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Emily Grabowsky
egrabowsky276@gmail.com

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UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

VENOM COMPOSITION OF LITTLE KNOWN MOUNTAIN RATTLESNAKES AND PREDATOR-PREY INTERACTIONS OF *CROTALUS PRICEI PRICEI* AND ITS NATURAL PREY, *SCELOPORUS JARROVII*

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

Emily Rose Grabowsky

College of Natural and Health Sciences
School of Biological Science

December 2018

This Thesis by: Emily Rose Grabowsky

Entitled: *Venom composition of little known mountain rattlesnakes and predator-prey interactions of Crotalus pricei pricei and its natural prey, Sceloporus jarrovii*

has been approved as meeting the requirement for the Degree of Master of Science in College of Natural and Health Sciences in the School of Biological Science

Accepted by the Thesis Committee:

Stephen Mackessy, Ph.D., Chair

Mitchell McGlaughlin, Ph.D., Committee Member

Jessica Salo, Ph.D., Committee Member

Richard Hyslop, Ph.D., Committee Member

Accepted by the Graduate School

Linda L. Black, Ed.D.
Associate Provost and Dean
Graduate School and International Admissions
Research and Sponsored Projects

ABSTRACT

Grabowsky, Emily Rose. *Venom composition of little known mountain rattlesnakes and predator-prey interactions of* Crotalus pricei pricei *and its natural prey,* Sceloporus jarrovii. Unpublished Master of Science Thesis, University of Northern Colorado, 2018.

The *Crotalus intermedius* clade is a small group composed of four species: *C. intermedius, C. transversus, C. pricei,* and the recently identified *C. tancitarensis* (Alvarado-Díaz and Campbell, 2004; Reyes-Valesco et al. 2013). Though these species are restricted to high elevations of Sky Islands of southern Arizona and throughout México, little has been reported about their natural history and basic biology, including venom composition. Specifically, the Western Twin-spotted Rattlesnake (*C. pricei pricei*) is a small lizard specialist restricted to the more northern Sky Islands of México, with isolated populations in southern Arizona, where they are a protected species. *Crotalus p. pricei* is restricted to high elevations, dispersal between mountain tops is impossible, and few studies have investigated venom composition, the predator-prey relationship between *C. p, pricei* and its primary prey source, Yarrow's Spiny Lizard (*Sceloporus jarrovii*), or ecological variables impacting distribution. This project aimed to characterize the venom of species within the *Crotalus intermedius* clade and the trophic and distributional relationships between *S. jarrovii* and *C. p. pricei* by using venom analysis techniques, lethal toxicity assays, and species distribution modeling techniques. Reverse-phase high-performance liquid chromatography (RP-HPLC), gel electrophoresis, and several enzyme

assays were used to identify compounds present in crude venom. Lethal toxicity assays were used to determine venom lethality towards *Hemidactylus frenatus.*(House Gecko) and *S. jarrovii*. Resource selection probability functions (RSPF) were evaluated to determine spatiotemporal ecological requirements of both *C. p. pricei* and *S. jarrovii*. The results of this study provides insight into the venom composition of little known, mountain rattlesnakes, the coevolutionary relationship between a lizard specialist (*C. p. pricei*) and its natural prey (*S. jarrovii*), and novel information on the likely distribution of both species based on ecological requirements. This information provides a solid basis for future land management and conservation plans concerning the unique habitats and fauna of the Sky Island ranges.

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INTRODUCTION

Background

There are approximately 200 species in the family Viperidae, 190 of which are within the subfamily Crotalinae (pit vipers) (Castoe and Parkinson 2006). Several of these snakes have been extensively studied due to the incidence of snake bites by these species (Gutiérrez et al., 2006; World Health Organization, accessed October 8, 2018). However, very little is known about many species of rattlesnakes found in the Americas, particularly those that are less common and have infrequent contact with humans, including many species in México. High elevation, montane rattlesnakes in the *Crotalus intermedius* group range from southeastern Arizona in the U.S. and through the Sierra Madre Occidental and Sierra Madre Oriental of México. To date, few studies have examined the venom composition and ecology of these species.

The evolution of venoms among squamate reptiles has allowed advanced Caenophidian viperid and elapid snakes to switch from a more primitive mechanical mode (constriction) to a chemical means (venom) of immobilizing prey (e.g., Kardong et al., 1997). These venoms are composed of various complex molecules including enzymatic proteins, non-enzymatic proteins, and peptides (Mackessy, 2008). This variety of components allows for the diverse and typically debilitating physiological effects seen upon envenomation. Patterns of toxin occurrence in rattlesnake species can be further

isolated to organize toxin abundances by characteristic activities and acute toxicity. Venom can generally be classified into one of two groups based on the toxins present and pathology of a bite: type I or type II venom (Mackessy, 2008; Mackessy, 2010a). These venoms can be differentiated by the presence of P-I, P-II, and P-III metalloproteases and lower toxicity (type I) or lack of significant metalloprotease activity and instead, high toxicity due to the presence of presynaptic neurotoxins and myotoxins (type II) (Mackessy, 2010a). Metalloproteases are responsible for tissue damage and hemorrhagic symptoms caused by lysis of blood cells, while myotoxins are responsible for rapid prey immobilization, causing muscle necrosis and paralytic effects (Mackessy, 2008; Mackessy, 1996; Gutierrez et al., 2000).

Mountain Rattlesnake Phylogeny

Rattlesnakes belong to the genera *Crotalus* and *Sistrurus* and range from southern Canada to Argentina. There are at least 47 species of rattlesnakes within these genera (Klauber, 1972; Reptile Database, accessed September 30, 2018), many of which remain poorly known. Approximately 42 species of rattlesnakes are native to México, with many of these species associated with the Méxican Plateau and other high elevation regions (The Reptile Database, accessed September 30, 2018). Recently, several studies have investigated the phylogeny of these species, which allows us to focus research on ecological relationships, potential medicinal applications, and conservation importance of Méxican rattlesnake species from an evolutionary perspective.

Based on several studies that used various mitochondrial and nuclear markers for phylogenetic analyses, the *Sistrurus* and *Crotalus* clade likely originated in the montane pine-oak forests associated with major mountain ranges in México and diversified

relatively quickly (Blair and Sánchez-Ramírez, 2016; Reyes-Velasco et al., 2013). This idea is supported by the basal phylogenetic placement of many high-elevation species such as *C. pricei, C. intermedius,* and *C. transversus* (Blair and Sánchez-Ramírez, 2016; Reyes-Velasco et al., 2013). Many of these species are endemic to these high elevation, often isolated, biodiversity hotspots (Peterson and Navarro-Sigüenza, 1999), another reason additional research and understanding are necessary.

Within these biodiversity hotspots in North and Central America, species are still being described, and in 2004 *Crotalus tancitarensis* was officially described by Alvarado-Díaz and Campbell (Alvarado-Díaz and Campbell, 2004). *Crotalus tancitarensis* is endemic to Cerro Tancítaro in Michoacán, México and was previously thought to be part of the *C. intermedius* species because of the apparent similarities in morphology and habitat. However, this isolated population of snakes occurs only on Cerro Tancítaro, the highest point in the state of Michoacán, over 300 km from the nearest population of *C. intermedius* (Campbell, 1982). Although research methods have advanced significantly and the understanding of rattlesnake ecology and evolution has improved in general, there are still many questions regarding these poorly understood montane rattlesnake species. In contrast, the venom composition and ecology of more broadly distributed rattlesnake species have been extensively explored (e.g., Mackessy 2008, 2010a, 2010b; Sanz et al., 2006; Saviola et al., 2015). However, few studies have explored ecological drivers of venom composition and the implications of these drivers (i.e., vegetation, precipitation, temperature, prey occurrence, etc.).

Crotalus pricei is divided into two subspecies, primarily inhabiting montane areas of the Sierra Madre Occidental. The nominate subspecies, *Crotalus pricei pricei*

(Western Twin-spotted Rattlesnake), is widely distributed in the Sierra Madre Occidental, from southeastern Arizona, USA, and northeastern Sonora/western Chihuahua, México, south to Aguascalientes and Durango, México (Campbell and Lamar, 2004). Within the United States, *C. p. pricei* is found exclusively in montane, talus ecosystems on Sky Islands of only four mountain ranges (Prival and Schroff, 2012). A second subspecies, *Crotalus pricei miquihuanus* (Eastern Twin-spotted Rattlesnake), is found in higher montane regions of the Sierra Madre Oriental in Coahuila, Nuevo Leon, Tamaulipas and San Luis Potosí, México (Armstrong and Murphy 1979; Campbell and Lamar, 2004). Although *C. p. pricei* is protected in Arizona and is threatened by climate change, human activity, and illegal collection (Prival and Schroff, 2012; Kupfer et. al., 2005), few studies have focused on ecology of these snakes.

Climate Change Effects on Rattlesnakes

As ectotherms thermoregulating via external environmental parameters, reptiles are particularly vulnerable to changing conditions resulting from rapid climate change. It is estimated that over half of the observed increase in global surface temperature between 1951 and 2012 was caused by anthropogenic factors, resulting in a net temperature increase of about 0.89 °C in the twentieth century (IPCC, 2013). This could have negative implications for ectotherms, such as rattlesnakes, if this warming trend continues, because adaptation to new conditions may not be possible. Relatively few studies examining possible effects of climate change on reptiles have been conducted, but those published have found that there is a clear correlation between temperature increase and either significant distribution shift or reduction in range size, depending on the

species (Barrows 2011; Boyle et al., 2016; Ceia-Hasse et al., 2014; Davis et al., 2015; Douglas et al., 2016; Lawing and Polly 2011; van Riper et al., 2014).

In particular, mountain rattlesnakes face environmental constraints from their upper and lower elevation limits. Madrean pine-oak ecosystems only exist as interspersed habitat on sky-islands in the southern United States and in México. The federally threatened New Mexico Ridge-nosed Rattlesnake (*Crotalus willardi obscurus*) is constrained to a narrow niche in several sky island mountains and is threatened by increases in both climate change-induced temperatures and wildfires (Davis et al., 2015). *Crotalus p. pricei* is restricted to elevations between 1600 and 2700 meters, and in the U.S., it is only distributed across four Sky Island ranges in Arizona, likely facing issues similar to the New Mexico Ridge-nosed Rattlesnake. *Crotalus p. pricei* inhabit and are restricted to higher elevations more than any other Arizona rattlesnake, making them a unique study organism. Previous studies have shown the species' northward range expansion into North America during the last glacial maximum (LGM) approximately 25,000 years BCE (Bryson et al., 2011). Some climate projection models expect temperatures to increase by 6 °C by the end of the 21st century, over six times as quickly as temperature rose in the 20th century (IPCC, 2013). Other predictive models suggest that climate change-driven precipitation variation could severely alter vegetation at higher elevations (Kupfer et. al., 2005). If these models are accurate, Madrean pine-oak forests may be greatly reduced in size over the next several decades. Though little is known about *C. intermedius, C. transversus,* and *C. tancitarensis,* these montane rattlesnakes could face similar threats.

Twin-Spotted Rattlesnake (*Crotalus pricei*)

Ecology. *Crotalus p. pricei* is a small-bodied member of the viper family best known for its unique morphology and habitat requirements. Because this rattlesnake is so unique, it has been a target for wildlife trafficking since before 1960 (Prival and Schroff, 2012). Few comprehensive studies have been conducted on *C. p. pricei* ecology, but thanks to a long-term study of a population in the Chiricahua Mountains (Prival and Schroff, 2012), we now have information that can be used to plan future studies and to further analyze the conservation status of this species.

Crotalus p. pricei are small snakes, reaching an average snout to vent length (SVL) of 388 mm and a mass of 40 g. There is apparent sexual dimorphism, with males being slightly longer and heavier than non-gravid females. This could be a result of sexes allocating energy resources to different biological functions such as reproduction, rapid growth, or other unknown variables (Amarello et. al., 2010; Prival and Schroff, 2012). *Crotalus p. pricei* use chemosensory cues to track prey, but this prey tracking method was not evident when tested on lab mice as compared to a small mammal specialist (*C. viridis*) (Cruz et. al., 1987). Gravid females eat only rarely (3.9% palpated had food boli) and move very little during their six-week gestation period. After these females give birth between early July and late August, they resume normal feeding patterns. Because reproduction is a biologically expensive task, females rarely give birth in consecutive years, and generally skip a year or two between litters (Prival and Schroff, 2012).

Crotalus p. pricei individuals seem to remain near the same southeast-facing talus slope but exhibit no obvious microhabitat site fidelity. Their high elevation habitats often experience extreme temperature fluctuations, and *C. p. pricei* have been observed to be

surface-active at air temperatures as low as 8.3 °C (Prival and Schroff, 2012). Despite their apparent hardiness, *C. p. pricei* are limited to very specific, higher elevation habitat and are most commonly associated with talus.

Diet

Evaluation of fecal samples determined that lizards (*Sceloporus sp.*) compose approximately 68%-87% of *C. p. pricei* diet, and mammals make up 13%-32%, depending on age class (Prival and Schroff, 2012). Adults are more likely than juveniles to consume mammals, possibly due to gape limiting prey size that can be accommodated. Approximately 18% of the fecal samples were identified to species level, and all were *Sceloporus jarrovii* (Prival and Schroff, 2012). Yarrow's Spiny Lizard (*Sceloporus jarrovii*) is broadly distributed throughout Arizona, but it is only one of five *Sceloporus* species (*S. slevini, S. jarrovii*, *S. magister*, *S. cowlesi*, *S. clarkii*) with a distribution overlap with *C. p. pricei* in Arizona and the only species that retains high population numbers at high elevations (Jones and Lovich, 2009). Of the five species, *S. jarrovii* is found most frequently at higher elevations, directly syntopic with *C. p. pricei.*

Distribution and Habitat

High elevation, montane regions inhabited by *C. pricei* are referred to as Sky Islands that range from Arizona through México, creating a fragmented landscape of higher elevation forests disconnected by lower elevation desert. A swift change in ecosystem vegetation occurs as elevation increases, making Sky Islands extremely species-rich. Desert scrub, chaparral, pine-oak scrub, montane scrub, and mixed deciduous forests can all be found in one mountain range (Arriaga et. al., 2005). Based upon packrat midden carbon dating, Sky Island geographic formations did not exist until

approximately 18,000 years before present (Thompson and Anderson, 2000). Before this, the southwestern landscape of North America was primarily forested, quite different from the current desert ecosystem, but as the Earth's climate changed, the lower elevation forests became deserts, isolating the more mesic forests on higher elevation Sky Islands (Thompson and Anderson, 2000). These unique ecosystems can present a number of challenges for native species, including geographically-limited genetic variation and restricted distributions due to rapidly changing climate gradient.

Conservation Concerns

Although specific climate change studies have not been conducted for high elevation rattlesnakes, we can anticipate that biome alterations will occur by using climate change models to predict effects on biodiversity. Climate changes can have varying impacts on vegetation distribution depending on the types and magnitude of changes. According to a vegetation climate model analysis by Kupfer et al. (2005), if only temperature were to increase, one would see an upward shift in montane habitat and an increase in desert scrub habitat. If only precipitation increased, a downward shift in montane habitat would be likely. If both temperature and precipitation were to increase, one would expect a mixture of these scenarios to occur. These complex changes would result in community disruption, depending on the magnitude and speed of the change (Kupfer et. al., 2005). Reports on negative repercussions of climate change on high elevation species are already evident in the primary literature. For example, Pika (*Ochotona princeps*) populations have been declining due to climate change and other human-induced impacts. Like *C. p. pricei*, Pikas specifically inhabit high elevation talus slopes and have little room to migrate higher in elevation to avoid warming temperatures

(Beever et al., 2003). As an ectotherm, *C. p. pricei* relies on external conditions to regulate body temperature and is already likely geographically and genetically isolated between mountain ranges (Fahrig 2003; Favé et al., 2015; Lomolino et al., 1989). This could lead to detrimental population impacts if habitat is negatively influenced by climate and human pressures, similar to the effects seen with Pika populations.

