistent with all six elution regions analyzed individually. Venom PC2 showed no association with geographic distance, which was consistent with both of the elution regions analyzed individually; it was negatively associated with genetic distance, which was consistent with one of the two elution regions analyzed individually; and it showed no association with environmental variables, though associations existed for both venom components analyzed individually. Venom PC3 yielded no associations at all, though associations with geographic distance, Environmental PC, and Vegetation PC existed for the two elutions regions when analyzed individually. Finally, Venom PC4 was significantly associated only with Environmental PC, which was fully consistent with the single elution region. The Mantel and partial Mantel results were largely congruent, except that the direction of association differed for geographic distance, which occurred also with analyses of the 11 elution regions.

**Distribution of Neurotoxicity**

Twitch height data from the chick biventer cervicis assays varied in intensity and in time to inhibition, even among replicate trials of the same venom sample. However, broad patterns of twitch response generally agreed among replicates. Consensus results for the replicate tests are summarized in Table 8, and their geographic distribution is mapped in Fig. 7. No clear pattern of geographic distribution could be discerned. The majority of samples (75.0% of 16) elicited an initial increase in twitch response followed
by gradual twitch decrease, an effect we termed "excitatory." Similarly, a majority of samples (87.5%) also exhibited some level of pre-synaptic, or beta, neurotoxicity, as indicated by substantial inhibition of stimulated twitches, but retention of sensitivity to exogenous ACh and CCh. Such inhibition of twitch height by toxins is commonly reported as either $t_{90}$ or $t_{50}$ (time required for toxin to inhibit original twitch height by 90% or 50%, respectively; Hodgson and Wickramaratna, 2002). Here, $t_{50}$ values are reported since some venom samples failed to achieve 90% inhibition within the timeframe of the assay. In many trials, the toxins’ excitatory effects complicated detection of β-neurotoxicity, as 50% inhibition was not achieved within the time duration.

**Table 8.** Consensus of replicate tests ($N = 3$ or 4) of chick biventer cervicis assays for neurotoxicity in crude venom of the Pacific Rattlesnake (*Crotalus oreganus* ssp.). Sample locations are arranged from north to south, with county abbreviations in parentheses. Results are also mapped across the geographic range in Fig. 7.

<table>
<thead>
<tr>
<th>Snake</th>
<th>Excitatory</th>
<th>β-Neurotoxic</th>
<th>Myotoxic</th>
<th>$t_{50}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alameda 1 (ALA)</td>
<td>+++</td>
<td>+</td>
<td>—</td>
<td>N/A</td>
</tr>
<tr>
<td>Modesto 1 (STA)</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>138</td>
</tr>
<tr>
<td>Bear Springs 1 (KER)</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>Valencia (LA)</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>N/A</td>
</tr>
<tr>
<td>Santa Monica (LA)</td>
<td>—</td>
<td>—</td>
<td>++</td>
<td>N/A</td>
</tr>
<tr>
<td>Cajon Pass 1 (SBD)</td>
<td>—</td>
<td>+</td>
<td>+++</td>
<td>N/A</td>
</tr>
<tr>
<td>Loma Linda 3 (SBD)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>Loma Linda 4 (SBD)</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>N/A</td>
</tr>
<tr>
<td>Snow Creek 1 (RIV)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>73</td>
</tr>
<tr>
<td>Idyllwild 2 (RIV)</td>
<td>++</td>
<td>++</td>
<td>—</td>
<td>44</td>
</tr>
<tr>
<td>Idyllwild 4 (RIV)</td>
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<td>—</td>
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<td>N/A</td>
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<td>Pinyon Pines (RIV)</td>
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<td>—</td>
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<tr>
<td>Bautista Canyon (RIV)</td>
<td>+</td>
<td>+</td>
<td>+++</td>
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</tr>
<tr>
<td>Carlsbad 1 (SD)</td>
<td>+++</td>
<td>+++</td>
<td>—</td>
<td>88</td>
</tr>
<tr>
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<td>+++</td>
<td>+</td>
<td>88</td>
</tr>
<tr>
<td>Ramona (SD)</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>N/A</td>
</tr>
</tbody>
</table>

— = No toxicity; + = Mild; ++ = Moderate; +++ = Strong; N/A = 50% reduction of original twitch intensity not achieved within trial duration.
Figure 7. Geographic distribution of excitatory activity, β-neurotoxicity, and myotoxicity of Pacific Rattlesnake (*Crotalus oreganus* ssp.) venom as indicated by replicate (n = 3 or 4) chick biventer cervicis assays. Only a subset of 16 of the 69 snakes were tested in this exploratory toxicity investigation.

of the run despite decreases in twitch response which were far too pronounced to reflect muscle fatigue. Many samples (68.8%) also showed myotoxic activity, as indicated by inhibition of muscle fiber response to exogenous KCl.

The stepwise logistic regression results reflected the general absence of associations between venom composition and excitatory, neurotoxic, and myotoxic activities. None of the three stepwise models tested provided a significant model.
Bivariate Relationships among Venom Components

Bivariate correlation analysis indicated a significant negative association between ER4 (predominantly MTA/GFs) and ER11 (predominantly SVMPs; $r_{67} = -0.571$, $p < 0.001$). A significant negative correlation was also detected between ER3 (predominantly non-croatmine SBPs) and ER11 ($r_{67} = -0.254$, $p = 0.035$), but no association existed between ER2 (predominantly crotamine) and ER11.

Discussion

Venom Composition Variation among Geographic Regions

Our results revealed substantial geographic variation in the relative abundance of numerous protein components (area under the curve for the 11 RP-HPLC elution regions) of *C. oreganus* venom. Discriminant analyses indicated a substantial distinction between venoms of northern (*C. o. oreganus*) and southern (*C. o. helleri*) populations, which underscores the subspecific differentiation of these snakes. Most notably, southern snakes had consistently higher expression of non-croatmine small basic peptides (Other SBPs; ER3) in their venom compared to northern snakes. Snakes from the San Jacinto Mountains (including Idyllwild) also possessed a highly distinctive venom composition, which included relatively high levels of Mojave toxin (MTA and MTB; ER4 and ER5), as documented previously (French et al., 2004; Sunagar et al., 2014), and relatively low levels of BIP/NP (ER2), LAAOs (ER10), and SVMPs (ER11). Although other populations had measurable levels of proteins within ER4 and ER5, the protein material undoubtedly represented other proteins such growth factors (GF; Sunagar et al., 2014, and Table 5). Thus, we have no reason to believe that intact Mojave toxin occurred in any
samples other than those from the San Jacinto Mountains. Mojave toxin, or its various homologs, occurs in numerous rattlesnake taxa (Table 2), and often only within a limited geographic range of a given species (Werman, 2008; Powell et al., 2008). Mojave toxin also occurs in the subspecies *C. oreganus concolor* (Pool and Bieber, 1981; Aird and Kaiser, 1985; Bieber et al., 1990), so it would not be surprising if further sampling reveals additional populations of *C. oreganus* that possess MT.

**Associations Among Venom Composition, Geographic Distances, Genetic Distances, and Environmental Variation**

Our Mantel analyses suggest that geographic distance, genetic relatedness, and environmental variation all exert some level of influence on venom composition. These three influences, of course, are not independent; however, our use of partial Mantel tests allowed us to test each one independently while controlling for the others. Environmental variation, measured in terms of elevation, temperature, precipitation, and relative vegetation density, appeared to have the strongest associations with venom composition.

The relatively strong association between venom composition and environmental variation adds to the growing body of evidence that selection can shape the pool of toxins within a snake population. Our previous transcriptomic study suggested that *C. o. helleri* venom-encoding genes have experienced differential evolutionary selection pressures both among toxin types within a population, and within the same toxin type among populations (Sunagar et al., 2014). For three toxins having sufficient full-length transcriptome sequences for analysis, we showed that: (1) positive selection is driving rapid evolution of serine proteases across populations but that the number of positively
selected amino acid sites varied widely among populations; (2) significant positive
selection appears to be driving rapid evolution in lectins except in the Catalina Island
population, where they are experiencing strong negative selection instead; and (3) β-
defensins in all four populations appeared to be subject only to weak positive selection.
However, because secreted regions were highly conserved, we suspect that evolutionary
constraints favor the preservation of those amino acid residues required for toxicity.
Given their apparent rapid rates of evolution, the failure of serine proteases and lectins to
explain much of the venom variation in the present study may be a function of their
relatively low abundance in the venom profiles. Our current findings that environment
and phylogeny substantially influence venom composition coincide well with the concept
of significant but highly variable selective pressures acting on individual venom
constituents among Pacific rattlesnake populations.