Illegal collection and human-perpetuated habitat destruction are two additional major concerns for the persistence of this species, even though *C. p. pricei* are protected in both Arizona and México. Talus slopes are an obvious landmark that are easy to pick out for those who know general habits and habitat preferences of *C. p. pricei*. During a two-year period, 24 people were seen searching for *C. p. pricei* at one site, and in 2007, a PIT-tagged snake from the Prival and Schroff (2012) study was offered for sale in a pet store on the east coast. It is predicted that up to 90 collectors could visit this study site per year using information that has been generally available for many decades (see, for example, Kauffeld, 1957). This long-term study site appears to maintain a stable population, but after extrapolating data onto all possible habitat within the Chiricahua Mountain Range, *C. p. pricei* population size may be between 1000-4000 individuals. However, this is a rough estimate, and it should be noted that the largest *C. p. pricei* population observed was approximately 64-77 individuals (Prival and Schroff, 2012). Besides collecting, humans have made a direct impact on *C. p. pricei* by increasing cattle grazing, mining, recreation, and vehicle traffic in the Sky Islands region. It was estimated that 300,000 people moved to the Sky Islands between 1994 and 2004 (Gottfried and Hodges, 2005), and that number has likely significantly increased since then.

Surprisingly, this species is not currently considered threatened or endangered according to the United States Fish and Wildlife Service (USFWS) or the International Union for Conservation of Nature Red List of Threatened Species (IUCN, 2017), despite specific habitat requirements, limited range, conservation concerns (including illegal collection), and predicted habitat reduction due to climate change (Prival and Schroff, 2012; Beever et. al., 2003). In comparison, the New Mexican Ridge-nosed Rattlesnake (*Crotalus willardi obscurus*) is listed as threatened under the U.S. Endangered Species Act. This species exhibits similar ecology and inhabits a restricted range in the southwestern U.S. within Sky Islands (Davis et al., 2015).

Venom

Very few studies have analyzed *C. p. pricei* venom. Fangs of this species are relatively short and straight compared to other rattlesnakes; generally, the longer the fang, the more curved it is (Ernst and Ernst, 2012). Although these snakes are small bodied and have small fangs, the venom is thought to be highly toxic. Minton and Weinstein (1984) conducted a preliminary analysis of *C. p. pricei* venom and found no protease activity, but high lethal toxicity (LD_{50}) for lab mice when injected intravenously. More recently, the high toxicity toward mice was confirmed, and numerous enzyme activities, including proteinase activity, were noted for venom of snakes originating in the Chiricahua Mountains (Mackessy, 2008). Because of the limited distribution and contact with humans, bites are not common, but the few that have been recorded reported both local and systemic effects that were more serious than expected (Minton and Weinstein, 1984). Because these preliminary reports indicate a high toxicity venom, and because *C. p. pricei* occurs on several isolated mountain ranges, it may be expected that regional

differences in venom composition may occur, as has been observed with other species (Glenn et al., 1983; Jiménez-Porras, 1964; Mackessy, 2010a).

Tancítaran Cross-Banded Rattlesnake (*Crotalus tancitarensis*)

Crotalus tancitarensis is endemic to Cerro Tancítaro in Michoacán, México and was first officially described by Alvarado-Díaz and Campbell in 2004. This species was previously considered to be part of the *C. intermedius* species, and the first publication to question the identity of this species (Campbell, 1982) discussed the geographically isolated nature of this population (nearest population of *C. intermedius* was 300 km away) and minute morphological differences from *C. pricei, C. intermedius,* and *C. transversus*.

Alvarado-Díaz and Campbell described *C. tancitarensis* using three specimens: one collected in 2002 by Javier Alvarado-Díaz and two previously identified as *C. intermedius*. Isolated geographic location and morphological measurements including body length, tail length, fang length, rattle length, and scale count were used to distinguish *C. tancitarensis* from the previous classification under *C. intermedius* (Alvarado-Díaz and Campbell, 2004). Apart from identifying morphological differences between rattlesnakes found on Cerro Tancítaro and similar species in the *Crotalus intermedius* clade, empirical information regarding *C. tancitarensis* is apparently lacking.

Specific Aims: Chapters II and III

Many venom constituents are shared amongst venomous reptiles. Toxin patterns tend to follow evolutionary lineages, with venoms of snakes in the family Viperidae (rattlesnakes included) sharing many toxins. Though venoms tend to be classified as either type I or type II, each venom contains a complex matrix of peptides and proteins,

and dominant physiological effects on prey are a result of individual toxin variation in a given venom (Mackessy, 2008). Although the generalized dichotomy of type I or type II venom is prevalent, there are several factors that determine venom composition, including prey availability, geographic region, and ontogeny, and exceptions to the rule exist (Mackessy et al., 2003; Mackessy, 2010b; Modahl et al., 2016). This makes placing venoms in one of two categories somewhat simplistic, as even venoms of genetically similar species or subspecies can vary drastically depending on the factors listed above (Sanz et al., 2006). In depth analyses of specific venoms are needed to fully understand their biological potential. Venoms are complex mixtures, and by performing specific biochemical analyses, one can identify compounds and begin to determine the potential biological role(s) of each component (Mackessy, 2008).

Venom composition can be analyzed through several processes used to characterize proteins, described in detail in many studies (e.g., Huang and Mackessy, 2004; Mackessy, 2008; Saviola et al., 2015; Smith and Mackessy, 2016). Gel electrophoresis is a tool used to separate proteins based on molecular weight (denatured with dithiothreitol and sodium dodecyl sulfate) and provides a molecular "fingerprint" of many samples simultaneously. Complementary methods, such as MALDI-TOF mass spectrometry, provide similar, but more precise, descriptive data about smaller peptides in the venoms. Reversed phase high performance liquid chromatography (RP-HPLC) is used to separate proteins based on hydrophobicity, molecule size, or charge. After isolating proteins, one can identify the types of toxins present in a venom sample and decipher the active components that assist in prey incapacitation. As discussed above, rattlesnake venoms can exhibit a variety of toxins that may target specific prey

homeostatic systems. For example, metalloproteases promote degradation of structural proteins and can aid in digestion, while another class of proteins, kallikrein-like serine proteinases, induce rapid hypotension leading to prey immobilization (Mackessy, 2008; Mackessy, 1996).

By analyzing venom samples using the techniques outlined above, we have isolated toxins and performed comparative analyses on toxins present in venom samples from species in the *Crotalus intermedius* group, including *C. pricei* and *C. tancitarensis*. Venom toxins can then be identified, and this process could facilitate discovery of novel toxins or regional and interspecific variation in venom composition. Additionally, lethal toxicity assays (LD_{50}) were performed to identify effect of *C. p. pricei* and *C. tancitarensis* venom on reptiles to address toxicity towards reptiles.

Specific Aims: Chapter IV

Sky Island ecosystems are characterized by high elevation Madrean pine-oak ecosystems surrounded by low elevation deserts, xeric shrubland, and grassland (Arriaga et al., 2005). *Crotalus p. pricei* is typically restricted in these habitats between elevations of 1600 and 2700 meters (Prival and Schroff, 2012). Ectotherms such as *C. p. pricei* are particularly vulnerable to rapid climate change at these high elevations as reported by the Intergovernmental Panel on Climate Change (IPCC 2013). Several studies have identified negative side effects of climate change on reptiles (Barrows 2011; Boyle et al., 2016; Ceia-Hasse et al., 2014; Davis et al., 2015; Douglas et al., 2016; Lawing and Polly 2011; van Riper et al., 2014), but none have examined climatic drivers of potential range shift of *C. p. pricei*. Niche models of high elevation reptiles, such as *C. p. pricei*, must be

conducted or updated to understand the current extent and predicted outcomes of climatic shifts.

In addition to abiotic factors limiting range, biotic factors should also be taken into consideration, particularly since this species has a restricted diet, specializing on lizards in the genus *Sceloporus.* Variation in snake venom composition is thought to be driven by adaptive evolution and can lead to development of prey-specific toxicity in species with specialized diets (Mackessy, 1988; Mackessy et al., 2003, 2006; Barlow et al., 2009). Coevolutionary patterns have been observed between venomous snakes and their prey, typically in areas of high predator and prey abundance or with specialist species (Barlow et al., 2009; Biardi et al., 2006; Poran et al., 1987). For example, California Ground Squirrels compose approximately 69% of the diet of *Crotalus o. oreganus,* and certain populations have developed resistance to *C. o. oreganus* venom toxins in areas of high predator density (Poran et al., 1987).

This type of predator-prey dynamic could prove to be an important driver in resource selection and adaptive venom evolution in the *C. p. pricei* and *S. jarrovii* system, considering the concentrated nature of *C. p. pricei* populations. An ongoing, 17-plus year study by Prival and Schroff has found that approximately 75-85% of *C. p. pricei's* diet consists of *Sceloporus* sp. (depending on age of snake), and all samples that were identified to species were from *Sceloporus jarrovii* (Yarrow's Spiny Lizard). By examining the effect of *C. p. pricei* venom on *S. jarrovii* (Chapter II), the effects of extreme habitat utilization and dependence on prey with a limited distribution can be evaluated for this geographically limited predator. Since the ecology of these two species appears to be intertwined, coevolutionary driving forces need to be studied further to

determine the extent of the relationship. Results of this study have provided empirical evidence outlining habitat requirements for both species and offer insight into climatic factors that may inhibit species persistence.

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CHAPTER II

CHARACTERIZATION OF THE VENOM OF A HIGH ELEVATION LIZARD SPECIALIST, *CROTALUS PRICEI PRICEI* (WESTERN TWIN-SPOTTED RATTLESNAKE)

Abstract

The Western Twin-spotted Rattlesnake (*Crotalus pricei pricei*) is a small lizard specialist restricted to the Sky Islands of Arizona and México. Though this species is restricted to high elevations and dispersal between mountain tops is impossible, few studies have investigated venom composition or the predator-prey relationship between *C. p. pricei* and its primary prey source, Yarrow's Spiny Lizard (*Sceloporus jarrovii*). This project aimed to characterize the venom of *C. p. pricei* and its relationship with *S. jarrovii* by using venom analysis techniques and lethal toxicity assays. Venom analysis included reverse-phase high performance liquid chromatography (RP-HPLC), gel electrophoresis, and several enzyme assays to identify compounds present in crude venom. Lethal toxicity assays were performed on both allopatric and sympatric populations of *S. jarrovii* to determine extent of venom lethality. Results indicate that there is geographic variation in venom composition, primarily between *C. p. pricei* from Durango, México and *C. p. pricei* from Arizona. Lethal toxicity results reveal that *S. jarrovii* has not developed resistance specific to *C. p. pricei* venom but do display a general tolerance to venom of several snakes in the genus *Crotalus*. These results provide insight into the evolutionary relationship between a lizard specialist and its natural prey

in addition to novel information on the venom composition of a little-studied species with a narrow range in the United States.

Introduction

Crotalus pricei (the Twin-spotted Rattlesnake) is a rarely-studied species endemic to México, with a restricted distribution in southeastern Arizona, U.S. (Campbell and Lamar, 2004). The United States distribution of this species is limited to four mountain ranges in southeastern Arizona and little is known about their natural history, population sizes, and venom composition. A handful of studies have provided insight into the demographics (Prival and Schroff et al., 2012) and venom composition on a very basic level (Mackessy, 2008; Minton and Weinstein, 1984), but further evidence is required if proper conservation plans and envenomation protocols are to be completed.

Crotalus pricei, is a small-bodied pit viper endemic to the Sky Islands of Arizona and México. This rattlesnake is unique in both morphology and habitat requirements. Consequently, it has been a target for wildlife trafficking since at least 1960 (Prival and Schroff, 2012). Thanks to a long-term study of a population in the Chiricahua Mountains of Arizona (Prival and Schroff, 2012), we now have information that can be used as a baseline for future studies and to further evaluate the status of this species throughout its range.

Crotalus pricei are small snakes, reaching an average snout to vent length (SVL) of 388 mm and a mass of 40 g, that appear to remain near the same southeast-facing talus slope, but exhibit no obvious microhabitat site fidelity (Prival and Schroff, 2012). Their habitat often experiences extreme temperature fluctuations due to the rapid elevation and climatic changes characteristic of Sky Island environments (Arriaga et al., 2005). These

snakes tolerate a wide temperature range (13.0 °C to 34.0 °C) and can be found basking in the open, moving across rocks, or coiled under vegetation (Campbell and Lamar, 2004; Prival and Schroff, 2012). Despite their apparent hardiness, *C. pricei* are limited to very specific, higher elevation habitat and are most commonly associated with talus.

Due to the habitat and morphological restrictions imposed on this species, *C. pricei* has a specialized diet composed primarily of lizards. Evaluation of fecal samples demonstrated that *Sceloporus sp.* compose approximately 68%-87% of *C. p. pricei* diet, and mammals make up 13%-32% depending on life stage. In contrast to juveniles, adults are more likely to consume mammals, likely due to prey size that can be accommodated. Approximately 18% of the fecal samples were identified to species level, and all were identified as *Sceloporus jarrovii* (Yarrow's Spiny Lizard; Prival and Schroff, 2012). Yarrow's Spiny Lizard is broadly distributed throughout Arizona, but only one of five *Sceloporus* species (*S. slevini, S. jarrovii*, *S. magister*, *S. cowlesi*, *S. clarkii*), has a distribution overlap with *C. p. pricei* in Arizona (Jones and Lovich, 2009). *Sceloporus jarrovii* is thought to retain high population numbers and is most common at higher elevations, directly overlapping with *C. p. pricei* in Arizona.

Crotalus pricei is divided into two subspecies, primarily in montane areas of the Sierra Madre Occidental. The nominate subspecies, *Crotalus pricei pricei*, is widely distributed in the Sierra Madre Occidental, from southeastern Arizona, U.S., and northeastern Sonora/western Chihuahua, México, south to Aguascalientes and Durango, México (Campbell and Lamar, 2004). Within the United States, *C. p. pricei* is found primarily in montane, talus ecosystems on Sky Islands of only four mountain ranges (Campbell and Lamar, 2004; Prival, 2013). A second subspecies, *Crotalus pricei*

miquihuanus (Eastern Twin-spotted Rattlesnake), is found in higher montane regions of the Sierra Madre Oriental in the states of Coahuila, Nuevo Leon, Tamaulipas and San Luis Potosí, México (Armstrong and Murphy, 1979; Campbell and Lamar, 2004).

These montane regions are referred to as Sky Islands and range from Arizona through México, creating a fragmented landscape of higher elevation forests separated by lower elevation desert. A rapid change in ecosystem vegetation occurs as elevation increases, making Sky Islands extremely species-rich and home to many endemic species (Peterson and Navarro-Sigüenza, 1999). Desert scrub, chaparral, pine-oak scrub, montane scrub, and mixed deciduous forests can all be found in one mountain range (Arriaga et al., 2005). Based on packrat midden radiocarbon dating, Sky Island geographic formations did not exist until approximately 18,000 radiocarbon years BP (^{14}C years before present) (Thompson and Anderson, 2001). Before this, the southwestern landscape of North American was primarily forested, quite different from the current xeric ecosystem. As the Earth's climate changed, the lower elevation forests became deserts, isolating the more mesic forests on higher elevation Sky Islands (Thompson and Anderson, 2000). These unique ecosystems can present a number of challenges for native species, including geographically-limited genetic variation and restricted distributions due to a rapidly changing climate and vegetation gradient. Given the unique habitat requirements of *C. p. pricei* and dynamic nature of Sky Island habitats, *C. p. pricei* may face similar threats to other high elevations species such as the Pika that have already experienced significant range decline due to climate change (Beever et al., 2003).

Although *C. pricei* population numbers and genetic diversity could be negatively impacted by various factors including climate change, illegal collection, human-

facilitated habitat destruction, and natural disasters, few studies have examined threats, let alone venom composition of these snakes. This species is not currently considered threatened or endangered according to the United States Fish and Wildlife Service or the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species (IUCN, 2017) despite specific habitat requirements, limited range, and conservation concerns including illegal collection and predicted habitat reduction due to climate change (Prival and Schroff, 2012; Beever et al., 2003). A species with similarly limited range and habitat requirements, the New Mexican Ridged-nosed Rattlesnake (*Crotalus willardi obscurus*), is listed as threatened under the U.S. Endangered Species Act (Davis et al., 2015).

The study of venom has resulted in better understanding of rattlesnake ecology as well as toxin pathology and drivers of venom evolution. The evolution of venom delivery systems have allowed viperid and elapid snakes to transition from mechanical to a chemical means of immobilizing prey (Kardong et al., 1997). These venoms are composed of an array of molecules including enzymatic and non-enzymatic proteins, peptides, and other small molecules (Mackessy, 2008). This variety of compounds produces a diverse range of physiological effects seen upon envenomation. Rattlesnake venoms can generally be classified into one of two groups based on toxins present and pathology of envenomation; type I or type II venom (Mackessy, 2008; Mackessy, 2010). These venoms can be differentiated by moderate to high metalloprotease activity and lower toxicity (type I) versus a near lack of metalloprotease activity and high lethal toxicity (type II) (Mackessy, 2010). Although this generalized dichotomy is commonly observed among rattlesnakes, there are several factors that determine venom composition,

including prey availability, geographic region, and ontogeny (Mackessy, 2010; Modahl et al., 2016). However, very few studies have analyzed *C. p. pricei* venom.

Fangs of this species are relatively short and straight compared to other rattlesnakes; generally, the longer the fang, the more curved it is (Ernst and Ernst, 2012). Although these snakes are small-bodied and have small fangs, the venom appears to be highly toxic. Minton and Weinstein (1984) conducted a preliminary analysis of *C. p. pricei* venom and found no protease activity, but high lethal toxicity (LD_{50}=0.95 µg/g) toward lab mice when injected intravenously. More recently, the high toxicity toward mice was confirmed, and numerous enzyme activities, including high metalloprotease activity, were noted for venom of snakes originating in the Chiricahua Mountains (Mackessy, 2008). Because of the limited distribution and contact with humans, bites are not common, but the few bites that have been recorded reported both local and systemic effects that were more serious than expected (Minton and Weinstein, 1984). These reports of relatively severe symptoms, along with high toxicity in mice, indicate *C. p. pricei* venom could contain toxins generally associated with type II neurotoxic venom. However, biochemical assays conducted on *C. p. pricei* venoms show conflicting results. Minton and Weinstein (1987) found that *C. p. pricei* venom had little protease activity, but Mackessy (2008) found both metalloprotease and serine protease activities to be relatively high. In addition to conflicting evidence of venom composition, these snakes may have venoms that vary in composition and correlate with differential prey use. Given that the diet of *C. p. pricei* primarily consists of *S. jarrovii* and these snakes are restricted to high elevation habitat (Prival and Schroff, 2012), adaptive evolution could be leading to either toxin resistance in *S. jarrovii* or increased toxicity of venom towards *S. jarrovii*.

Tight coupling between two species, especially in a predator-prey relationship, may result in coevolution of traits related to predation and escape from predator pressures (Arbuckle et al., 2017; van Valen, 1973), and such a relationship between *S. jarrovii* and *C. p. pricei* may be apparent when venom toxicity and prey susceptibility are analyzed. Because *C. p. pricei* occurs on several isolated mountain ranges, it may also be expected that regional differences in venom composition exist, as has been observed with other species (Mackessy, 2010; Oliveira et al., 2013; Fry et al., 2003).

Coevolution is defined as reciprocal interaction between two or more organisms that leads to evolutionary change (Ehrlich and Raven, 1964). Although difficult to quantify, many studies focus on defining coevolutionary relationships, along with genotypic and phenotypic drivers of these relationships, including those between predator and prey. Predator-prey coevolution, also referred to as the Red Queen Hypothesis (van Valen, 1973), provides a theoretical basis for describing how predator and prey reciprocally adapt to each other, leading to increased overall fitness. This results in continuous change; the predator may not survive without a specific prey item, but the prey species will not persist if it cannot escape the predator (Ehrlich and Raven, 1964; van Valen, 1973). In predator-prey arms race scenarios, it is thought that the selection pressure is stronger on the prey species, given that the deleterious effects of being caught by a predator is immediate (death), while the consequences for the predator not acquiring prey are delayed (hunger) (Brodie III and Brodie Jr., 1999). Many studies have focused on theoretical models and used simple systems to support the theory of reciprocal evolution in antagonistic relationships (Mougi, 2011; Yoder and Nuismer, 2010; Yoshida et al., 2003).

While coevolution as a driving force in an ecological system can be hard to identify conclusively, evidence of prey-specific venom toxicity (Heyborne and Mackessy, 2013; Mackessy et al., 2006; Pawlak et al, 2006, 2009) and venom resistance via multiple physiological mechanisms has been demonstrated in multiple systems (Arbuckle et al., 2017; Barlow et al., 2009; Poran et al., 1987). One of the most studied systems is resistance of garter snakes (*Thamnophis sirtalis*) to tetrodotoxin (TTX) found in their newt prey (*Taricha sp.*) (Brodie III and Brodie Jr., 1990; Feldman et al., 2015). This species, as well as several others that prey on amphibians with TTX, has evolved physiologically altered sodium channels that prevent TTX from binding to the outer pore and inhibiting sodium uptake (Feldman et al., 2012; 2015; Geffeney et al., 2005).

Conversely, toxin resistance has been observed in prey as an adaptation to counter attacks from venomous animals. California Ground Squirrels (*Otospermophilus beecheyi*) exhibit venom resistance when sympatric with large populations of Northern Pacific Rattlesnakes (*Crotalus oreganus*), but they show less resistance when *C. oreganus* population numbers are low (Biardi et al., 2006; Poran et al., 1987). In these populations, *O. beecheyi* comprise up to 69% of *C. oreganus* diet (Poran et al., 1987), indicating that resistance in these squirrels has evolved in response to greater predation pressures by *C. oreganus.*

In both the *T. sirtalis* and *C. oreganus* scenarios, adaptive toxin resistance shows a geographic mosaic pattern similar to other coevolutionary systems (Brodie Jr. et al, 2002; Poran et al., 1987). Given that toxin resistance as an adaptation in predator-prey relationships is present in multiple systems and considering *Sceloporus* comprise

approximately 67.6 % of *C. p. pricei* diet, there is a chance this dynamic is driven by adaptive toxin resistance (Prival and Schroff, 2012).

Hypotheses

To address the knowledge gaps outlined above and to add to the empirical evidence regarding predator-prey relationships and venom composition of *C. pricei*, several hypotheses have been developed.

H_1 *Crotalus pricei* venom will show most characteristics of type I venom (more degradative and highly enzymatic), but may retain properties associated with type II venom (i.e., rapid immobilization/high toxicity).

Prediction Based on previous preliminary work completed by Mackessy (2008), *C. p. pricei* exhibits venom with high enzymatic activity and high toxicity towards mice. Results are expected to be similar between *C. p. pricei* from the various regions analyzed and *C. p. miquihuanus* because of their close phylogenetic relationship (Blair and Sánchez-Ramírez, 2016; Bryson et al. 2010).

H_2 *Crotalus p. pricei* venom will be more toxic to a non-native species (House Geckos; *Hemidactylus frenatus*) than toward *S. jarrovii.*

H_0 There will be no significant difference in *C. p. pricei* venom toxicity towards *Hemidactylus frenatus* and *S. jarrovii.*

Prediction *Crotalus p. pricei* venom will be more toxic to a reptile model organism, *Hemidactylus frenatus*, than to prey found within their range, *S. jarrovii*. Since *Sceloporus sp.* are known to constitute a large portion of the *C. p. pricei* diet, toxin tolerance/resistance may be occurring.

H_3 *Crotalus p. pricei* venom will be more toxic to *S. jarrovii* that are allopatric than *S. jarrovii* that are sympatric with *C. p. pricei.*

H_0 There will be no difference between *C. p. pricei* venom toxicity to *Sceloporus jarrovii* that occur sympatrically and allopatrically.

Prediction *S. jarrovii* individuals within *C. p. pricei* range will show greater tolerance to venom than those outside of the normal *C. p. pricei* range due to resistance mechanisms in prey (Red Queen hypothesis; van Valen, 1973).

H_4 Venom from *C. p. pricei* will show taxon-specific toxicity – that is, lizard prey (*Hemidactylus frenatus* and *S. jarrovii*) will be differentially more sensitive to toxic effects of venom than will mammalian prey model species (*Mus musculus*).

H_0 *Crotalus p. pricei* venom will show similar levels of toxicity to mammalian prey models (*Mus musculus*) and lizard prey models (*Hemidactylus frenatus* and *S. jarrovii*).

Prediction Taxon-specific toxicity is observed in several species of colubrid snakes, and venoms are more toxic to target prey (lizards, birds) than toward mammals. It is predicted that *C. p. pricei* venom contains lizard-specific toxins that facilitate prey capture in this most common natural prey.

Materials and Methods

Supplies and Reagents

Protein concentration reagents were obtained from BioRad, Inc. (Hercules, CA, USA). NuPage gels, buffers and standards for gel electrophoresis were obtained from Life Technologies, Inc. (Grand Island, NY, USA). All reverse phase-high performance liquid chromatography materials (515 HPLC Pump, Fraction Collector II, and 2487 Dual λ Absorbance Detector) were purchased from Waters Corporation (Milford, MA, USA), and Jupiter 5 μm C_{18} 300Å 250 x 4.60 mm reversed phase columns were purchased from Phenomenex, Inc (Torrance, CA, USA). All fast protein liquid chromatography equipment (ÄKTApurifier P-900 and Superdex™ 75 10/300 GL column) was purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). Sigma Biochemical Corp. (St. Louis, MO, USA) supplied all other reagents (analytical grade or higher). All absorbances for enzyme assays were measured using a Genesys™ 10 Series spectrophotometer purchased from Thermo Electron Corporation (Madison, WI, USA).

Animals and Venoms

Crotalus pricei pricei specimens or venoms were collected from three of the four Sky Island locations in the United States with known populations: Chiricahua Mountains, Huachuca Mountains, Santa Rita Mountains, and Pinaleño Mountains (Figure 2.1); unfortunately, venoms samples from the Huachuca Mountains were not obtained.. Venom samples from Durango, México were extracted from three snakes housed at the Chiricahua Desert Museum. The two *C. p. pricei* venom samples from the Santa Rita Mountains were obtained from snakes housed at Sternberg Museum of Natural History. *Crotalus p. miquihuanus* samples and samples from Barfoot (Chiricahua Mountains) were previously acquired by the Mackessy Venom Analysis Lab and are included in this study. Venoms of fourteen individual snakes from the Chiricahua Mountains were the primary samples for venom analysis, and two venom samples were collected from snakes from the Pinaleño Mountains and from the Santa Rita Mountains in order to obtain preliminary data on geographic venom variation. All venom samples were mechanically extracted from *C. pricei,* lyophilized, and stored in a -20 °C freezer until analyzed.

Sixty *S. jarrovii* individuals were collected from two locations: one within *C. p. pricei* habitat in the Chiricahua Mountains, and one in the Dragoon Mountains (southeast of Tucson, AZ), outside of the known range of *C. p. pricei.* All animals were collected in Arizona in accordance with the scientific collecting license guidelines provided by Arizona Game and Fish Department (AGFD) under Dr. Stephen Mackessy's AGFD Scientific Collection Permit (#SP591359). Live animals were held in the Animal Research Facility at the University of Northern Colorado. All experimental protocols

were completed in accordance with the Institutional Animal Care and Use Committee (IACUC protocols 1302D-SM-16 and 1701D-SM-S-20).

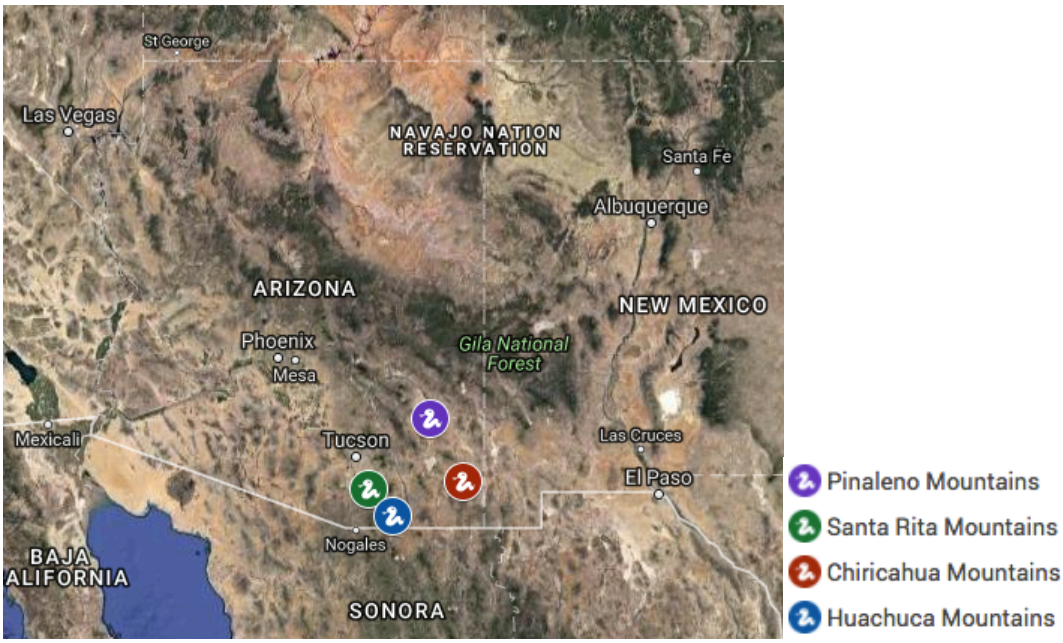

Figure 2.1. Arizona mountain ranges with known populations of *C. p. pricei.*

Protein Concentration Determination

Lyophilized venom samples were reconstituted at an approximate concentration of 4.0 mg/mL in Millipore-filtered water. Protein concentration of the crude venom samples was determined using the Thermo Scientific Pierce® BCA Protein Assay kit, with bovine gamma globulin as the standard.

Venom Analysis

For all venom samples collected, protein concentration (BCA) assays were used to standardize protein amounts used in all assays. Several biochemical assays (described in Smith and Mackessy, 2016) were completed to determine enzymatic activity in crude *C. pricei* venom. These assays detect enzyme activities common to rattlesnake venoms and include metalloproteinase, thrombin-like and kallikrein-like serine proteinases,

phosphodiesterase, phospholipase A_2, and L-amino acid oxidase; protein concentration (BCA) assays were used to standardize protein amounts used in all assays. In addition, RP-HPLC, fast protein liquid chromatography (FPLC), and gel electrophoresis were used to separate and identify venom toxin families following protocols outlined in Saviola et al. (2015).

Gel Electrophoresis

Crotalus p. pricei crude venom samples, RP-HPLC fractions, and FPLC fractions (reduced) were electrophoresed on reduced NuPAGE Novex bis-tris 12% acrylamide mini gels with MES running buffer to provide a "molecular fingerprint" of dominant venom components. Mark 12 standards were run on each gel to provide an estimate of molecular weight. Crude venom (20 µg) or RP-HPLC/FPLC-fractionated proteins (approximately 5 µg), and 7 µg Mark 12 protein standards were loaded into wells and gels were electrophoresed at 150 volts, 125 mA for about 90 minutes. Gels were then stained in 0.1% Coomassie Brilliant Blue R-250 and placed on a gyrating shaker overnight. Excess stain was removed the next day and gels were placed in rapid destain (30% methanol, 7% glacial acetic acid in water) for approximately two hours; gels were then imaged on an HP Scanjet.