If other toxins are experiencing strong selection similar to those documented for
serine proteases and lectins, and if their expression is consistently correlated with
environment and genetic distance, it seems likely that these proteins may play a key role
in adaptation of venom profiles in response to regional selective pressures. Our data
support previous studies documenting associations between venom components and
environmental factors such as habitat type, elevation, annual temperature and
precipitation extremes, diet, and exposure to predation (e.g., Mackessy, 2003, 2008).
*Crotalus viridis viridis*, which ranges far north and experiences a short active season and
extreme daily and seasonal temperature fluctuations, exhibits exceptionally high venom
concentrations of metalloproteinases (Gleason et al., 1983; Ownby and Colberg, 1987),
which Mackessy suggests can benefit digestive activity when processing large and bulky
meals. Similar evidence of the interplay between ecological pressures and venom composition is illustrated by increases in venom proteolytic activity following ontogenetic shifts in diet from lizard to rodent prey, but retention of more toxic venom in populations which continue to rely on reptile prey (Fiero et al., 1972; Mackessy, 1988, 1993; Mackessy et al., 2003). Gibbs and Mackessy (2009) demonstrated a positive correlation between mammalian-specific venom toxicity and extent of incorporation of mammalian prey in the diet of Sistrurus rattlesnake species. In South America, populations of Bothrops pitvipers that continue to rely on ectothermic prey into adulthood retain their metalloproteinase-dominated juvenile venoms rather than shifting to the more complex venoms typical of populations where suitable mammalian prey are available (Núñez et al., 2009; Alape-Giron et al., 2008). Additional studies have suggested similar correlations between geographic variation in diet and venom in snakes (e.g., Salazar and Lieb, 2003; Gibbs et al., 2009). Even very localized differences in prey behavioral ecology and physiology can apparently influence snake venom evolution, as illustrated by the increased mammalian toxicity in the venom of rattlesnakes living near ground squirrel colonies exhibiting increased anti-snake aggression and venom resistance (Poran et al., 1987; Biardi, 2008). It is interesting to note that whole venom electrophoresis profiles (e.g., Mackessy, 2008) often exhibit greater complexity in more xeric species, such as C. tigris (six bands), C. durissus terrificus (seven bands), and Sistrurus catenatus edwardsii (nine bands), often have less complex venom profiles, whereas mesic species such as C. h. horridus (14 bands), C. polystictus (14 bands), and C. pusillus (15 bands) often possess more complex venom profiles. This pattern is imperfect, however, as illustrated by the complex venom profiles of some xeric species such as C. mitchellii pyrrhus (14 bands)
and *C. ruber* (15 bands). Perhaps the diversity of prey types available in mesic environments often preserves more diverse venom profiles, whereas less varied prey assemblages in xeric habitats allow a thinning of venom constituents.

The influence of environmental pressures and diet on venom composition have been contested by some (e.g., Jones, 1976; Boche et al., 1981; Gregory-Dwyer et al., 1986; Williams et al., 1988; Chippaux et al., 1991), and are certainly not the only factors driving venom variation. The predigestion hypothesis (Mackessy et al., 2003), for example, would predict that the Mt. San Jacinto snakes in our study collected at over 1600 m, should express especially high levels of metalloproteinase. And given the inverse correlation between neurotoxicity and proteolytic activity (Mackessy, 2008), we might expect snakes in the San Jacinto Mountains to lack significant expression of neurotoxins. Both predictions fail, however, as venoms in the San Jacinto Mountains are nearly void of metalloproteases (the single most abundant toxin family in most *C. o. helleri* venoms; Tables 5 and 6) and exhibit neurotoxicity unmatched among all tested populations. In the absence of a distinct diet (see Chapter 4), we suspect that the unique venom composition of *C. o. helleri* in the San Jacinto Mountains may reflect a population founder effect or some other past genetic bottleneck event. We question whether there is anything unique about the diet or predators of these snakes that would favor a more toxic and less digestive venom. Further study is needed to determine whether the venom composition represents an alternative adaptive peak.

We incorporated multiple permutations in our Mantel analyses to gauge congruence among the different approaches, maximize our use of environmental information, and give us confidence in our conclusions. The permutations included two
groups of snakes (all 69 snakes and a subset of only 38 southern snakes having NDVI data), two levels of association (Mantel tests for bivariate matrix associations and partial Mantel tests for bivariate matrix associations while controlling additional variables), and two data sets for venom components (all 11 elution regions and four PCs for the reduced set of toxins). Although this approach resulted in a large number of statistical tests with high experimentwise error, the percentage of significant tests (e.g., 64.9% of 154 tests involving venom elution ranges, and 37.5% of 56 tests involving venom principle components) greatly exceeded that expected by chance (5% of all tests), which provides compelling evidence that real associations exist. General agreement between the full sample of 69 snakes and the subset of 38 southern snakes suggests that smaller data sets of future studies may be sufficient to identify associations. General agreement between the Mantel and partial Mantel tests underscores the strength of some associations, while occasional disagreement, particularly for associations between venom composition and geographic distance, suggests substantial covariance between geographic distance and other variables (genetic distance and environmental variation). General agreement between analyses of venom protein elution regions and venom principle components validates both approaches, but occasional disagreement emphasizes the greater resolution derived from analysis of individual venom components.

**Distribution of Neurotoxicity**

Neurotoxins may act either pre-synaptically or post-synaptically. Pre-synaptic neurotoxins infiltrate the synaptic nerve terminal and block the release of acetylcholine (ACh) from the vesicles into the synaptic cleft. Thus, following inhibition of twitches by
venom exposure, a contractile response after addition of exogenous ACh and its analog carbachol (CCh), indicates venom pre-synaptic neurotoxicity since the nerve fiber’s motor end plate remains responsive. Post-synaptic neurotoxins act by binding to acetylcholine receptors on the motor end plate, inhibiting the effect of acetylcholine released from the motor neuron axon across the synaptic cleft. Therefore, a decrease in response to exogenous ACh and CCh after treatment with venom indicates the presence of post-synaptic toxicity in the venom. Potassium chloride (KCl) elicits muscle contraction via a separate receptor, and is therefore unaffected by either pre- or post-synaptic neurotoxins. Accordingly, decreased muscle response to KCl at the conclusion of the experiment indicates either fatigue of the muscle tissue or myotoxic activity in the venom.

Results of nerve-muscle assays for neurotoxicity vary among mammalian, avian, or reptilian/amphibian tissue preparations. Although extrapolations between in vitro results and clinical observations are imperfect, mammalian tissues reasonably approximate the physiological effects of human envenomation due to general homology of nicotinic receptors (Hodgson and Wickramaratna, 2002). Avian tissues differ somewhat from mammalian tissues in their receptor morphology and response to toxins, but their multiply innervated muscle fibers afford a key advantage for preliminary analysis of venom toxicity by facilitating the simultaneous identification of post-synaptic and pre-synaptic toxicity as well as myotoxicity. The chick biventer cervicis nerve-muscle assay, therefore, serves as an appropriate method for initial survey of snake populations exhibiting unknown neurotoxic or myotoxic activity (Hart et al., 2013).
Variability among trials is typical in the chick biventer assay due to differences among individual tissue samples, sample preparation, and electrode placement, so standard protocol requires a minimum of three replicate trials per venom sample. Interpretation of our dataset is further complicated by the observed excitatory activity, which often increased twitch response far beyond initial levels. This prevented inhibition of twitch intensity to 50% within the time duration of the assays, despite twitch reduction too rapid to be explained by muscle fatigue, and accounts for the apparent discrepancies between reported β-neurotoxicity and \( t_{50} \) data.

Interestingly, only two of the four Mt. San Jacinto-area venom samples analyzed exhibited pre-synaptic neurotoxicity, despite all four containing substantial protein concentration in the elution regions dominated by the acidic and basic subunits of Mojave toxin. However, the Mt. San Jacinto samples that did exhibit neurotoxicity showed the most potent twitch inhibition of any samples tested, with the next most potent sample collected nearby at the base of the same mountain. It is possible that post-translational modifications may explain the reduced toxicity of the remaining San Jacinto-area venoms through inactivation of one or both of the Mojave toxin subunits.