Enzyme Assays

Azocasein proteolytic assay. Azocasein protein substrate (sulfanilamide-azocasein) was used to determine metalloprotease activity of *Crotalus p. pricei* venom samples following a procedure from Aird and da Silva (1991). All samples and blank controls were run in duplicate. Five µL (20 µg) of each sample was resuspended in 450 µL buffer (50 mM HEPES, 100 mM NaCl at pH 8.0). Substrate solution (500 µL, 2

mg/mL buffer) was then added to each sample (tubes on ice). Samples were vortexed and immediately transferred to a water bath incubator (37 °C for 30 min). After 30 min, tubes were placed on ice and trichloroacetic acid (TCA, 0.5 M) stop solution was added (250 μL) to each tube to stop the reaction. Tubes were vortexed, brought to room temperature, and then centrifuged at 2,000 rpm for 10 minutes. UV absorbance of samples was recorded at 342 nm with a Genesys™ 10 Series spectrophotometer, and specific activities (adjusted for protein concentration) were expressed as $\Delta A_{342\ nm}$/min/mg protein after absorbance of buffer control was subtracted.

L-amino acid oxidase assay. Crude venom samples were assayed for L-amino acid oxidase activity using the method of Kishimoto and Takahashi (2001); all samples and controls were run in duplicate. Ten-fold concentrated stock solutions of reagents were prepared: L-methionine (MET) substrate was dissolved at 7.46 mg/mL in buffer (50 mM borax, pH 8.5), o-phenylenediamine (OPD)-coupled substrate was resolubilized in buffer at 2.16 mg/mL, and horseradish peroxidase (HRP) was resolubilized at 8.1 U/mL. Crude venom samples (10.0 μL at 0.1 mg/mL) were added to each well on a 96-well plate. Ninety μL master mix (70% buffer, 10% MET, 10% OPD, and 10% HRP solutions) were then added to each well. The plate was then incubated at 37 °C for 30 minutes before returning to a cold surface and rapidly adding 50 μL termination solution (2.0 M sulfuric acid). Sample absorbance was read at 492 nm on a SpectraMax platereader, and specific activity was expressed as ΔA_{492nm}/min/mg protein after absorbance of buffer control was subtracted.

Phosphodiesterase assay. Phosphodiesterase assays were conducted on all *C. p. pricei* crude venom samples using methods outlined by Bjork (1963) and modified by

Laskowski (1980). All samples and controls were run in triplicate. Two hundred and twenty μL buffer (100 mM tris-HCl pH 9.0 with 10 mM $MgCl_2$) was combined with 5.0 μL crude venom. Sample tubes were then placed in an ice bath for three minutes before 150 μL substrate (1.0 mM bis-p-nitrophenylphosphate dissolved in buffer; 0.3402 mg/mL) was added to begin the reaction. Samples were incubated at 37 °C for 30 minutes, returned to the ice bath and reactions stopped by adding 375 μL termination solution (100 mM NaOH with 10 mM disodium-EDTA) to each tube. Samples were then vortexed, brought to room temperature and absorbance read at 400 nm with a Genesys™ 10 Series spectrophotometer. Absorbance of buffer control was subtracted from samples and specific activity was expressed as ΔA_{400nm}/min/mg protein.

Phospholipase A_2 assay (PLA_2). *Crotalus p. pricei* crude venom samples were analyzed for PLA_2 activity using 4-nitro-3-(octanoyloxy)benzoic acid dissolved in 100% acetonitrile (Holzer and Mackessy, 1996). Fifty μL of venom sample (4.0 mg/mL) were combined with 500 μL buffer (10 mM Tris-HCl (pH 8) containing10 mM $CaCl_2$ and 100 mM NaCl) and tubes places in an ice bath. Fifty μL substrate was then added to each sample, vortexed and immediately incubated at 37 °C for 30 min. The reaction was terminated with 50 μL 2.4% Triton X-100 in ddH_2O. Absorbance of samples was recorded at 425 nm with a Genesys™ 10 Series spectrophotometer. Absorbance of buffer control was subtracted from samples and specific activity was expressed as nmol product/min/mg protein (based on a standard curve).

Thrombin-like and kallikrein-like assays. *Crotalus p. pricei* crude venom samples were tested for thrombin-like and kallikrein-like serine proteinase activity using methods modified from Mackessy (1993). Samples and controls were run in duplicate.

Substrate (1.0 mM) was first dissolved in DMSO and then Millipore-filtered water added to achieve the 1.0 mM concentration (1% DMSO – final concentration). Kallikrein-like substrate (benzoyl-Pro-Phe-Arg-paranitroaniline) or thrombin-like substrate (benzoyl-Phe-Val-Arg-paranitroaniline) was used depending on assay. One µL crude venom at a concentration of 4.0 mg/mL and 374 µL of buffer (50 mM HEPES pH 8.0 with 100 mM NaCl) were combined before incubating at 37 °C for 3.0 min. Reactions were initiated by adding 50 µL substrate and vortexing, and samples were incubated at 37 °C for 3.0 min. The reaction was then terminated with 75 µL 50% (v/v) acetic acid and absorbance of samples was recorded at 405 nm with a Genesys™ 10 Series spectrophotometer. Absorbances of buffer control was subtracted from samples and specific activity was based on a standard curve for p-nitroaniline (Mackessy, 1993) and was expressed as nmol product/min/mg protein.

Fast Protein Liquid Chromatography

Three Chiricahua Mountain *C. p. pricei* samples were combined (approximately 2 mg each) and fractionated using size-exclusion fast protein liquid chromatography (FPLC). Crude venom (6.42 mg) was resuspended in 321 µL buffer (20 mM HEPES with 100 mM NaCl and 5 mM $CaCl_2$) to achieve a concentration of 20 mg/mL and 250 µL were injected onto a 10/300 GL Superdex™ 75 column. Fractions were collected at a rate of 0.4 mL/min for 75 minutes. All 75 fractions were collected and stored at -20 °C before further evaluation via electrophoresis and enzyme assays.

Reverse-Phase High Performance Liquid Chromatography

Crotalus p. pricei crude venom samples were analyzed using reverse-phase high performance liquid chromatography (RP-HPLC). Methods were consistent for all samples and modeled after procedures outlined in Smith and Mackessy (2016). Two milligrams crude venom was resuspended in 200 µL Millipore-filtered water. Samples were then centrifuged at 10,000 x g for 5 minutes and filtered through a 0.45 µm syringe tip filter before injection into a Jupiter 5 µm C_{18} 300Å 250 x 4.60 mm RP-HPLC column. One minute fractions were collected at a rate of 1 mL/min for 104 minutes. Venoms were fractionated using a gradient of 0.1% trifluoroacetic acid in Millipore-filtered 18.2 MΩ water (solution A) and 0.1% trifluoroacetic acid in 100% acetonitrile (solution B). Proteins were eluted with solution A at 95% and solution B at 5%. From 0-10 minutes, flow of B increased to 10%. Between 10-20 minutes, flow of B changed to 25%. From minutes 20-80, B ramped to 45%. Solution B continued to ramp to 70% between minutes 80-92. Finally, solution B ramped to 95% between minutes 92-95. Eluting proteins and peptides were then detected at 220 nm and 280 nm. Fractions corresponding to protein/peptide peaks were collected and placed in a -80 °C freezer overnight, then lyophilized for at least 12 hours.

Lethal Toxicity Assays

Lethal toxicity (LD_{50}) assays with crude *C. p. pricei* venom were performed on *S. jarrovii* and *Hemidactylus frenatus* to determine acute (lethal) toxicity in a native prey and a model lizard species. *Sceloporus jarrovii* individuals were wild-caught in Cochise County, Arizona. Two experimental groups were used - one from high elevation habitat within known *C. p. pricei* range in the Chiricahua Mountains, and one group from lower

elevation outside of known *C. p. pricei* range from the Dragoon Mountains. *Hemidactylus frenatus* were ordered from Bushmaster Reptile (Boulder, CO, USA). These results were compared to LD_{50} assays previously performed on Non-Swiss Albino mice (Mackessy, 2008).

Venom toxicity methods were adapted from Mackessy (2008). Three subadult lizards (ranging from 3.0 to 6.0 g) were used at each dose level, and six adult *C. p. pricei* venom samples from the Chiricahua Mountains were combined for use in order to obtain an average lethal toxicity measure for this population. Lyophilized venom was reconstituted in Millipore-filtered water to a concentration of 1.0 mg/mL. Doses of 0.5, 1.0, and 5.0 μg venom/g body weight were administered initially, with intervening doses as needed to determine LD_{50}. Doses appropriately adjusted to individual lizard mass were injected intraperitoneally anterior to the right hind leg using a 28G x ½ in. needle and 0.5 mL syringe. A 24-hour time frame was used to determine lethal toxicity: if a lizard survived after 24 hours, that dose was considered non-fatal.

Statistical Analyses

Significance was calculated for enzymatic activities using a 2-way Analysis of Variance (ANOVA). A Tukey HSD test was completed post-hoc to determine differences between all combinations of means. P-values < 0.05 were considered statistically significant. However, very limited number of samples were available for several locations, and due to violation of certain assumptions of ANOVA tests relating to sample size, statistical results should not be considered statistically rigorous.

Results

Gel Electrophoresis of Crude Venom

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of crude venom showed the presence of nine bands for most individuals analyzed (Figure 2.2). Toxins typical of type I venoms, PIII snake venom metalloprotease (SVMP) and PI SVMP, were visible at ~53 kDa and between 23 kDa, respectively (Figure 2.2). The greatest variation was present in the SVMP PI band (~23 kDa) and the PLA_2 bands (~14 kDa; Figure 2.2); these differences appeared to vary randomly and there is no clear correlation with region. However, all Durango *C. p. pricei* samples showed very prominent double PLA_2 bands, with masses slightly lower than most other samples, and PI SVMPs were absent from almost half of all venom samples.

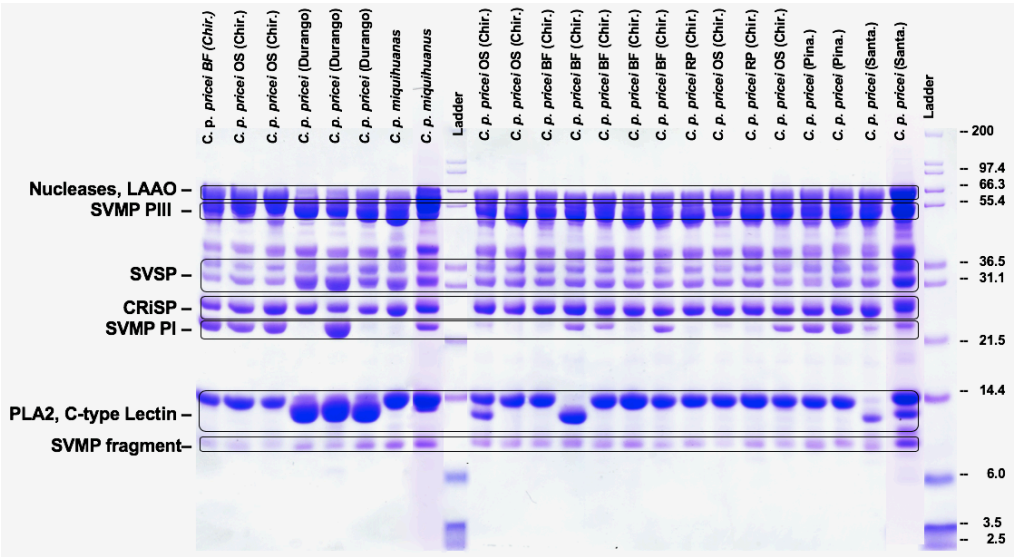

Figure 2.2. SDS-PAGE gel of *Crotalus pricei pricei* (and two *C. p. miquihuanus*) venom samples from different populations and geographic locations: Chiricahua Mountains (Chir.), Pinaleño Mountains (Pina.), Santa Rita Mountains (Santa.), and Durango, México. Venom samples from the Chiricahua Mountains were collected from multiple locations including Onion Saddle (OS), Rustler's Park (RP), and Barfoot (BF). Approximate molecular weight is displayed to the right and shown in kDa.

Enzyme Activities

Average specific activities were calculated for six different enzyme assays. Venom samples included 14 *C. p. pricei* from the Chiricahua Mountains, two from the Pinaleño Mountains, two from the Santa Rita Mountains, three from Durango, México, and two *C. p. miquihuanus* from Nuevo León, México (Table 2.1). Because of the larger sample size of the Chiricahua region, the range of specific activities likely represents snakes within this mountain range more accurately than populations in other regions (Figure 2.3; 2.4). In general, snakes from México of both subspecies showed the highest values of snake venom serine proteases (SVSP) and phosphodiesterase (PDE) activities.

Table 2.1. Enzyme activities of *Crotalus pricei* venoms ($\bar{x}\pm$SD) originating from five different localities.

Species	Location	Thr (nmol/min/mg)	Kal (nmol/min/mg)	MPr (ΔA_{342} nm/min/mg)
Crotalus p. pricei	Chiricahuas n= 14	1859 ± 719	1996 ± 1202	1.40 ± 0.12
	Pinaleño n= 2	1704 ± 339	1426 ± 218	1.56 ± 0.16
	Santa Ritas n= 2	2231 ± 1018	2374 ± 1256	1.43 ± 0.09
	Durango, MX n= 3	3333 ± 44	5419 ± 107	1.19 ± 0.15
Crotalus pricei miquihuanus	Nuevo León, MX n= 2	3236 ± 29	5201 ± 196	1.19 ± 0.06

Species	Location	PLA_2 (nmol/min/mg)	PDE (ΔA_{400} nm/min/mg)	LAAO (ΔA_{492}nm/min/mg)
Crotalus p. pricei	Chiricahuas n= 14	58.2 ± 8.7	0.033 ± 0.017	14.37 ± 2.61
	Pinaleño n= 2	53.2 ± 1.9	0.013 ± 0.009	17.92 ± 0.42
	Santa Ritas n= 2	53.2 ± 50.9	0.044 ± 0.003	22.87 ± 2.52
	Durango, MX n= 3	57.9 ± 9.4	0.184 ± 0.066	24.11 ± 3.16
Crotalus pricei miquihuanus	Nuevo León, MX n= 2	79.6 ± 11.4	0.232 ± 0.124	16.78 ± 3.34

Abbreviations: thrombin-like (Thr), kallikrein-like (Kal), metalloproteinase (MPr), phospholipase A_2 (PLA_2), phosphodiesterase (PDE), and L-amino acid oxidase (LAAO).