Small basic peptides have been shown to exhibit myotoxic activity (Cameron and Tu, 1977; Maeda et. al., 1978; Fox et al., 1979; Bieber et al., 1987; Samejima et al., 1991; dos Santos et al., 1993; Allen et al., 1996; Ownby, 1998). Thus, given their prominent expression in the majority of venom samples analyzed, the wide distribution of myotoxicity observed is unsurprising. The observed excitatory activity, by contrast, was unexpected. To our knowledge, such excitatory activity is unusual in snake venoms but closely resembles activity sometimes observed in arachnid venoms (e.g., Rash et al.,
2000). We suspect this could be a result of the direct potentiation of sodium or potassium channels in the muscle, but it remains unknown which particular toxin(s) may be involved. This, together with observed pre-synaptic neurotoxicity in venom profiles lacking Mojave toxin subunits, underscores the need for further investigation of neurotoxicity in the species.

Although the chick biventer assay offers the important advantages for initial analysis of unknown neurotoxins, the assay imperfectly reflects toxicity in mammalian tissue. It would be ideal to expand on our preliminary findings by subjecting all remaining populations to chick biventer analysis, as well as performing parallel rat phrenic nerve assays on corresponding venom samples to gain improved insight into mammalian toxicity.

**Acknowledgments**

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Appendix 1. Results of simple and partial Mantel analyses of associations among centered-log ratio Pacific Rattlesnake (Crotalus oreganus ssp.) venom protein concentrations (corresponding to elution regions, ER) and geographic distance, genetic relatedness, and environmental principal components. Matrices held constant as controls are indicated within parentheses.

<table>
<thead>
<tr>
<th></th>
<th>ER1</th>
<th>ER2</th>
<th>ER3</th>
<th>ER4</th>
<th>ER5</th>
<th>ER6</th>
<th>ER7</th>
<th>ER8</th>
<th>ER9</th>
<th>ER10</th>
<th>ER11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Snakes: Simple Mantel (N = 69)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GeogDist</td>
<td>-0.07</td>
<td>-0.03</td>
<td>0.13**</td>
<td>0.01</td>
<td>0.03</td>
<td>-0.10</td>
<td>0.07</td>
<td>-0.13**</td>
<td>0.15*</td>
<td>0.05</td>
<td>-0.11**</td>
</tr>
<tr>
<td>GenDist-cyt-b</td>
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<td>-0.44***</td>
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<td>-0.08*</td>
<td>0.05</td>
<td>-0.03</td>
<td>0.16***</td>
<td>-0.07</td>
<td>0.02</td>
<td>0.43***</td>
</tr>
<tr>
<td>Enviro PC</td>
<td>-0.12**</td>
<td>-0.14**</td>
<td>0.07*</td>
<td>0.17***</td>
<td>0.32***</td>
<td>-0.03</td>
<td>-0.04</td>
<td>-0.26**</td>
<td>0.15***</td>
<td>-0.29**</td>
<td>-0.34**</td>
</tr>
<tr>
<td><strong>All Snakes: Partial Mantel (N = 69)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.03</td>
<td>-0.15**</td>
<td>-0.16**</td>
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<td>0.12**</td>
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<tr>
<td>GenDist-cyt-b (x Enviro PC x GeogDist)</td>
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<td>0.03</td>
<td>-0.46***</td>
<td>-0.31***</td>
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<td>0.02</td>
<td>-0.01</td>
<td>0.09**</td>
<td>0.02</td>
<td>0.03</td>
<td>0.44***</td>
</tr>
<tr>
<td>Enviro PC (x GenDist-cyt-b x GeogDist)</td>
<td>-0.12*</td>
<td>-0.16***</td>
<td>0.04</td>
<td>0.18***</td>
<td>0.32***</td>
<td>-0.02</td>
<td>-0.04</td>
<td>-0.25***</td>
<td>0.15**</td>
<td>-0.30***</td>
<td>-0.37***</td>
</tr>
<tr>
<td><strong>South Snakes: Simple Mantel (N = 38)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GeogDist</td>
<td>-0.12*</td>
<td>-0.07</td>
<td>0.34***</td>
<td>0.15**</td>
<td>0.13*</td>
<td>-0.04</td>
<td>0.02</td>
<td>-0.09**</td>
<td>0.00</td>
<td>-0.09</td>
<td>-0.23***</td>
</tr>
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<td>-0.20***</td>
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<tr>
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<td>0.21***</td>
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<td>0.16*</td>
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<td>-0.28***</td>
<td>-0.01</td>
<td>-0.23***</td>
<td>-0.40***</td>
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<tr>
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<td></td>
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</tr>
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<td>-0.05</td>
<td>0.00</td>
<td>0.12**</td>
<td>-0.04</td>
<td>0.06</td>
<td>0.18**</td>
</tr>
<tr>
<td>GenDist-cyt-b (x Enviro PC x Veg PC x GeogDist)</td>
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<td>0.02</td>
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<td>-0.09</td>
<td>-0.05</td>
<td>0.04</td>
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<td>0.17*</td>
<td>0.50***</td>
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<tr>
<td>Enviro PC (x GenDist-cyt-b x GeogDist x Veg PC)</td>
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<td>-0.19**</td>
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<td>0.16*</td>
<td>0.30***</td>
<td>-0.16*</td>
<td>0.06</td>
<td>-0.16**</td>
<td>0.32***</td>
<td>-0.19**</td>
<td>-0.44***</td>
</tr>
<tr>
<td>Veg PC (x GenDist-cyt-b x GeogDist x Enviro PC)</td>
<td>-0.17*</td>
<td>-0.10</td>
<td>0.13*</td>
<td>0.20***</td>
<td>0.36***</td>
<td>0.16*</td>
<td>-0.04</td>
<td>-0.27***</td>
<td>0.00</td>
<td>-0.23***</td>
<td>-0.44***</td>
</tr>
</tbody>
</table>

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

Protein contents of individual elution regions (ER) are provided in Table 5.

GeogDist = Geographic distance between snake locations

Genetic distances are computed from mtDNA gene cyt-b (GenDist-cyt-b)

Environment principle components correspond to the following factors (with negative or positive association with PC indicated parenthetically): Environmental PC (EnvironPc) elevation(+), precipitation(+), temperature(-); Vegetation PC (VegPC, for southern snakes only) = normalized difference vegetation index, NDVI(+).
Appendix 2. Results of simple and partial Mantel analyses of associations among Pacific Rattlesnake (Crotalus oreganus ssp.) venom principal components (PCs) and geographic distance, genetic relatedness, and environmental principal components. Matrices held constant as controls are indicated within parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Venom PC1</th>
<th>Venom PC2</th>
<th>Venom PC3</th>
<th>Venom PC4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Snakes: Simple Mantel (N = 69)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GeogDist</td>
<td>-0.11*</td>
<td>0.09</td>
<td>-0.11</td>
<td>0.00</td>
</tr>
<tr>
<td>GenDist-cyt-b</td>
<td>0.36***</td>
<td>-0.29***</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Enviro PC</td>
<td>-0.34***</td>
<td>0.02</td>
<td>0.03</td>
<td>-0.11**</td>
</tr>
<tr>
<td><strong>All Snakes: Partial Mantel (N = 69)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GeogDist (x Enviro PC x GenDist-cyt-b)</td>
<td>0.07</td>
<td>-0.10</td>
<td>-0.10</td>
<td>0.07</td>
</tr>
<tr>
<td>GenDist-cyt-b (x Enviro PC x GeogDist)</td>
<td>0.36***</td>
<td>-0.30***</td>
<td>-0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Enviro PC (x GenDist-cyt-b x GeogDist)</td>
<td>-0.36***</td>
<td>0.01</td>
<td>0.05</td>
<td>-0.14**</td>
</tr>
<tr>
<td><strong>South Snakes: Simple Mantel (N = 38)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GeogDist</td>
<td>-0.25**</td>
<td>0.05</td>
<td>0.13*</td>
<td>0.04</td>
</tr>
<tr>
<td>GenDist-cyt-b</td>
<td>0.52***</td>
<td>-0.03</td>
<td>-0.14</td>
<td>-0.09</td>
</tr>
<tr>
<td>Enviro PC</td>
<td>-0.33***</td>
<td>0.12*</td>
<td>-0.11</td>
<td>-0.12</td>
</tr>
<tr>
<td>Veg PC</td>
<td>-0.41***</td>
<td>-0.12</td>
<td>0.15*</td>
<td>-0.02</td>
</tr>
<tr>
<td><strong>South Snakes: Partial Mantel (N = 38)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GeogDist (x Enviro PC x Veg PC x GenDist-cyt-b)</td>
<td>0.16**</td>
<td>-0.01</td>
<td>0.02</td>
<td>-0.01</td>
</tr>
<tr>
<td>GenDist-cyt-b (x Enviro PC x Veg PC x GeogDist)</td>
<td>0.48***</td>
<td>-0.03</td>
<td>-0.08</td>
<td>-0.09</td>
</tr>
<tr>
<td>Enviro PC (x GenDist-cyt-b x GeogDist x Veg PC)</td>
<td>-0.37***</td>
<td>0.13</td>
<td>-0.14</td>
<td>-0.14*</td>
</tr>
<tr>
<td>Veg PC (x GenDist-cyt-b x GeogDist x Enviro PC)</td>
<td>-0.44***</td>
<td>-0.12</td>
<td>0.12</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

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

Venom principle components correspond to the following elution regions (ER) named in Appendix 1 (negative or positive association with Venom PC is indicated parenthetically): PC1 = ER11(+), ER1(+), ER8(+), ER10(+), ER4(-), ER5(-); PC2 = ER7 (+), ER3(+); PC3 = ER6(+), ER9(-); PC4 = ER2(-). Protein contents of individual elution regions are provided in Table 5.