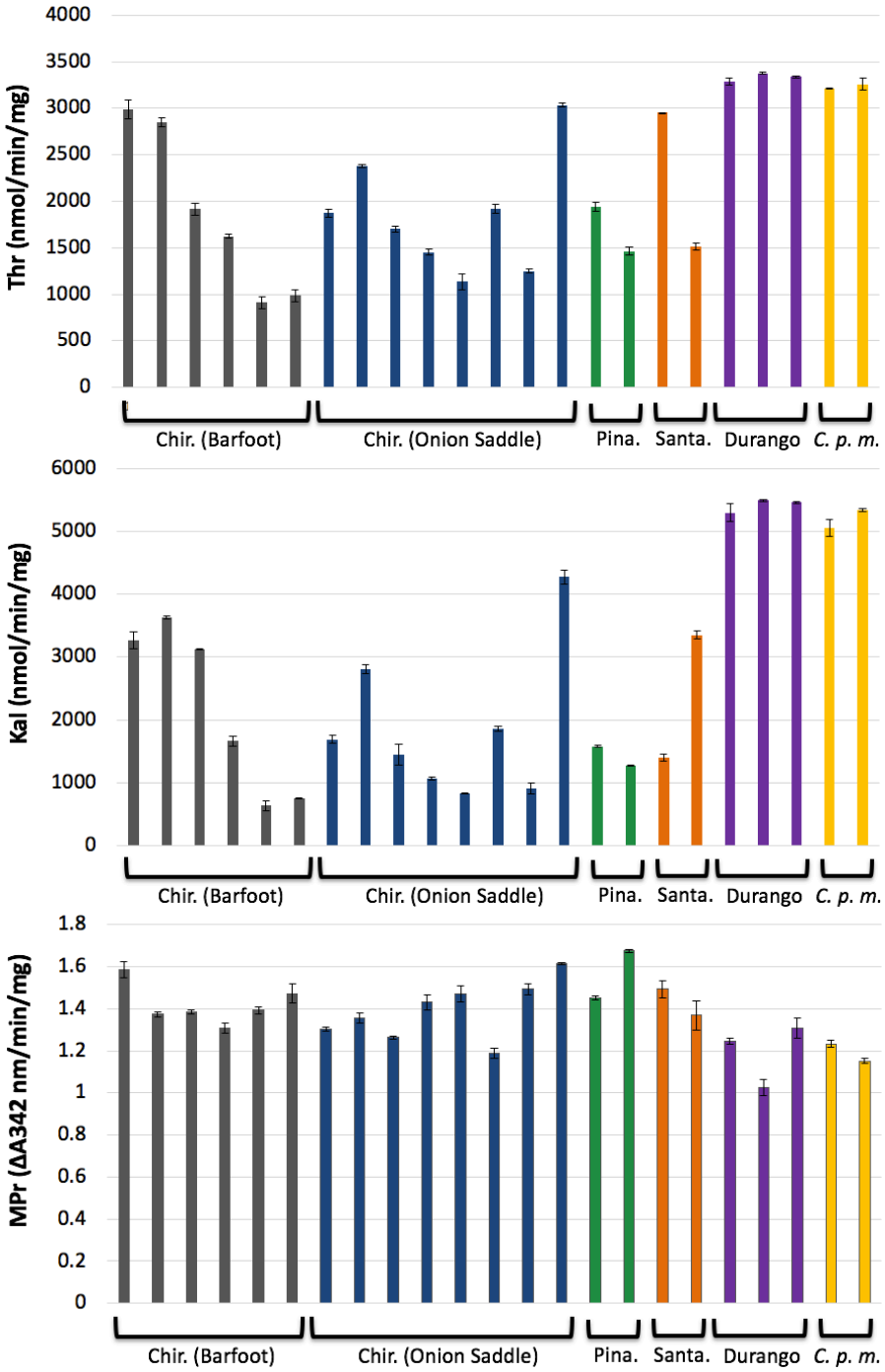

Figure 2.3. Enzyme activities of *C. p. pricei* crude venoms from the Chiricahuas (Chir. (Barfoot), Chir. (Onion Saddle)), Pinaleño (Pina.), Santa Ritas (Santa.), Durango, and *C. p. miquihuanus* (C. p. m.). Assays included thrombin-like serine protease (Thr), kallikrein-like serine protease (Kal), and azocasein metalloprotease (MPr). Each bar represents an individual venom with brackets representing ± one standard deviation.

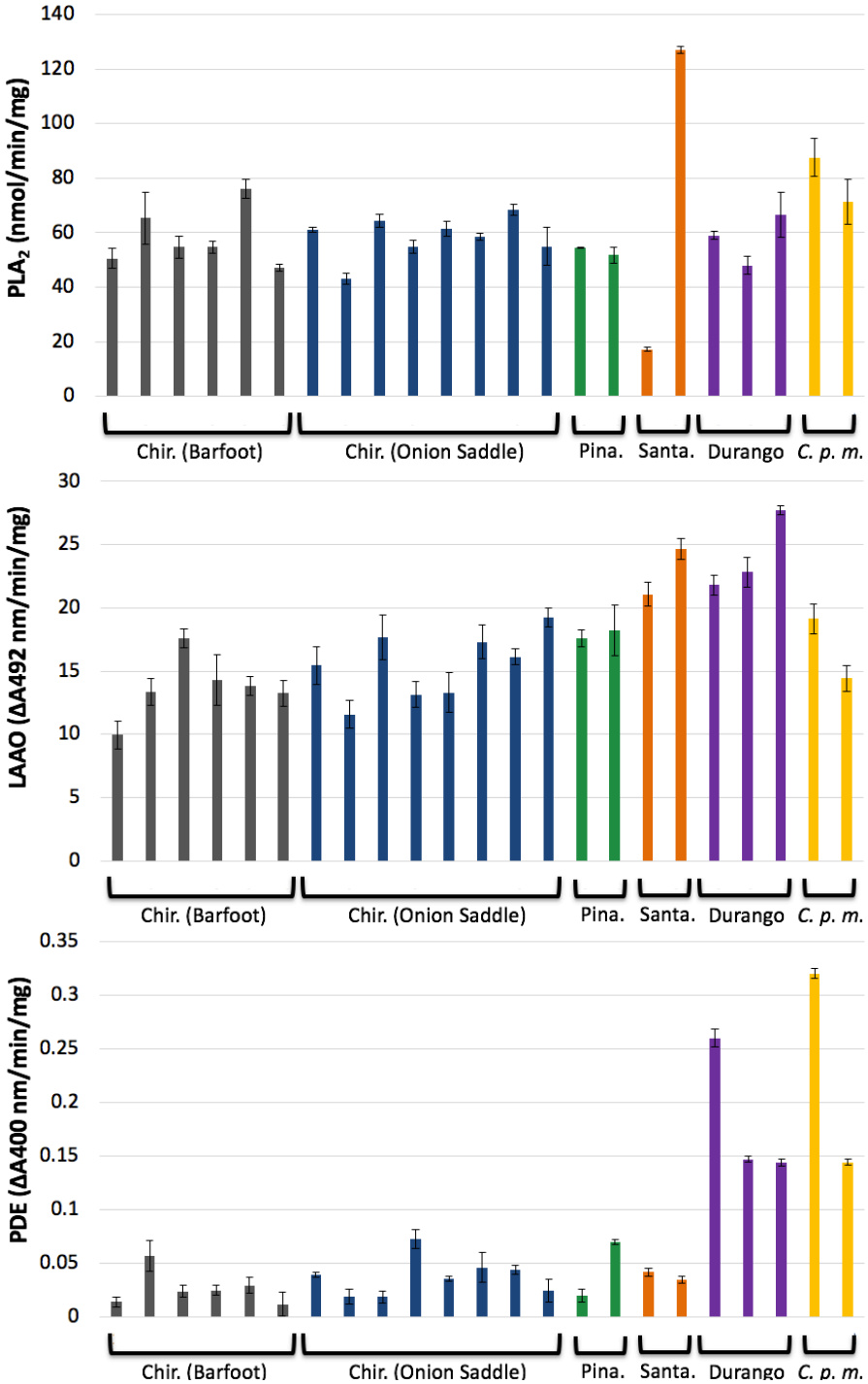

Figure 2.4. Enzyme activities of *C. p. pricei* crude venom from the Chiricahuas (Chir. (Barfoot), Chir. (Onion Saddle)), Pinaleño (Pina.), Santa Ritas (Santa.), Durango, and *C. p. miquihuanus* (C. p. m.). Assays included phospholipase A_2 (PLA_2), L-amino acid oxidase (LAAO), and phosphodiesterase (PDE). Each bar represents an individual venom with brackets representing $\pm$ one standard deviation.

High activity was observed for SVSPs compared to other species in the genus *Crotalus*, as previously reported by Mackessy (2008). Chiricahua snakes also displayed the widest range of specific activity values; thrombin-like SVSP ranged from 910 to 2990 nmol/min/mg and kallikrein-like SVSP ranged from 633 to 4278 nmol/min/mg. Durango snakes had the highest overall SVSP activity and the Pinaleño snakes had the lowest (Table 2.1; Figure 2.3). Kallikrein-like SVSP activities from *Crotalus p. miquihuanus* and samples from the Durango regions were significantly higher than samples from the Chiricahuas and Pinaleños ($p < 0.05$), and Durango samples were also significantly higher than Santa Ritas samples ($p < 0.05$).

Overall SVMP was also relatively high compared to other *Crotalus* species (Mackessy, 2008) and varied slightly between individuals from the Chiricahuas with a range from 1.19 to 1.62. Lower specific activity was apparent in *C. p. miquihuanus* individuals and *C. p. pricei* individuals from Durango (Table 2.1; Figure 2.3) and these values were significantly lower than individuals from the Pinaleños ($p < 0.05$).

Phospholipase A_2 (PLA_2) activity was relatively high as well, and showed similar results to previous studies (Mackessy, 2008). Activities of individuals from the Chiricahuas ranged from 43.1 to 68.5 nmol/min/mg. *Crotalus p. miquihuanus* samples were substantially higher compared to *C. p. pricei* samples, and the Santa Ritas samples had the greatest range in values with a high of 89 nmol/min/mg and low of 17.2 nmol/min/mg (Table 2.1; Figure 2.4). Despite the apparent differences, PLA_2 activity levels were not statistically different between regions.

Phosphodiesterase (PDE) activity was variable and relatively low for all individuals when compared to other *Crotalus* sp. (Mackessy, 2008). Individuals from the

Chiricahuas ranged from 0.012 to 0.072 $\Delta A_{400\ nm}$/min/mg. *Crotalus p. miquihuanus* activity was considerably higher at 0.32 $\Delta A_{400\ nm}$/min/mg and snakes from the Pinaleños appeared to have the lowest amount of activity (Table 2.1; Figure 2.4). Samples from Durango and Nuevo León (*C. p. miquihuanus*) were significantly higher than all regions in AZ ($p < 0.01$).

L-amino acid oxidase (LAAO) activity was moderate compared to species in the same genus (Mackessy, 2008). Individuals from Durango had the highest activity, while those from the Chiricahuas had the least amount of activity with 14.37, with *C. miquihuanus* samples displaying a slightly higher value at 16.78 (Table 2.1; Figure 2.4). Samples from the Chiricahuas were significantly lower than those from the Santa Ritas and Durango ($p < 0.05$) and samples from Durango were significantly higher than Nuevo León ($p < 0.05$).

Fast Protein Liquid Chromatography

Size exclusion FPLC resolved six peaks, with clustering of the major groups of enzymatic proteins within the first 3 major peaks (Figure 2.5). Enzymatic activities of each peak were analyzed using biochemical assays targeting LAAO, PLA_2, thrombin-like, kallikrein-like, and PDE toxins. The highest LAAO specific activity was recorded for fraction 30, though repeated freeze-thaw cycles may have contributed to low values observed. An SDS-PAGE gel of individuals fractions showed significant overlap of several major toxin groups, with elution of larger mass nucleases and PIII SVMPs appearing in the first peak, and smaller PI SVMP, PLA_2, and SVSP enzymatic proteins eluting in peaks two and three (Figure 2.5). No disintegrins were found when fraction 32 was analyzed using RP-HPLC (data not shown), and so the 9 kDa band (between 14.4

and 6.0 kDa std.) was likely an SVMP fragment. Peaks 4-6 (fractions 40-49) likely contained small peptides such as BPPs and tripeptide metalloprotease inhibitors, as no bands were observed on SDS-PAGE.

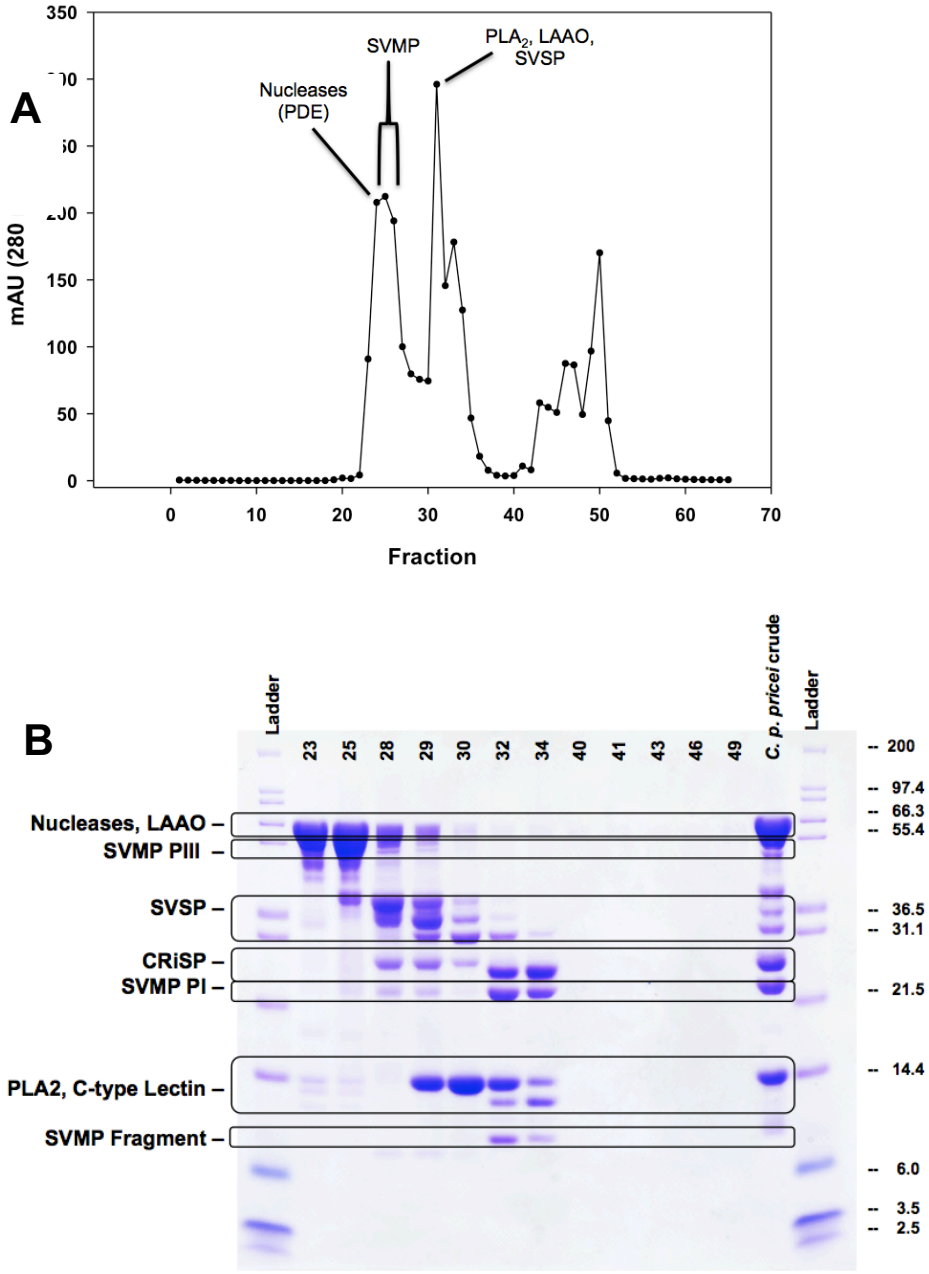

Figure 2.5. A. Size exclusion FPLC chromatogram of three combined venom samples (6.4 mg; *C. p. pricei,* Chiricahua Mountains) showing distributions of major enzyme activities as a function of size. B. SDS-PAGE gel of corresponding size exclusion FPLC fractions.

Reverse-Phase High Performance Liquid Chromatography

Venom samples from the Chiricahua Mountains showed near-identical RP-HPLC profiles based on five individuals (Figures 2.6, 2.7 A). Arizona populations of *C. p. pricei* (Chiricahua, Santa Rita, and Pinaleño Mountains) showed similar RP-HPLC profiles, with only minor variation in frequency and elution time of toxin peaks (Figure 2.7 B). SDS-PAGE gel electrophoresis was completed for two venom samples with different RP-HPLC profiles. Gel electrophoresis was completed for only one Chiricahua sample, due to similarities between HPLC profiles of all Arizona samples, and one Durango sample. Crude *C. p. pricei* venom from the Chiricahua Mountains and *C. p. pricei* venom from Durango displayed similar venom profiles, but two distinct peaks in the Durango samples (minutes 44-47) did not occur in *C. p. pricei* venoms from other U.S. localities (Figure 2.8). These two peaks contain primarily PLA_2 toxins (Figure 2.8), while all PLA_2 proteins for Chiricahua Mountain venom samples elute in the peaks between minutes 59-65 (Figure 2.6).

Chromatograms of venoms from the Durango, MX population of *C. p. pricei* and the *C. p. miquihuanus* population from Nuevo Léon showed similarities to each other. Similarly, no differences in major peaks were identified when crude *C. p. pricei* venom samples were compared between and within populations located in Arizona (Figure 2.7 B). Differences in peak height was apparent between some samples, indicating differences in concentrations of specific components, and these differences were also reflected in differences observed in enzyme activity assays (Table 2.1) Presence of a distinct peak at minute 44 was noted for *C. p. pricei* from Durango and *C. p. miquihuanus* (Figure 2.9, B).