GeogDist = Geographic distance between snake locations

Genetic distances are computed from mtDNA gene cyt-b (GenDist-cyt-b)

Environment principle components correspond to the following factors (with negative or positive association with PC indicated parenthetically): Environmental PC (EnvironPc) = elevation(+), precipitation(+), temperature(-); Vegetation PC (VegPC, for southern snakes only) = normalized difference vegetation index, NDVI(+).
CHAPTER FOUR

DIET AND VENOM ONTOGENY IN INSULAR AND HIGH-ALTITUDE POPULATIONS OF THE SOUTHERN PACIFIC RATTLESNAKE (CROTALUS OREGANUS HELLERI)

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Abstract

As one of the most medically significant snake species in North America, the southern Pacific rattlesnake (*Crotalus oreganus helleri*) exhibits unusually pronounced geographic variation in venom composition. Ontogenetic variation in lethality and enzyme activities has also been documented in the taxon. Transition from the more toxic, less proteolytic venom of juveniles to the less toxic, more proteolytic venom of adults has been associated with a shift from lizard to rodent prey as the snake grows. However, detailed analyses of diet and venom composition at the population level are lacking. In this study, we compared the diet and venom composition of two age classes of *C. o. helleri* from two populations occurring in distinctly different environments and having very distinctive venoms. Adult *C. o. helleri* on Santa Catalina Island express proteolytic venom typical of the species, whereas adults in the San Jacinto Mountains possess neurotoxic venom that has been interpreted as paedomorphic—the retention of a juvenile characteristic (highly toxic venom) into adulthood. We hypothesized that 1) if diet influences venom composition, then diet ontogeny will differ substantially between the proteolytic and neurotoxic populations, and 2) that venom of the neurotoxic population will exhibit less ontogenetic change between juvenile and adult snakes than that of the proteolytic population. Stomach and fecal samples indicated that snakes from the insular population (Santa Catalina Island) and the montane population (San Jacinto Mountains) had similar diets, consuming mainly lizards when young and incorporating more rodents as adults. Reversed-phase HPLC chromatograms confirmed that venom composition differed substantially between populations, with both juvenile and adult Santa Catalina Island snakes expressing high levels of metalloproteinases and no PLA$_2$ neurotoxins,
whereas trace amounts of metalloproteinases and substantial expression of Mojave toxin were seen in both age classes in the San Jacinto Mountains population. Snakes in both populations also showed fairly substantial changes in venom composition during ontogeny, but with similar effect sizes. High toxicity (expression of PLA$_2$ neurotoxins) in the venom of both juvenile and adult rattlesnakes has been interpreted as paedomorphism. However, we question this interpretation because the overall level of ontogenetic change in venom was similar in the two populations. The question remains whether venom paedomorphism should be interpreted on the basis of ontogenetic changes in overall venom composition, or ontogenetic changes in venom function (relative toxicity and proteolytic activity).

**Introduction**

Pitvipers rely on venom for both prey acquisition and defense against predators. These essential ecological functions have allowed selection to shape pit viper venoms into complex cocktails of toxins that can be fine-tuned to the snakes’ specific predatory or defensive needs. On a broad scale, venom composition of viperid snakes is strongly influenced by phylogeny (e.g., Tan and Ponnadurai, 1990, 1991). Nevertheless, intraspecific venom variation occurs among geographic localities (Glenn et al., 1983; Alape-Girón et al., 2008; Núñez et al., 2009), between sexes (Marsh and Glatston, 1974; Mebs and Kornalik, 1984; Menezes et al., 2006), and within individuals over time (Mackessy, 1985, 1988, 1993a,b; Mackessy et al., 2003; Alape-Girón et al., 2008; Calvete et al., 2010, Gao et al., 2013).

Snake venom proteins exhibit variable effects and toxicities upon injection into
different animal taxa (e.g., Heatwole et al., 1995, 1998; Daltry et al., 1996b; Mackessy et al., 2006; Barlow et al., 2009; Gibbs and Mackessy, 2009). Venom composition, therefore, can be optimized for primary prey types, and prey type availability has been proposed as a primary driver of venom variation (e.g., Daltry et al., 1996a,b, Barlow et al., 2009). Rattlesnakes, like many other snakes, often exhibit an ontogenetic shift from preying predominantly on small ectotherms as juveniles, toward larger endothermic prey as adults (e.g., Mackessy, 1988; Taylor, 2001; Hollycross and Mackessy, 2002; Mackessy et al., 2003; LaBonte, 2008; Glaudas, et al., 2008). In type I rattlesnakes, juveniles possess highly toxic venom profiles thought to be especially effective in immobilizing ectothermic prey, whereas adult venoms are dominated by metalloproteases and serine proteases which may facilitate venom distribution and pre-digestion of larger prey items (Mackessy, 2003). The ontogenetic shift in venom generally commences a short time after the ontogenetic shift in prey preference (Mackessy, 2003). Type II rattlesnakes express highly toxic venoms both as juveniles and as adults. This phenomenon, has been interpreted as paedomorphosis—the retention of juvenile characteristics into adulthood (Mackessy et al., 2003).

As one of the most medically significant snake species in North America (Parrish et al., 1964; Wingert and Chan, 1988; Bush et al., 2002; Seifert et al., 2009), the southern Pacific Rattlesnake (Crotalus oreganus helleri) ranges from southern California (USA) southward to Baja California Norte (Mexico). An isolated population also occurs on the Pacific Ocean island of Santa Catalina (Los Angeles County, California; Klauber, 1997), approximately 35 km offshore. Although some rattlesnake species within the region lack an ontogenetic shift in diet (Dugan and Hayes, 2011), a shift from lizards to rodents with
snake growth has been documented in *C. o. helleri* for specimens collected from the mainland (Mackessy, 1988). Prior studies have also revealed ontogenetic changes in toxicity, enzymatic activity, and relative expression of key proteins in this and several other *C. oreganus* subspecies (Mackessy, 1988, 1993, 1996). More recent work has shown substantial geographic variation in the venoms of adult *C. o. helleri* (Sunagar, 2014). However, no detailed whole venom protein profiles have been described for juveniles in the species.

In this study, we compared the diet and venom composition of two age classes of *C. o. helleri* from two populations (Fig. 8) experiencing very different climates and having highly distinctive venoms. Snakes from Santa Catalina Island live in a Mediterranean climate dominated by coastal sage scrub interspersed with chaparral and oak woodland (Schoenherr, 1992). They also average smaller body size than conspecifics on the mainland (Carl Person et al., unpubl. data). Although the island has never been connected to the mainland, fossil evidence from nearby islands suggests that rattlesnakes have been present since the Pleistocene (Guthrie, 1993). Venom composition of adult snakes resembles that of type I viperid venoms with relatively high metalloprotease activity and low toxicity (*LD*$_{50}$ >1 µg/g mouse body weight; Sunagar et al., 2014).

Mainland snakes from the San Jacinto Mountains (Riverside County, California), in contrast, live at much higher elevation (1,000-3,000+ m) in a highly variable thermal environment (hot summers, cold winters) where the habitat consists of chaparral and oak forest at lower elevations and coniferous forest at higher elevations (Schoenherr, 1992). Venom composition of adult snakes from the San Jacinto Mountains (including the Idyllwild area) matches that of type II viperid venoms with low metalloprotease activity.
and higher toxicity ($\text{LD}_{50} < 1.0 \ \mu\text{g/g mouse body weight}; \text{French et al., 2004; Sunagar et al., 2014}$). Like other rattlesnakes having type II venoms, they produce a phospholipase A$_2$ (type IIA; Davidson and Dennis, 1990) heterodimeric $\beta$-neurotoxin in their venom (French et al., 2004).

We tested two hypotheses that derive from the profound ecological and venom composition differences between these snake populations. First, we hypothesized that $C. \ o. \ helleri$ from Santa Catalina Island feeds largely on lizards throughout their life, whereas those from the San Jacinto Mountains shift their diet from lizard to rodent prey as they grow in size. Heretofore, no study has examined the diet of snakes from either of these two populations. However, $C. \ o. \ caliginis$ on nearby Isla Coronado Del Sur (Baja
California Norte, Mexico), only 136 km from Santa Catalina Island, feeds primarily on lizards, even as adults (Klauber, 1949, 1997). Given the similarities in climate (offshore islands often enshrouded in mist), prey base (ample lizards and rodents present), and small body size, it seemed reasonable that the two insular populations would have a similar diet that differed from that of mainland snakes. Second, we hypothesized that the venom differences between juvenile and adult snakes would be greater for the type I venoms from Santa Catalina Island than those of the type II venoms from the San Jacinto Mountains.

Materials and Methods

Diet Sampling

We used an integrated approach (Saviozzi and Zuffi, 1997) to collect diet information from preserved museum specimens and from live specimens collected in the field. We obtained 11 samples from Santa Catalina Island snakes, including 10 items from 17 preserved specimens at the Los Angeles County Museum of Natural History (LACM; institutional codes follow Leviton et al., 1985), and another from one of two road-killed specimens donated to us by the Catalina Island Conservancy (Catalina Island, California, USA). We procured 17 fecal samples from 19 live snakes captured in the San Jacinto Mountains. We gently palpated fecal material (from the lower abdomen) through the cloaca of snakes restrained within clear plastic tubes (Hardy and Greene, 1999) and anesthetized with sevoflurane (Halocarbon Products Corp., River Edge, New Jersey, USA). Several snakes, however, simply defecated when brought into the lab. We recorded the snout-vent length (SVL), sex, collection locality, and number and identity of
prey items for each snake. We did not record snake mass since those of preserved specimens were considered unreliable. We stored all diet samples in 70% ethanol.

All prey items were either lizards or mammals. Although lizard scales generally could not be identified to species, we determined the identity of mammal dorsal guard hairs to the lowest taxonomic level possible. We mounted the hairs on glass slides with a cover slip using clear nail polish, and viewed the slides under a light microscope. We compared these with images from published sources (Mayer, 1952; Moore et al., 1974) and with reference samples similarly prepared from a museum teaching collection at Loma Linda University. We used regional faunal lists to ensure consideration of all potential prey items.

**Venom Sampling**

We collected venom via voluntary expulsion (Glenn and Straight, 1982) from two juvenile (28–39 cm SVL) and five adult (60–74.5 cm SVL) *C. o. helleri* from Santa Catalina Island, and from seven juvenile (26–45 cm SVL) and six adult (68–109 cm SVL) snakes from the San Jacinto Mountains. Most of the latter snakes were captured in the Idyllwild area, which is approximately 1600 m above sea level and surrounded by high altitude pine and cedar montane forests. We considered snakes <60 cm snout-vent length (SVL) to be juveniles, and those ≥60 cm to be adults (c.f. Aldridge, 2002).

**RP-HPLC Analysis and Mass Spectrometry**

Following the methods of Sunagar et al. (2014; Chapter 2 of this dissertation), we used reversed-phase high-pressure liquid chromatography (RP-HPLC) to fractionate each
venom sample and generate a protein profile (chromatogram). The RP-HPLC fractions from the venoms of four individuals from four geographic locations were subjected to proteomic analyses (LC-MS and MALDI-ToF/ToF MS/MS) to identify the toxins present. The results, reported elsewhere (Chapter 2 and Sunagar et al., 2014), were used to establish 11 arbitrary RP-HPLC elution regions that corresponded reasonably well (but imperfectly) to 13 major toxins and toxin families identified by mass spectrometry (Fig. 5; Table 5). For each chromatogram, we integrated the area under the absorbance trace to determine the percent of total protein for each elution region (i.e., sum of the area of all peaks in each region relative to total area of the entire chromatogram). We did this after minor visual alignment of individual chromatograms.

**Analyses**

We used binomial logistic regression to compare diet composition (lizards versus mammals) between the two populations while simultaneously assessing snake body size. The sample size (N = 28 prey items) was certainly adequate for this test, but we consider the analysis to be preliminary, as we plan to acquire additional diet samples in the next field season and achieve greater statistical power. We conducted this and all other analyses using SPSS 20.0 for Macintosh (Statistical Package for the Social Sciences, Inc., Chicago, 2011), with alpha = 0.05.

To assess the influence of population and age on venom composition, we subjected protein concentration (integrated peak area) for each of 11 elution ranges to a $2 \times 2 \times 11$ (population $\times$ age $\times$ elution range) analysis of variance model (ANOVA; Green and Salkind, 2005), treating population and age class as between-subjects factors and
protein family as a within-subjects factor. We rank-transformed the data to avoid analysis of percentage data that summed to 100 for each individual. We also ran all tests using log_{10}-transformed data, but the ranks better met normality and homoscedasticity, and results were essentially identical (the one difference is mentioned in Results). The omnibus three-way ANOVA yielded a prohibitively complex three-way interaction. We therefore chose to perform a 2 × 2 (population × age class) ANOVA for each of the 11 elution ranges individually. Data were again rank transformed, but within each elution range individually. Resulting ranks were homoscedastic for every elution range. To examine simple main effects, we conducted independent-samples \( t \)-tests (Green and Salkind, 2005) of the rank-transformed data for pairwise comparisons of age within each population. Following Nakagawa (2004), we chose not to apply Bonferroni adjustments to multiple tests.

Finally, to compare the relative amount of ontogenetic change in each population, we computed the effect size (Cohen's \( d \); Cohen, 1988; Nakagawa and Cuthill, 2007) of age for each of the 11 elution regions separately for each population (using the rank-transformed data). Effect sizes of \(-0.2, \sim 0.5, \geq 0.8\) can be loosely interpreted as small, medium, and large, respectively (Cohen, 1988). We then compared these effect sizes (for all 11 elution ranges) between populations using a paired \( t \)-test (Green and Salkind, 2005). In contrast to statistical significance, measures of effect size are independent of sample size and are especially appropriate here because sample size differed between the two populations. Comparing the number of elution regions having significant age differences could give a misleading comparison of the two populations.
Results

Snake Diet

Rattlesnakes from both Santa Catalina Island and the San Jacinto Mountains consumed a diverse diet that included both lizard and mammal prey (Table 9). The logistic regression model was non-significant ($\chi^2 = 0.13$, $N = 28$, $p = 0.13$, Nagelkerke $R^2 = 0.20$), despite the relatively large effect size ($R^2$ value; Cohen, 1988), modest success in predicting prey type of individual snakes (69.4%), and an effect of snake size that approached significance (Wald = 2.93, df = 1, $p = 0.087$, odds ratio = 1.037, 95 C.I. of odds ratio = 0.995–1.081). Thus, no difference existed between the two populations in primary prey type ($p = 0.22$), as both populations had similar proportions of lizard and mammalian prey (63.6% and 58.8% mammals for Santa Catalina Island and San Jacinto Mountains, respectively; Table 9). Although a larger data set might show an ontogenetic shift in the diet from lizards to mammals, individuals of both juvenile and adult size classes consumed mammals (Fig. 9).

The species of mammals preyed upon differed between the two populations, which reflected availability. However, mice from the genus *Peromyscus* comprised a high proportion of mammalian prey in both populations (57.1%, Santa Catalina Island; 60.0%, San Jacinto Mountains; Table 9).

Venom Composition

Substantial differences in venom composition existed between the two populations. Dominant protein families identified by mass spectrometry in each elution region are presented on representative chromatograms in Figure 10. Chromatograms of
Table 9. Prey items obtained from two populations of the southern Pacific rattlesnake (*Crotalus oreganus helleri*).