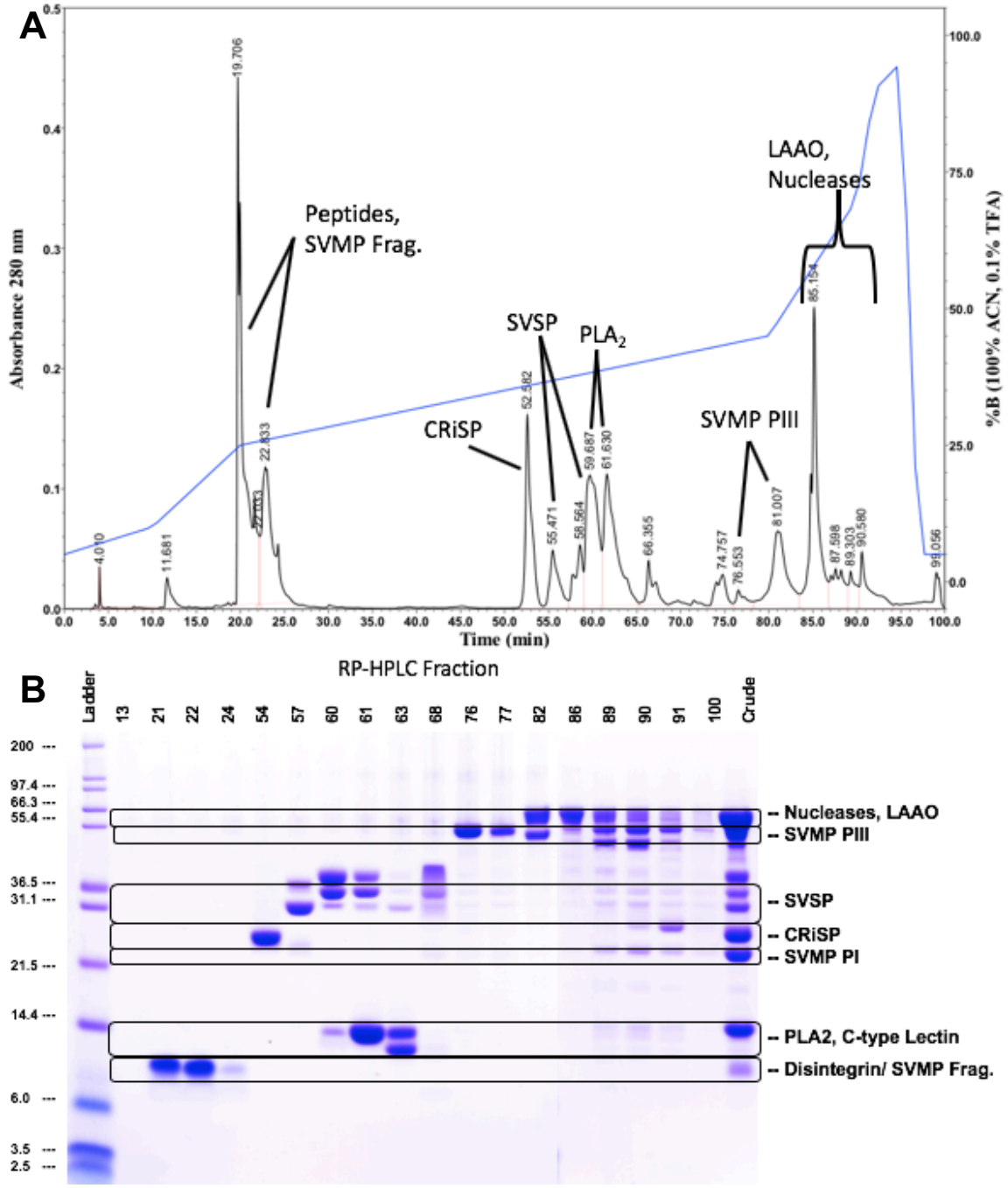

Figure 2.6. **A**. Reverse-phase HPLC chromatogram and peak elution times of 2.0 mg crude *C. p. pricei* venom from the Chiricahua Mountains, AZ (Onion Saddle, snake 493). Elution gradient is displayed to the right of chromatogram. **B**. SDS-PAGE gel of each fraction peak. Protein families are displayed to the right of the gel and fraction numbers displayed at the top of the gel represent each fraction peak.

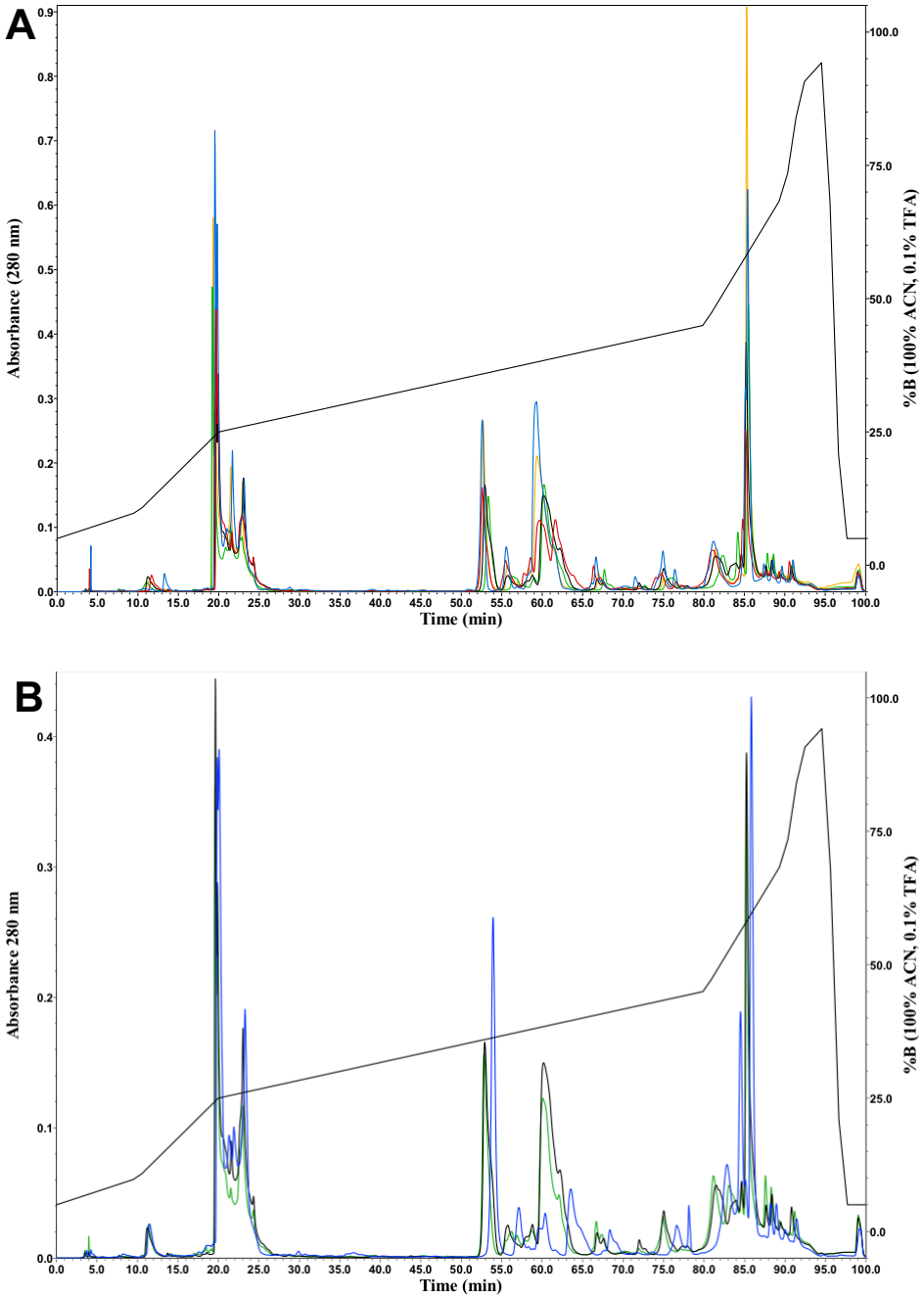

Figure 2.7. Reverse-phase HPLC chromatogram overlays and peak elution times of 2.0 mg crude *C. pricei* venom samples. **A**. Samples from five individuals from the Chiricahua Mountains; note similarities in elution profiles. **B**. Samples from the Pinaleño Mountains (green), Chiricahua Mountains (black), and Santa Rita Mountains (blue). Elution gradient is indicated by the black line, with concentrations displayed on the right side of the chromatograms.

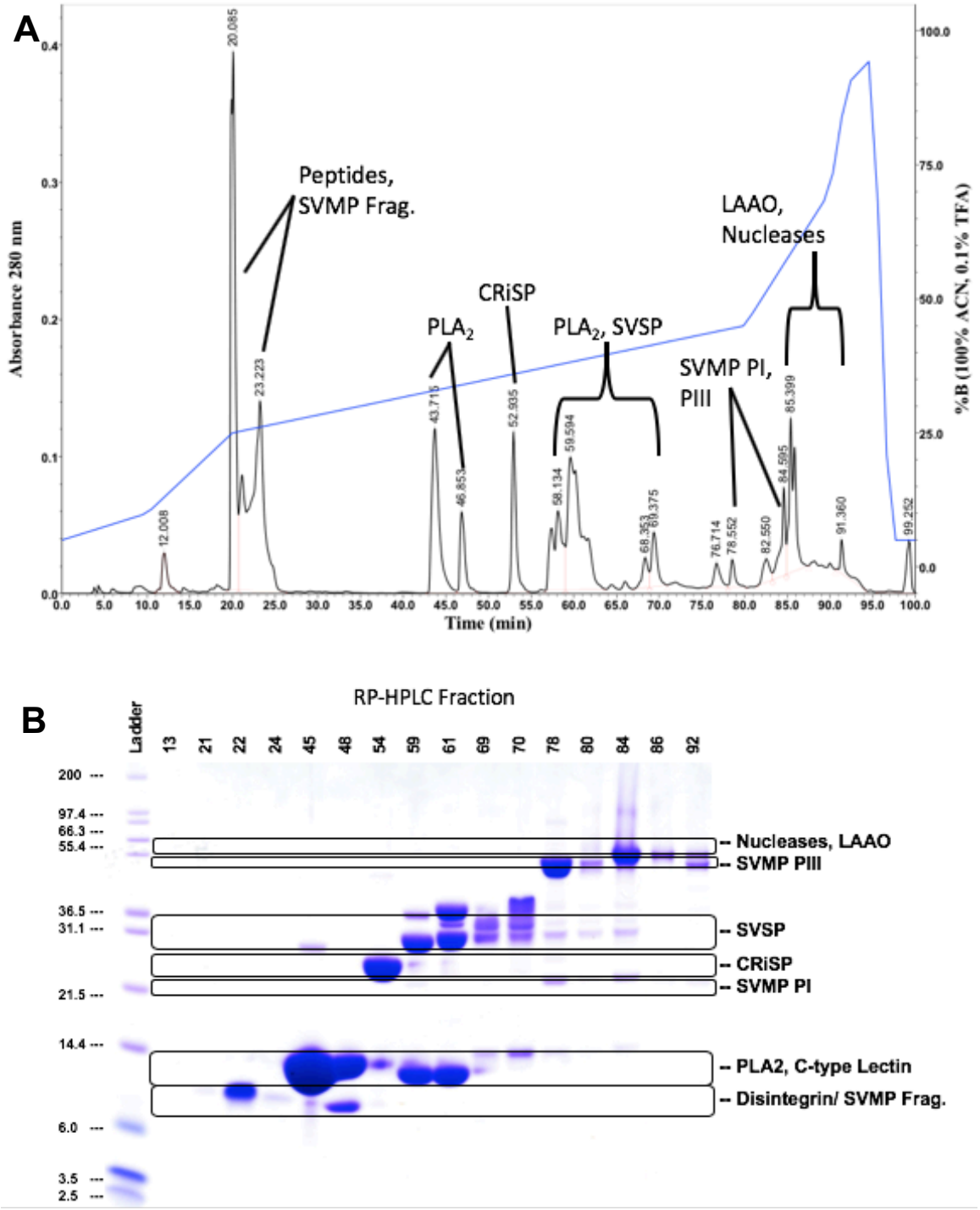

Figure 2.8. **A**. Reverse-phase HPLC chromatogram and peak elution times of 2.0 mg crude *C. p. pricei* venom from Durango, México (#2, extracted from captive specimen at the Chiricahua Desert Museum) and **B.** SDS-PAGE gel of each fraction peak. Elution gradient is displayed to the right of chromatogram. Protein families are displayed to the right of the gel and fraction numbers displayed at the top of the gel represent each fraction peak.

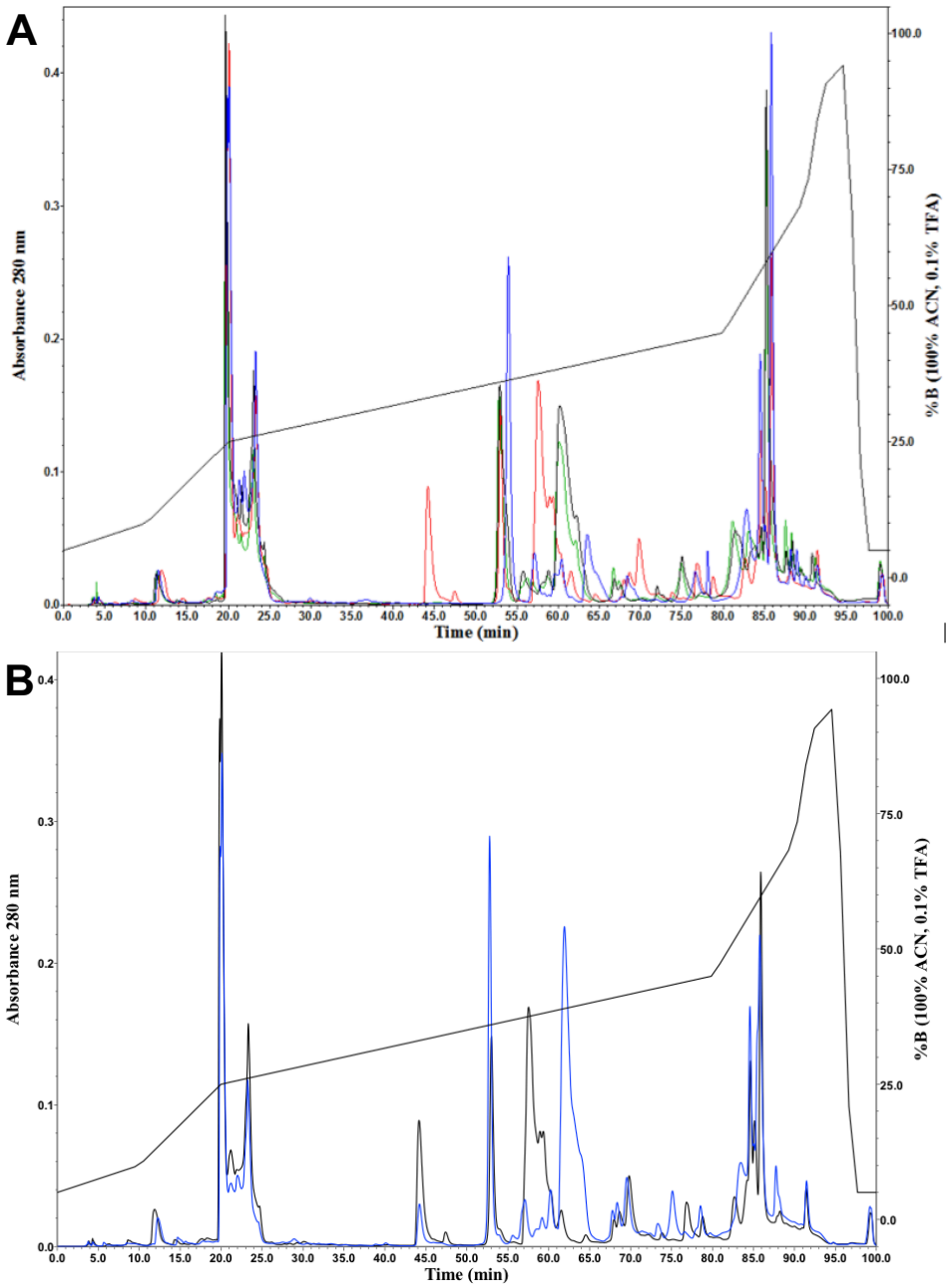

Figure 2.9. Reverse-phase HPLC chromatogram overlays and peak elution times of 2.0 mg crude *C. pricei* venom. **A**. Samples from the Pinaleño Mountains (green), Chiricahua Mountains (black), Santa Rita Mountains (blue), and Durango, MX (red). **B.** Samples from Durango, MX (black) and *C. p. miquihuanus* from Nuevo León, MX (blue). Note the unique peak at approx. 44 min in samples from México. Elution gradient is indicated by the black line, with concentrations displayed on the right side of the chromatogram.

Lethal Toxicity Assays

There was a slight difference in toxicity of *C. p. pricei* venom toward sympatric *S. jarrovii* and allopatric *S. jarrovii,* with values of 6.9 μg/g and 7.2 μg/g, respectively (Figure 2.10); however, in terms of envenomation potential by *C. pricei*, these differences were not biologically significant. In comparison, the lethal dose of *C. p. pricei* venom was substantially lower when tested on NSA lab mice (1.25 μg/g; Mackessy, 2008) and *Hemidactylus frenatus* geckos (0.82 μg/g). Additional LD_{50} assays were performed on Chiricahua Mountain *S. jarrovii* using *Crotalus lepidus klauberi* venom (also from the Chiricahuas) which resulted in an LD_{50} value of 7.9 μg/g (Figure 2.10). The assay of *C. l. klauberi* venom allowed for comparison of *C. p. pricei* results to those of a second rattlesnake that is sympatric with *S. jarrovii* throughout most of its range.

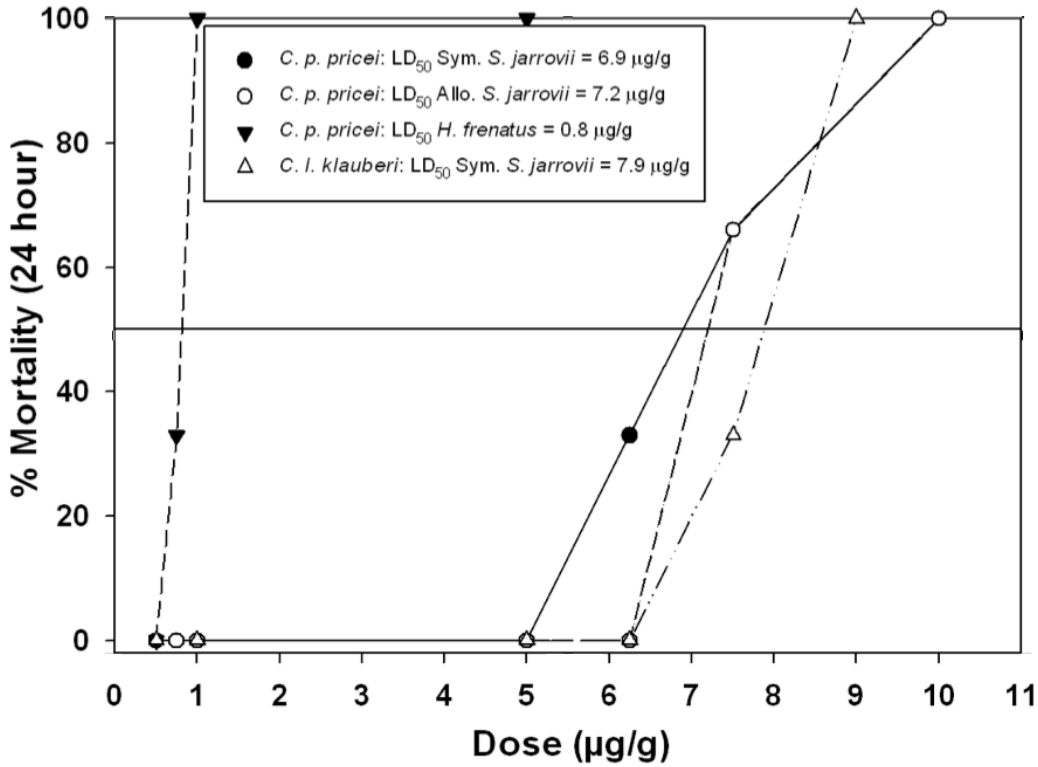

Figure 2.10. Lethal toxicity (μg/g) of *C. p. pricei* and *C. l. klauberi* venom from the Chiricahua Mountains on sympatric (Sym.) and allopatric (Allo.) *Sceloporus jarrovi* and toward *Hemidactylus frenatus* geckos.

Discussion

The Sky Islands and Méxican highlands are unique habitats due to their isolated nature and stratification of climate and vegetation components as elevation increases. These distinctive geographic structures are considered biodiversity hotspots and the study of species endemic to these regions have provided essential information regarding species divergence and origin in México (Bryson et al., 2011a, b; Coblentz and Riitters, 2005; Gottfried and Hodges, 2005; Mastretta-Yanes et al., 2015; Peterson and Navarro-Sigüenza, 1999). Due to their isolated nature and the dramatic climatic shifts that occurred during the last glacial maximum (LGM) (approximately 23,000 – 10,000 yr BP; Bryson et al. 2011a; Metcalfe et al., 2000), apparent rapid radiation occurred throughout various taxonomic groups, including *Sistrurus* and *Crotalus* (Blair and Sánchez-Ramírez, 2016; Castoe and Parkinson, 2005; Gottfried and Hodges, 2005; Mastretta- Yanes et al., 2015; Place and Abramson, 2004). Many of these divergences can be partially attributed to differentiation in physiological adaptation as the Méxican Plateau transitioned from a more mesic forest ecosystem to xeric deserts and grassland (Arriaga et al., 2005; Thompson and Anderson, 2000). This xerification of México and the southwestern United States led to the unique, stacked biotic communities and unique fauna present within and between Sky Islands. *Crotalus pricei* has likely been isolated between these Sky Islands since the LGM (Bryson et al., 2011a) and venom differentiation seemed likely. Other species of high elevation rattlesnakes, such as those in the *C. lepidus* and *C. willardi* species groups, show differential levels of certain toxins (SVSP and SVMP), but not others (PLA_2) (Saviola et al., 2017).

For some enzyme activities (thrombin-like and kallikrein-like SVSP, SVMP, PLA_2), there appears to be a geographic pattern of specific activity differences, and *C. p. pricei* venoms from Durango and *C. p. miquihuanus* seem more similar in their activity levels than their Arizona counterparts. Thrombin- and kallikrein-like SVSPs, SVMP toxins, and enzymatic PLA_2 toxins are primarily lytic and hemorrhagic compounds that, together, result in incapacitation of prey via circulatory collapse, hemorrhage and myonecrosis, and they also likely aid in prey predigestion (Mackessy, 2010). In an ecological context, higher enzymatic activity may be required to immobilize prey in certain regions based on presumed ability to escape in complex environments (for example, incline of talus habitat) or prey toxicity tolerance. Due to lack of natural history information regarding regional variation in ecology, it can only be predicted that *C. pricei* prefers to prey on *Sceloporus* lizards throughout their range, based on snakes in the Chiricahua Mountains (Prival and Schroff, 2012). The regional differences in *C. p. pricei* and *C. p. miquihuanus* in comparison to the Arizona populations could also be attributed to glacial patterns leading to distinct populations and genetic isolation, resulting in slight variation between populations due to random mutations or selective pressures, climate patterns favoring higher enzymatic protein activity, or perhaps simply as a consequence of smaller sample sizes in several populations sampled. Overall, all venoms analyzed show high levels of metalloprotease activity, typical of type I venoms. These results are consistent with the hypothesis that *C. pricei* will display properties of type I venoms.

Toxin isolation via reverse phase HPLC and gel electrophoresis allowed for analysis/identification of individual toxin peaks, resulting in identification of differences in toxin presence and molecular weight between venoms of *C. p. pricei* individuals from

Arizona populations and *C. p. pricei* individuals from Durango, México, primarily in the presence of unique PLA_2 toxins in Mexican snakes (Figures 2.6 and 2.8). The structural and functional differences of these PLA_2 toxins have yet to be determined, but these differences may be related to phylogeographic distribution patterns of *C. pricei.* Sky Island habitats allows for little, if any, gene exchange currently between mountain ranges (Favé et al., 2015; Lomolino et al., 1989; Thompson and Anderson, 2000), and based on predicted historical distribution patterns, fragmentation of *C. pricei* populations likely occurred after the LGM, when pine-oak corridors connecting the Méxican Plateau were present (Metcalfe et al., 2000). During this time, it is estimated that dominant vegetation communities were approximately 1000 m lower than present day, associated with the cooler, wetter montane climate and more aligned with apparent physiological needs of *C. p. pricei* and other high elevation herpetofauna (Bryson et al., 2011; McDonald, 1993; Prival and Schroff, 2012; Thompson and Anderson, 2000). Bryson et al. (2011) analyzed mitochondrial DNA and found that *C. p. pricei* from Durango and *C. p. miquihuanus* from the northern Sierra Madre Occidental were genetically more similar, based on a mixed-model Bayesian approach. This apparent genetic similarity, inconsistent with current taxonomy, could account for the venomic similarities between *C. p. pricei* from Durango and *C. p. miquihuanus* and differences between these groups and *C. p. pricei* from Arizona. Additional samples from the Pinaleño, Santa Rita, and Huachuca Mountain ranges are needed to provide substantial support for toxin variation and differentiation within Arizona populations of *C. p. pricei,* though no consistent differences were apparent from the samples analyzed in the present study. Furthermore, most venom samples collected from snakes originating in the Chiricahua Mountains were

located in only two distinct areas, and sampling multiple areas within each mountain range could provide important information about levels of local variation in venom composition. However, multiple sampling visits in Arizona Sky Island habitats for *C. p. pricei* in two distinct seasons in 2018 yielded no specimens, so the vicariant nature of field sampling can limit broader interpretation of results.

Lethal toxicity results indicate that some level of resistance to venom exists among populations of *Sceloporus jarrovii,* the dominant prey of *C. p. pricei.* These results are consistent with hypothesis 2, that *C. p. pricei* venom would be more toxic to a model prey organism (*Hemidactylus frenatus*) than natural prey (*S. jarrovii*). Results were similar between venom tested on allopatric *S. jarrovii* from the Dragoon Mountains, outside of the known range of *C. p. pricei,* and *S. jarrovii* from the Chiricahua Mountains that are sympatric with *C. p. pricei,* contrary to predicted results and hypothesis 3, indicating that local adaptation (resistance to *C. p. pricei* venom) has not occurred. However, toxicity of *C. l. klauberi* venom toward *S. jarrovii* from the Chiricahuas was also similar to that observed for *C. pricei* (6.9 μg/g vs 7.9 μg/g). We hypothesize that *S. jarrovii* may not be specifically adapted to resist *C. p. pricei* venom, but instead has evolved a more generalized resistance to venom of snakes from the genus *Crotalus*. These data also indicate that these lizards may be "evolutionarily ahead" of high elevation rattlesnake species, evolving a general adaptive resistance to toxins present in venoms of species that target them most frequently, namely *C. p. pricei* and *C. l. klauberi*. This phenomenon of toxin resistance has been reported in several taxonomic groups to varying degrees (Arbuckle et al., 2017). Specifically, the correlation between high toxicity towards model lizards (*Hemidactylus frenatus*) and a diet composed

primarily of lizard prey is also apparent in the Desert Massasauga (*Sistrurus catenatus edwardsii*) (Gibbs and Mackessy, 2009; Holycross and Mackessy, 2002), a species with a similar natural diet and body size to that of *C. p. pricei*.

Conclusions

Overall, there are well-defined similarities in venom composition between the five *C. pricei* populations surveyed, including the distribution of protein families common to all samples, as demonstrated by SDS-PAGE, enzyme assays, and comparative HPLC, but there are also several obvious differences that should be explored further. These venoms show type I venom characteristics with relatively high SVMP, SVSP, and enzymatic PLA_2 activities present, but the venom is quite toxic to mammal and lizard model species. However, native lizards (*S. jarrovii*) are an order of magnitude less sensitive to the venoms, consistent with an emerging pattern of general resistance in native prey species relative to model species (Smiley-Walters et al., 2018). The few regional difference noted in venom composition could be explained by the long period of time that these populations have been isolated and/or slight differences in natural history characteristics of each region that have yet to be explored. The differences seen between Arizona/U.S. *C. pricei* and México *C. pricei* venoms should be evaluated further, as the PLA_2 toxin unique to the southern populations may have biological and functional significance.

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CHAPTER III

VENOM CHARACTERIZATION OF *CROTALUS TANCITARENSIS* AND RELATED SPECIES WITHIN THE *CROTALUS INTERMEDIUS* GROUP

Abstract

The *Crotalus intermedius* group is a clade of rattlesnakes typically categorized as basal within the *Crotalus* phylogeny. *Crotalus tancitarensis* was previously classified as *C. intermedius,* until individuals occurring on Cerro Tancítaro in Michoacán, México were reevaluated and classified as a new species (*C. tancitarensis*) based on scale pattern and geographic location. This study aimed to characterize the venom of *C. tancitarensis* and compare the general venom profile to those of other species within the *Crotalus intermedius* group using gel electrophoresis, biochemical assays, reverse-phase high performance liquid chromatography, and lethal toxicity (LD_{50}) assays. Results show that the venom profiles of species within the *Crotalus intermedius* group are similar, but with distinct differences specifically in phospholipase A_2 (PLA_2), metalloprotease PI (SVMP PI), and kallikrein-like serine protease (SVSP) activity and relative abundance. The overall venom profile of *C. tancitarensis* appears most similar to *C. transversus,* which is consistent with a previous mitochondrial DNA analysis of the *Crotalus intermedius* group.

Introduction

There are at least 43 species of rattlesnakes native to México, with many of these species associated with the Méxican Plateau and other high elevation regions (The Reptile Database, http://www.reptile-database.org/, accessed August 2018). Although venomous snakes, especially rattlesnakes, are of particular importance because of the incidence of snakebite and potential development of therapeutics from venom toxins, little is known about many higher elevation rattlesnake species. Recently, several studies have investigated the phylogeny of these species, but there is a need for research with a focus on ecological relationships, medicinal applications, and conservation of Méxican rattlesnake species. Although research methods have advanced and knowledge of Méxican rattlesnake ecology and evolution is improving, there are still many questions regarding little-known species, particularly diminutive species of the Mexican highlands.

The diversity of species in México can be partially attributed to the apparent rapid radiation of rattlesnakes (*Sistrurus* and *Crotalus* genera) throughout México, as suggested by several more recent phylogenetic and biogeographic analyses (Blair and Sánchez-Ramírez, 2016; Castoe and Parkinson, 2005; Place and Abramson, 2004). Based on several studies that used various mitochondrial and nuclear markers for investigating phylogeny, the *Sistrurus* and *Crotalus* clade likely originated in the montane pine-oak forests associated with major mountain ranges and then diversified relatively rapidly (Blair and Sánchez-Ramírez, 2016; Reyes-Valesco et al., 2013). This idea is supported by the basal location of many high-elevation species such as *C. pricei, C. intermedius* and *C. transversus* within rattlesnake phylogenies (Blair and Sánchez-Ramírez, 2016; Reyes-Valesco et al., 2013). Many of these mountain species are endemic to high elevation,

often isolated, biodiversity hotspots (Peterson and Navarro-Sigüenza, 1999), another reason additional information about these species is necessary for conservation of these ecosystems. In addition, long evolutionary history and isolation may result in local adaptation that may also be reflected in venom phenotype.