<table>
<thead>
<tr>
<th>Prey species</th>
<th>Catalina Island</th>
<th>San Jacinto Mountains</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reptilia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unidentified lizard</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td><strong>Mammalia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Microtus californicus</em></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><em>Otospermophilus beecheyi</em></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><em>Peromyscus californicus</em></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><em>Peromyscus maniculatus</em></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td><em>Peromyscus truei</em></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Rattus</em> sp.</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>Sciurus griseus</em></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>Sorex ornatus</em></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Unidentified rodent</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Total Reptilia</strong></td>
<td>4 (36.4%)</td>
<td>7 (41.2%)</td>
</tr>
<tr>
<td><strong>Total Mammalia</strong></td>
<td>7 (63.6%)</td>
<td>10 (58.8%)</td>
</tr>
<tr>
<td><strong>Total items</strong></td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td><strong>Total snakes</strong></td>
<td>11</td>
<td>16</td>
</tr>
</tbody>
</table>

Additional individuals are shown in Figure 11. Analysis of variance results indicated significant differences between either populations or age groups for nine of the 11 elution regions (Fig. 12). Significant interactions existed between population and age for three elution regions, suggesting contrasting ontogenetic trajectories for the corresponding toxin families within the two populations. Cysteine-rich secretory proteins (CRiSPs) were relatively more prominent in adult venoms from Idyllwild and in juvenile venoms from Santa Catalina Island ($F_{1,16} = 6.45$, $p = 0.022$, partial $\eta^2 = 0.29$). Phospholipases A$_2$ (PLA$_2$) were relatively more abundant in juvenile venoms from Idyllwild and in adult venoms from Santa Catalina Island ($F_{1,16} = 6.99$, $p = 0.018$, partial $\eta^2 = 0.31$). L-amino acid oxidases (LAAO) were also relatively better represented in juvenile venoms from Idyllwild and in adult venoms from Santa Catalina Island ($F_{1,16} = 14.64$, $p = 0.001$, partial $\eta^2 = 0.48$). The population x age interaction approached significance for crotamine ($F_{1,16} = 4.24$, $p = 0.056$, partial $\eta^2 = 0.21$), with the toxin possibly more prominent in adult
Figure 9. Relationships between prey item types (lizards versus mammals) and body size (snout-vent length) of southern Pacific rattlesnakes (*Crotalus oreganus helleri*) from two populations.

Venoms from Idyllwild and in juvenile venoms from Santa Catalina Island. Significant population differences existed among five elution regions (Fig. 12) corresponding largely to bradykinin-potentiating peptides/natriuretic peptide (BIPNP; $F_{1,16} = 5.04$, $p = 0.039$, partial $\eta^2 = 0.24$), non-crotamine small basic peptides (SBP; $F_{1,16} = 14.38$, $p = 0.002$, partial $\eta^2 = 0.47$), Mojave toxin-A (MT-A; $F_{1,16} = 29.10$, $p < 0.001$, partial $\eta^2 = 0.65$), Mojave toxin-B (MT-B; $F_{1,16} = 35.69$, $p < 0.001$, partial $\eta^2 = 0.70$), and snake venom
Figure 10. Annotated RP-HPLC chromatograms of samples subjected to LC-MS/MS analysis. Colored regions represent dominant protein families identified: bradykinin-potentiating peptide/natriuretic peptide (35-56 mL), crotamine (56-61 mL), small basic peptides other than crotamine (61-78 mL), Mojave toxin A subunit/growth factors (78-87 mL), Mojave toxin B (87-95 mL), cysteine-rich secretory proteins (95-97 mL), snake venom serine proteases (97-105 mL), phospholipases A\textsubscript{2} (105-109 mL), lectins (109-111 mL), L-amino acid oxidases (111-123 mL), and snake venom metalloproteases (123-end mL).

Idyllwild snakes produced substantial MT (both MT-A and MT-B subunits) and minute quantities of SVMPs, whereas Santa Catalina Island snakes exhibited only trace amounts of protein in the MT elution region (which likely belonged to other toxin families) but produced amounts of substantial SVMPs. The Santa Catalina population had greater quantities of BIPNP, whereas the Idyllwild population exhibited more SBP.

The ANOVA models yielded one significant main effect of age, and this was for the elution region corresponding to snake venom serine proteases (SVSPs; $F_{1,16} = 12.77$, $p < 0.001$, partial $\eta^2 = 0.80$). Most notably, Idyllwild snakes produced substantial MT (both MT-A and MT-B subunits) and minute quantities of SVMPs, whereas Santa Catalina Island snakes exhibited only trace amounts of protein in the MT elution region (which likely belonged to other toxin families) but produced amounts of substantial SVMPs. The Santa Catalina population had greater quantities of BIPNP, whereas the Idyllwild population exhibited more SBP.

metalloproteases (SVMP; $F_{1,16} = 63.40, p < 0.001$, partial $\eta^2 = 0.80$). Most notably, Idyllwild snakes produced substantial MT (both MT-A and MT-B subunits) and minute quantities of SVMPs, whereas Santa Catalina Island snakes exhibited only trace amounts of protein in the MT elution region (which likely belonged to other toxin families) but produced amounts of substantial SVMPs. The Santa Catalina population had greater quantities of BIPNP, whereas the Idyllwild population exhibited more SBP.
Adults of both populations had significantly greater quantities than juveniles. Based on t-tests within each population, three elution regions differed between age groups in Idyllwild, with fractions corresponding to SBP \((t_{11} = 3.76, p = 0.003)\) and CRiSP \((t_{11} = 2.66, p = 0.027)\) increasing and LAAO decreasing \((t_{11} = 4.24, p < 0.001)\) with age (Fig. 12). Two elution regions differed between age groups for Santa Catalina Island, with fractions corresponding to crotamine decreasing \((t_{5} = 2.89, p = 0.034)\) and SVSP increasing \((t_{5} = 2.89, p = 0.034)\) with age (Fig. 12).

No significant main effects or interactions of population and age were found for the elution regions corresponding largely to crotamine and lectins. However, a significant main effect of age for the MT-B fraction was detected with log\(_{10}\)-transformed data, constituting the only observed difference between rank- and log\(_{10}\)-transformed data. This latter result suggested that MT-B was present in greater quantities in juvenile snakes of both populations, but protein in this elution region for the Santa Catalina population likely belonged to other toxin families.

Effect sizes (Cohen's \(d\)) for age groups among the 11 elution regions are provided for each of the two populations in Table 10. Effect sizes were large (>0.80) for six of the 11 elution regions in each population. A paired t-test comparing effect sizes between the two populations for all elution regions suggested that the magnitude of ontogenetic change in overall venom composition was similar for the two populations \((t = 0.72, p = 0.49, \text{Cohen's } d = 0.31; \text{note the small effect size})\)
Figure 11. Representative RP-HPLC chromatograms of crude venom from each population and age class sampled (only two Catalina Island juveniles were sampled).
Figure 12. Mean (± 1 SE) protein composition of juvenile and adult *Crotalus o. helleri* venom from two populations (Idyllwild, in the San Jacinto Mountains: $N = 7$ and 6, respectively; Catalina Island: $N = 2$ and 5, respectively), as indicated by integration of RP-HPLC peak areas among 11 RP-HPLC elution regions (ER). Dominant protein constituents for each elution region are presented in Table 5. Crosses reveal significant interactions between age class and population (based on ANOVAs); chevrons indicate significant variation between populations (based on ANOVAs); and stars denote significant variation between age classes (based on $t$-tests).

**Discussion**

**Diet Composition**

Our data fail to support our first hypothesis, that Santa Catalina Island *C. o. helleri* feed largely on lizards throughout their lives, whereas those from San Jacinto Mountains shift their diet from lizard to rodent prey as they grow. We observed reptile and mammal prey in similar proportions among all snakes, though this may have been influenced by our relatively small sample size. The effect of snake size on diet, which approached significance, suggested that smaller snakes relied more heavily on reptiles than adult snakes, supporting the results of previous studies (e.g., Mackessy, 1988;
Table 10. Cohen's $d$ effect sizes for venom components (rank-transformed percentages of 11 RP-HPLC elution regions [ER] corresponding to major toxins and toxin families) of two age classes (juvenile vs. adult) of Southern Pacific Rattlesnakes (*Crotalus oreganus helleri*) from two populations.

<table>
<thead>
<tr>
<th>Elution Region</th>
<th>Santa Catalina</th>
<th>San Jacinto Mountains</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-1</td>
<td>1.01</td>
<td>0.00</td>
</tr>
<tr>
<td>ER-2</td>
<td>2.42</td>
<td>0.35</td>
</tr>
<tr>
<td>ER-3</td>
<td>0.00</td>
<td>1.81</td>
</tr>
<tr>
<td>ER-4</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td>ER-5</td>
<td>1.63</td>
<td>1.05</td>
</tr>
<tr>
<td>ER-6</td>
<td>1.06</td>
<td>1.52</td>
</tr>
<tr>
<td>ER-7</td>
<td>2.42</td>
<td>1.17</td>
</tr>
<tr>
<td>ER-8</td>
<td>1.01</td>
<td>1.17</td>
</tr>
<tr>
<td>ER-9</td>
<td>1.53</td>
<td>0.56</td>
</tr>
<tr>
<td>ER-10</td>
<td>1.01</td>
<td>2.69</td>
</tr>
<tr>
<td>ER-11</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Mean ± 1 SE  \[1.20 ± 0.24\]  \[0.94 ± 0.26\]

See Table 5 for elution region protein constituents. Paired $t$-test to compare effect sizes for overall ontogenetic change between Idyllwild and Santa Catalina Island, California, populations: $t = 0.72, p = 0.49$, Cohen's $d = 0.31$.

LaBonte, 2008), but this shift was similar in both populations. We intend to obtain more data to achieve greater statistical power.

Population Differences in Venom

Our study confirmed the previously identified differences in venom composition between these populations (Sunagar et al., 2014). Most notably, Idyllwild snakes produced substantial quantities of both MT-A and MT-B subunits, and only minute quantities of SVMPs, whereas Santa Catalina Island snakes exhibited only trace amounts
of protein in the MT elution region (which likely belonged to other toxin families) but produced substantial SVMPs. The Santa Catalina population had greater quantities of BIPNP, whereas the Idyllwild population exhibited more SBP. When both are expressed, MT-A and MT-B subunits form the fully functional Mojave toxin dimer and produce pronounced pre-synaptic neurotoxicity upon envenomation. Introduction of SVMPs into mammalian tissue elicits hemorrhage and proteolysis, resulting in destruction of recipient tissue (e.g. Gutiérrez et al., 2005; Ramos and Selistre-de-Araujo, 2006; Fox and Serrano, 2009; Sajevic et al., 2013; Bernardoni et al., 2014). The two dominant peaks in the BIPNP elution region correspond to non-enzymatic disintegrins crotatroxin 1 and 2, which were previously demonstrated to serve as relocator proteins which allow rattlesnakes to relocate prey following envenomation and release (Saviola, 2014). It would be interesting to learn whether these venom composition differences relate to functional differences in venom effectiveness for securing lizard versus rodent prey.

Similar intraspecific variation in expression of proteolytic (type I) or neurotoxic (type II) venom profiles has also been described in other pitviper taxa including *C. scutulatus* (Glenn and Straight, 1978; Glenn et al., 1983; Massey et al., 2012), *C. mitchellii* subspecies (Glenn and Straight, 1983, 1985), *C. oreganus concolor* (Mackessy, 2003), *C. lepidus* (Borja et al., 2013), *Sistrurus* subspecies (Sanz et al., 2006), *C. horridus atricaudatus* (Minton, 1967; Glenn et al., 1994), *C. durissus* (Calvete et al., 2009b) and *Bothrops atrox* (Núñez et al., 2009; Calvete et al., 2011). Such cases are often interpreted as paedomorphism (e. g. Mackessy, 2003), possibly resulting from geographic dispersal into areas where venom neurotoxicity confers a selective advantage, such as
through more effective immobilization of new or variable prey types (Calvete et al., 2009b; Núñez et al., 2009; Calvete et al., 2011).

**Ontogenetic Differences**

The data failed to support our hypothesis that the venom differences between juvenile and adult snakes would be greater for the type I venoms from Santa Catalina Island than those of the type II venoms from the San Jacinto Mountains. Snakes in both populations showed fairly substantial changes in venom composition during ontogeny, but the overall change in venom composition between age classes was similar for the two populations. Three elution regions differed significantly between age groups for Idyllwild snakes and two for Santa Catalina Island snakes. The difference in significant elution regions may be explained, however, by the larger sample size for the San Jacinto Mountains snakes, which provided greater statistical power. More importantly, the effect sizes, which are independent of sample size, indicated that both Idyllwild and Santa Catalina venoms exhibited similar levels of overall ontogenetic change in venom. This finding suggests that population differentiation likely has a greater influence on adult venom type than ontogenetic change.

Ontogenetic shifts in venom composition are widely documented across various snake taxa. Among pitvipers, classic examples include shifts from highly toxic venom profiles in juvenile *Crotalus simus* (Calvete et al., 2010), *Bothrops atrox* (Guércio et al., 2006), *B. asper* (Alape-Girón et al., 2008), and *B. jararaca* (Zelanis et al., 2011) to less toxic venom in adults. Such shifts in venom composition often follow ontogenetic shifts in prey preference, supporting claims of the influence of diet on venom.
**Statistical Inferences**

Our analyses of venom composition involved a high number of statistical tests, of which 5% (based on alpha of 0.05) would be expected to be significant by chance alone. Following Nakagawa (2004), we chose not to control for experimentwise error 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). In spite of the high experimentwise error, we feel that our conclusions are robust for each of the two major sets of analyses (ANOVAs of age class × population, and t-tests of age within populations). For the 11 ANOVA models (one for each elution region), three (27.3%) showed interaction between age class and population, which far exceeded that expected by chance, and five (45.5%) showed significant variation between populations. Three (27.3%) of the 11 t-tests for the effect of age class were significant for Idyllwild snakes, and two (18.2%) were significant for the Santa Catalina Island snakes.

**Defining Paedomorphism**

Our data raise the question, what constitutes a paedomorphic phenotype in snake venom? Should we define venom paedomorphism as the relative lack of overall venom composition change, or as a change in the function of venom (e.g., its toxicity or digestive capacity)? In our study, the venoms of both populations changed to a similar degree, with neither showing full retention of juvenile venom composition into adulthood.
Zelanis et al. (2011) demonstrated that N-deglycosylation resolves differences between 2D electrophoretic profiles of juvenile and adult venoms, indicating that N-deglycosylation can be a key source of ontogenetic changes in venom. Such post-translational modifications may not be readily detected by standard proteomic techniques. Functional activity, which will presumably reflect even minor alterations in protein structure, may therefore constitute more reliable criteria for the designation of venom “types.” As such, the purely proteomic data presented in the current study may be insufficient to test whether Idyllwild *C. o. helleri* venom profiles represent a paedomorphic phenotype or simply populational variation. The present study may therefore benefit from the addition of toxicity data to yield a more comprehensive investigation such as those by Mackessy (2003) and Calvete (2011).

**Conclusions**

Our study confirmed the previously identified differences in venom composition between Idyllwild and Santa Catalina Island *C. o. helleri* populations but failed to show significant population differences in diet. The possible role of diet as a driving force for venom differences warrants further study in this group, and a larger diet sample would be informative. Idyllwild snakes produced substantial quantities of both MT-A and MT-B subunits and only minute quantities of SVMPs, whereas Santa Catalina Island snakes exhibited only trace amounts of protein in the MT elution region (which likely belonged to other toxin families), but produced substantial SVMPs. The Santa Catalina population had greater quantities of BIPNP, whereas the Idyllwild population exhibited more SBP. Snakes in both populations also showed fairly substantial changes in venom composition.
during ontogeny, with similar effect sizes. Designation of paedomorphic venom phenotypes based solely on proteomic analysis in the absence of functional data is tenuous due to the substantial influence of post-translational modifications of protein structure to ontogenetic venom variation. Further investigation of the enzymatic activity and general toxicity of the venom samples is, therefore, required in order to establish with certainty whether Idyllwild *C. o. helleri* venoms are a result of paedomorphism or populational variation.

**Acknowledgments**

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References


CHAPTER FIVE

CONCLUSIONS

In this dissertation, I examined the extent of venom variation and neurotoxicity in the Pacific rattlesnakes (*Crotalus oreganus oreganus* and *C. o. helleri*), and assessed factors influencing the observed variation. Together, these studies represent the most thorough investigation of venom composition and variation in the species conducted to date. In this chapter, I will briefly review the principal conclusions drawn from each of my studies and suggest directions for future research.

In *Chapter 2*, I assessed intraspecific variation of venom protein composition among four populations of the southern Pacific rattlesnake, *Crotalus oreganus helleri*. These analyses constituted the proteomics portion of a proteomic/transcriptomic collaboration we conducted with researchers at the University of Queensland, Australia.

As expected, venom profiles varied substantially among the four populations but variation among individual snakes within each population were minor. The most striking disparity in venom composition among the sampled populations was the reduced SVMP expression and expression of large amounts of functional neurotoxin in the San Jacinto mountains population. We documented strong selective pressures acting on all venom protein families tested, yet the strength and direction of these pressures varied dramatically among protein families within a population, as well as among populations within protein families.

These results support those of previous studies. Salazar et al. (2009) documented substantial geographic variation of venom enzymatic activity among *Crotalus oreganus helleri* populations in Southern California which correlate well with the venom
composition we saw in nearby populations. Although precise location data for the snakes were not provided, their map suggests that four of their snakes were collected near populations we investigated in Chapter 2. A snake collected near Loma Linda exhibited pronounced coagulation and fibrinolytic activity, potent lethality, and a notable lack of hemorrhagic activity, consistent with the low metalloproteinase, high serine proteinase, high crotamine expression we noted in our Loma Linda snakes. Similarly, a snake from the high desert near Phelan showed pronounced hemolytic and hemorrhagic activity, and a lack lack of thrombin-like activity and clotting, in line with the phospholipase $A_2$ content, abundant and varied metalloproteinase expression, and serine protease expression that, although similar in abundance to other populations, was less diverse in terms of the number of peaks. The high serine protease, low metalloproteinase content, and high expression of both Mojave Toxin subunits we saw in our Idyllwild snakes coincide well with the pronounced coagulation, low hemorrhagic activity, and potent lethality Salazar et al. noted in a nearby snake, as well as with the venom composition findings of French et al. (2004).

The ecological significance of the noted variation in venom composition among *C. o. helleri* populations remains unclear but the strong selective pressures we saw suggest that contemporary adaptation does seem to be a factor. Phylogeny undoubtedly also plays a role, so it is likely that not every disparity in venom composition among populations will have a clear ecological application. Nevertheless, further investigation of specific toxins’ ecological functions, such as toxicity assays in local prey species as suggested by Sasa (1999), are warranted. It would be interesting, for example, to see whether the serine proteases missing from our Phelan venom profiles but present in the other populations are
responsible for thrombin-like activity or clotting and whether crotamine explains neurotoxicity in snakes lacking Mojave Toxin.

In *Chapter 3*, I conducted a more thorough examination of intraspecific variation of venom composition and neurotoxicity among *C. o. helleri* and *C. o. oreganus* across their ranges in California. Our results revealed substantial geographic variation in the relative abundance of numerous toxin components in *C. oreganus* venom. Southern snakes had consistently higher content of small basic peptides other than crotamine (Other SBPs) in their venom compared to northern snakes. Snakes from the San Jacinto Mountains also possessed a highly distinctive venom composition, which included relatively high levels of Mojave toxin and relatively low levels of BIP/NP, LAAOs, and SVMPs. We showed that environmental factors exert the greatest influence on venom composition, followed by genetic relatedness, and geographic distance, and environmental factors. The results of our bioassay for neurotoxicity varied among the replicates for each sample, failing to consistently identify neurotoxicity even in samples confirmed to contain both Mojave Toxin subunits, and should therefore be considered preliminary.

The observed north-south sorting of venom profiles in this study resembles patterns of venom variation seen in other taxa including the Mojave Rattlesnake, *Crotalus scutulatus scutulatus* (Glenn and Straight, 1978; Glenn et al., 1983; Massey et al., 2012), Tamaulipan Rock Rattlesnake, *Crotalus lepidus morulus* (Borja et al., 2013), and South American Rattlesnake, *Crotalus durissus* spp. (Boldrini-França et al., 2010). Calvete et al. (2012) suggest these trends appear to correlate with the dispersal of rattlesnakes north and south (Klauber, 1997; Campbell and Lamar, 2004) from their

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origin in the Sierra Madre Occidental in north-central Mexico Plateau (Place and Abramson, 2004). The development of neurotoxicity in adult venoms has been interpreted as a paedomorph trend characterized by increased expression of crotoxin along the axis of Crotalus radiation in South America (Calvete et al., 2010; Boldrini-França et al., 2010) and of the closely-related Mojave toxin from Mexico northward (Werman, 2008). A lack of phylogenetic clustering among rattlesnakes with neurotoxin PLA2 molecules in their venoms has been cited as evidence that phylogeny is not a key factor in the development of rattlesnake type II venoms (Powell et al., 2008). Instead, the evolution of neurotoxic venom profiles have been suggested to represent local adaptation to novel prey bases (Powell and Lieb, 2008).

While our results do suggest that environment has a greater effect on venom composition in Pacific Rattlesnakes than phylogeny, and showed no clear geographical pattern in the distribution of neurotoxicity, these data should be considered preliminary and require further investigation. It is possible, for example, that analysis of all populations for neurotoxicity may reveal a trend that was indiscernable in our initial limited data set. Importantly, certain snake venom toxins are known to function differently in avian and mammalian tissues so, although the chick biventer cervicis assay is appropriate for initial analysis of unknown neurotoxins (see Chapter 4 Discussion), parallel trials with the alternative rat phrenic nerve assay may be warranted.

Remote sensing is a powerful tool that has been used extensively for at least four decades to assess distribution of vegetation (Rouse et al., 1974) and, more recently, animal populations on broad spatial extents outside the scope of field-based investigation (Kerr and Ostrovsky, 2003; Turner et al., 2003). Recent advances in the field have made
more fine-scale analyses feasible; accurate assessments of plant-animal associations on even a 2 m scale have been performed (see, for example, Machault et al., 2014). However, to our knowledge, remote sensing remains under-utilized in fine-scale analysis of snake habitats (see Erbas-White, 2014). This may represent skepticism in the method’s reliability and ecologically relevancy. Verification of our habitat analysis by ground-truthing at each location site may confer greater confidence in our data and contribute to the increasing acceptance of the use of remote sensing in the field.

In Chapter 4, I investigated the influence of snake age and diet on the venom of individual _C. o. helleri_ in an insular population (Santa Catalina Island) and a high-altitude montane population (San Jacinto Mountains). Snakes from both populations had similar diets, consuming mainly lizards when young and incorporating more rodents as adults. Venom composition differed substantially between populations. Snakes in both populations also showed fairly substantial changes in venom composition during ontogeny, with similar effect sizes.

Venoms high in protease activity and low in toxicity, termed ‘Type I’ venoms, are common among adult rattlesnakes, especially in heavy-bodied species. ‘Type II’ venoms, by contrast, are low in protease activity and high toxicity (often neurotoxicity), and are less common in adult rattlesnake venoms. Most, if not all, rattlesnake venoms exhibit ontogenetic change in venom composition, typically from more Type II-like profiles to less toxic Type I-like profiles (Minton, 1967; Fiero et al., 1972; Reid and Theakston, 1978; Lomonte et al., 1983; Minton and Weinstein, 1986; Mackessy, 1988; Gutiérrez et al., 1991; Mackessy et al., 2003; Mackessy, 2008). Retention of highly toxic, non-proteolytic venom in adults, therefore, has been interpreted as a paedomorphic trait.
Type I venoms presumably aid in the digestion of the larger prey items typically consumed by adult heavy-bodied snakes, while Type II venoms are suspected to aid in the rapid immobilization of small ectothermic prey typically consumed by juveniles and smaller adults. These hypotheses seem to be supported by ontogenetic shifts from ectotherm-dominated diets in many juvenile rattlesnakes to mammalian-dominated diets in adults, which are often followed by subsequent shifts from highly toxic to proteolytic venoms.

Although Mackessy (2008) notes that Type II species do exhibit a degree of ontogenetic change in composition, references to paedomorphism have, to our knowledge, been limited to the context of Type I species. The lack of obvious Type II-like venom profiles in juvenile snakes on Catalina Island, together with the substantial ontogenetic shift in venom composition in the classic Type II San Jacinto Mountains population (nearly equal to that of the Catalina Island venoms), lead us to question the validity of the current paedomorphism paradigm. Are all highly toxic rattlesnake venoms to be considered ‘juvenile’ and all proteolytic profiles ‘adult?’ Given the high proteinase expression in the venom of our juvenile Santa Catalina Island *C. o. helleri,* and the substantial ontogenetic venom shift in the supposed paedomorphic San Jacinto Mountain population, we agree with Mackessy (2008) that the current current Type I-Type II model drastically oversimplifies the issue.
References


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