Crotalus tancitarensis is endemic to Cerro Tancítaro in Michoacán, México and was first officially described by Alvarado-Díaz and Campbell in 2004. This species was previously considered to be part of the *C. intermedius* species group, and the first publication to question the identity of this species (Campbell, 1982) discussed the geographically isolated nature of this population (nearest population of *C. intermedius* was 300 km away) and several minor morphological features that show similarities to *C. pricei, C. intermedius* and *C. transversus*. Alvarado-Díaz and Campbell (2004) described *C. tancitarensis* using three specimens: one collected in 2002 by Javier Alvarado-Díaz and two collected from Cerro Tancítaro previously identified as *C. intermedius*. Isolated geographic location, and morphological measurements including body length, tail length, fang length, rattle length, and scale counts, were used to distinguish *C. tancitarensis* from *C. intermedius* (Alvarado-Díaz and Campbell, 2004). More recently, mitochondrial DNA analysis has revealed that *C. tancitarensis* is likely nested with *C. transversus* rather than *C. intermedius* (Figure 3.1; Bryson et al., 2011). This relationship may be reflected in venom composition.

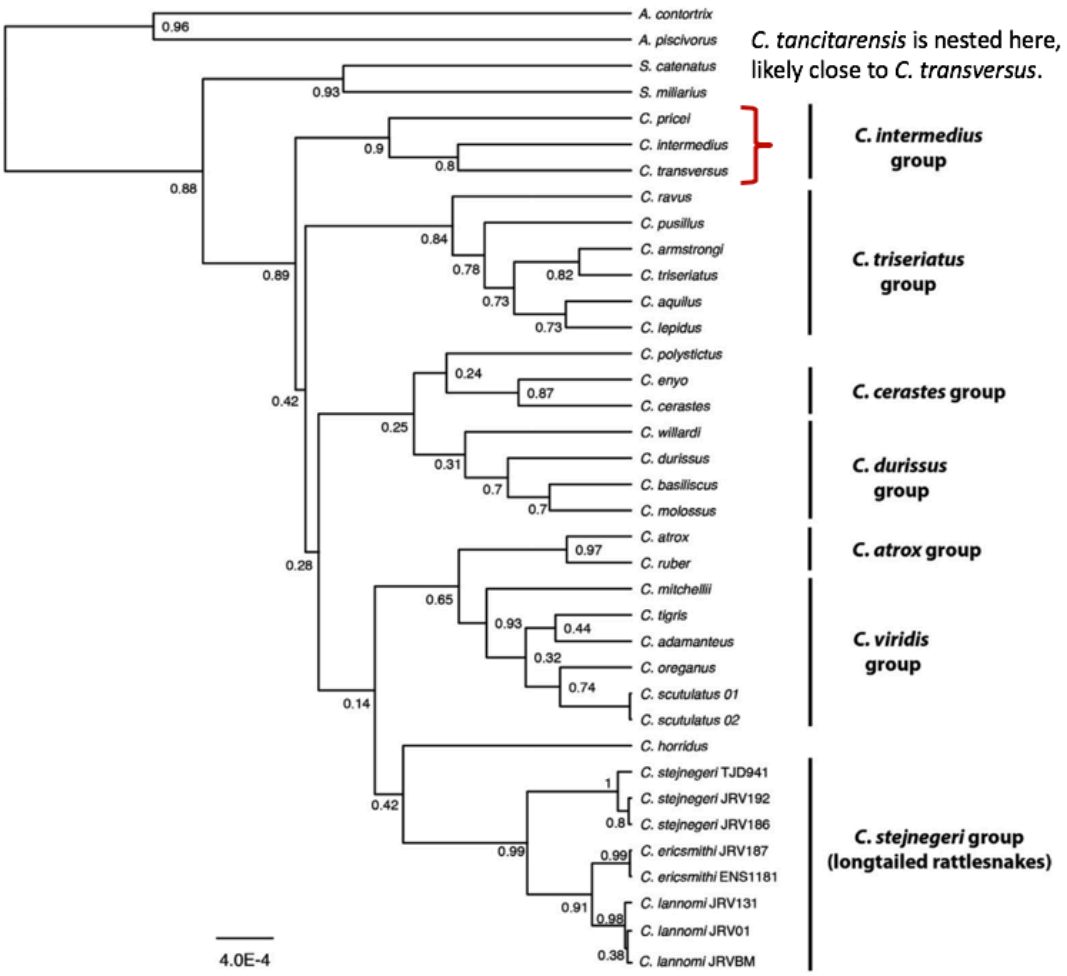

Figure 3.1. Rattlesnake phylogeny adapted from Reyes-Velasco et al., 2013. *Crotalus tancitarensis* placement in the phylogeny is estimated.

The evolution of venom has allowed for snakes in the families Elapidae and Viperidae, including rattlesnakes, to exploit a chemical means of acquiring prey as opposed to mechanical methods used by other families such as Pythonidae, Boidae and many "colubrid" snakes (Kardong et al., 1997). These venoms are composed primarily of proteins and peptides and produce a wide array of pathologies (Mackessy, 2008). Rattlesnake venoms tend to fall into two categories based on biochemistry, pathology and toxicity: type I venoms are generally more enzymatic and result in tissue damage caused by high levels of metalloprotease activity, and type II venoms are highly toxic and cause

neurological symptoms from presynaptically neurotoxic phospholipase A_2 toxins (Mackessy, 2008, 2010). Despite this dichotomy of venom composition and pathology, venoms are often quite variable, and intraspecific variation often exists, likely due to geographic, ontogenetic, and prey variability factors (Mackessy, 2010).

Individual species of rattlesnakes often have specific phenotypic characters in the form of dominant venom toxins, and sometimes these can be quite distinct, particularly when comparing type I and type II venoms (e.g., Smith and Mackessy, 2016). Often, but not always, more closely related species of rattlesnakes have more similar venoms, and because *C. tancitarensis* is suspected to be closely related to *C. pricei,* the two venoms may be relatively similar. *Crotalus pricei pricei* venom has been found to have moderate to high protease activity and relatively high toxicity (LD_{50} of 1.25 μg/g) when tested on lab mice (see Chapter II; Mackessy, 2008).

This study aimed to analyze venom composition in *C. tancitarensis* and several members of the *Crotalus intermedius* group (including *C. pricei, C. transversus,* and *C. intermedius*). By identifying toxins and enzyme activities of *C. tancitarensis* venom and comparing these results to other species within the *Crotalus intermedius* group and with an outgroup (*C. triseriatus*), one can add venom phenotype as an additional set of characteristics in support of *C. tancitarensis* relationship to other members of the *C. intermedius* group.

Hypotheses

To approach the question of venom characterization of *C. tancitarensis* and where this venom profile falls in the *Crotalus intermedius* group, two hypotheses have been generated.

H_1 *Crotalus tancitarensis* venom composition will be most similar to that of *C. pricei*, given its hypothesized position in the *Crotalus* phylogeny.

Prediction Dominant venom phenotypic characters, such as metalloproteinase and serine proteinase levels, are predicted to be similar between *C. tancitarensis* and *C. pricei.*

H_2 *Crotalus tancitarensis* venom will show characteristics of type I venom (more degradative and highly enzymatic).

Prediction *Crotalus pricei* produces a venom characteristic of type I venom with moderate to high metalloprotease activity and relatively low toxicity (Mackessy, 2008). *Crotalus tancitarensis* is predicted to show similar enzymatic and toxicity patterns.

Materials and Methods

Supplies and Reagents

Protein concentration reagents were obtained from BioRad, Inc. (Hercules, CA, USA). NuPage gels, gel standards, and buffers for electrophoresis were obtained from Life Technologies, Inc. (Grand Island, NY, USA). All reverse phase-high performance liquid chromatography materials (515 HPLC Pumps, Fraction Collector II, and 2487 Dual λ Absorbance Detector) were purchased from Waters Corporation (Milford, MA, USA), and Jupiter 5 µm C_4 300Å 250 x 4.60 mm reversed phase columns were purchased from Phenomenex, Inc (Torrance, CA, USA). All other reagents not specified (analytical grade or higher) were purchased from Sigma Biochemical Corp. (St. Louis, MO, USA). All absorbances were measured using a Genesys™ 10 Series Spectrophotometer purchased from Thermo Electron C rporation (Madison, WI, USA).

Animals and Venoms

Crotalus tancitarensis venoms samples were extracted from snakes collected on Cerro Tancítaro and held at IRENA in Michoacan; these were the same individuals used to characterize the species in 2004 (Alvarado-Díaz and Campbell, 2004). The female was gravid, and both neonate venom samples analyzed were collected from these offspring. *Crotalus pricei* specimens or venoms were collected in the Chiricahua Mountains, Cochise Co., Arizona, in accordance with the scientific collecting license guidelines provided by Arizona Game and Fish Department (AGFD) under Dr. Stephen Mackessy's AGFD Scientific Collection Permit (#SP591359). Live *C. p. pricei* were held in the Animal Research Facility (ARF) at the University of Northern Colorado (UNC) and extracted at regular intervals (at least two months between extractions). All methods were approved by the UNC Institutional Animal Care and Use Committee (IACUC; protocols 1302D-SM-16 and 1701D-SM-S-20). Venom was mechanically extracted from snakes, lyophilized or air dried over desiccant and stored in a -20 °C freezer until analyzed. In addition, several samples of *C. p. miquihuanus* and *C. p. pricei* venoms were collected from captive snakes at several institutes (see Chapter II). *Crotalus intermedius* and *C. triseriatus* venoms were also collected from captive specimens held at IRENA in Michoacan, and one sample of C. transversus was collected by Dr. Robert Bryson; all are included in analyses reported here. Venoms from *C. triseriatus* and *C. pusillus*, likely more closely related to *C. lepidus* than the *C. intermedius* group (Figure 3.1), were included in this study as an outgroup.

Gel Electrophoresis

Reduced venom samples were electrophoresed on a NuPAGE Novex bis-tris 12% acrylamide mini gel with MES running buffer to provide a "molecular fingerprint" comparison of numerous venom samples. One Mark 12 standard (7 μL) was run concurrently with the venom samples (20 μg) to estimate molecular weight. Gels were electrophoresed at 150 volts, 125 mA for approximately 90 minutes. Gels were then stained in 0.1% Coomassie Brilliant Blue R-250 and placed on a gyrating shaker overnight to facilitate even staining. Excess stain was removed the following day and gels were placed in rapid destain (30% methanol, 7% glacial acetic acid in water) for approximately two hours or until sufficiently destained; images of gels were then transferred to 7% acetic acid in water and scanned using an HP Scanjet.

Enzyme Assays

Enzymatic activities of crude venoms were determined based on methods described in Smith and Mackessy (2016) and in Chapter II. Assays included metalloproteinase, thrombin-like and kallikrein-like serine proteinases, phospholipase A_2, phosphodiesterase and L-amino acid oxidase. Protein concentration (BCA) was used to standardize protein amounts used in all assays. Reverse-phase high performance liquid chromatography (RP-HPLC), fast protein liquid chromatography (FPLC), and gel electrophoresis were used to identify venom toxins using modified protocols outlined in Saviola et al. (2015).

Protein concentration determination. Lyophilized and air dried venom samples were dissolved at an approximate concentration of 4.0 mg/mL in Millipore-filtered water.

Thermo Scientific Pierce® BCA Protein Assay kit, with bovine gamma globulin as the standard was used to determine protein concentration of the crude venom samples.

Azocasein proteolytic assay. Venom samples and two controls were analyzed for metalloproteinase activity using an azocasein assay procedure from Aird and da Silva (1991). All samples were run in duplicate. Twenty µg of each sample were resuspended in 450 µL buffer (50 mM HEPES, 100 mM NaCl at pH 8.0). Substrate solution (sulfanilamide-azocasein, 2 mg/mL buffer; 500 µL) was then added to each sample. Samples were then vortexed and immediately transferred to a water bath incubator at 37 °C for 30 min. Two hundred and fifty µL trichloroacetic acid (TCA, 0.5 M) stop solution was added to each tube to stop the reaction. Tubes were vortexed, allowed to sit at room temperature for 10 minutes and then centrifuged at 2,000 rpm for 10 minutes. Absorbance of each tube was recorded at 342 nm using a Genesys™ 10 Series Spectrophotometer and buffer control absorbance was subtracted from samples, and specific activities were expressed as ΔA_{342} nm/min/mg protein.

Thrombin-like and kallikrein-like assays. Venom samples were tested for thrombin-like and kallikrein-like serine proteinase activity using methods modified from Mackessy (1993). All samples and controls were run in duplicate. One mM pNA substrate was first dissolved in DMSO and Millipore-filtered water was added to achieve final concentration (final - 1% DMSO). One µL crude venom at a concentration of 4 mg/mL and 374 µL of buffer (50 mM HEPES pH 8.0 with 100 mM NaCl) were combined before incubating at 37 °C for 3.0 min. Kallikrein-like substrate (benzoyl-Pro-Phe-Arg-paranitroaniline) or thrombin-like substrate (benzoyl-Phe-Val-Arg-paranitroaniline) was used depending on assay. The reaction was started by adding 50 µL

substrate and samples were incubated at 37 °C for 3.0 min. The reaction was then terminated with 75 µL 50% (v/v) acetic acid stop solution and absorbance of samples was recorded at 405 nm using a Genesys™ 10 Series Spectrophotometer and buffer control absorbance was subtracted from samples. Specific activity was expressed as nmol product/min/mg protein.

Phospholipase A_2 assay (PLA_2). Crude venom samples and controls were evaluated for PLA_2 activity in triplicate. Buffer used for analysis was 10 mM Tris-HCl (pH 8) containing 10 mM $CaCl_2$ and 100 mM NaCl. The substrate used was 4-nitro-3-(octanoyloxy)benzoic acid (Enzo Life Sciences, Inc., Farmingdale, NY, USA) dissolved in 100% acetonitrile. 50 µL of venom sample were combined with 500 µL buffer and placed in an ice bath. Fifty µL substrate was then added to each sample, vortexed and immediately incubated at 37 °C for 30 min. The reaction was then terminated with 50 µL 2.4% Triton X-100 in ddH_2O. Absorbance of samples was recorded at 425 nm using a Genesys™ 10 Series Spectrophotometer and buffer control absorbance was subtracted from samples, and specific activity was expressed as nmol product/min/mg protein.

L-amino acid oxidase assay. Venom samples were assayed for L-amino acid oxidase activity using methods outlined in Kishimoto and Takahashi (2001); all samples and controls were run in triplicate. L-methionine (MET) substrate (10x) was resolubilized at 7.46 mg/mL in buffer (50 mM borax, pH 8.5), o-phenylenediamine (OPD)-coupled substrate was dissolved in buffer at 2.16 mg/mL (10x), and horseradish peroxidase (HRP) was resolubilized at 8.1 mg/mL (10x). Crude venom sample at 1.0 mg/10 mL (10.0 µL) was added to each well on a 96-well plate. Ninety µL master mix (70% buffer, 10% MET, 10% OPD, and 10% HRP, v/v) were then added to each well to initiate reaction.

The plate was incubated at 37 °C for 30 minutes before returning to a cold surface and rapidly adding 50 µL termination solution (2.0 M sulfuric acid). Sample absorbance was read at 492 nm using a Genesys™ 10 Series Spectrophotometer and buffer control absorbance was subtracted from samples. Specific activity was expressed as ΔA_{492nm}/min/mg protein.

Phosphodiesterase assay. Phosphodiesterase assays were conducted in triplicate on adult *C. tancitarensis, C. pricei,* and *C. triseriatus* venom samples using methods outlined by Bjork (1963) and modified by Laskowski (1980). 220 µL buffer (1.0 mL 100 mM Tris-HCl pH 9.0 with 10 mM $MgCl_2$) was combined with 20 µg (5 µL) crude venom. Samples were then placed in an ice bath for three minutes before 150 µL substrate (0.3402 mg/mL; 1.0 mM bis-p-nitrophenylphosphate dissolved in buffer) was added to initiate reaction. Samples were then incubated at 37° C for 30 minutes, returned to the ice bath and reactions were stopped by adding 375 µL termination solution (100 mM NaOH with 10 mM disodium-EDTA) to each tube. Samples were brought to room temperature before absorbance was read at 400 nm using a Genesys™ 10 Series Spectrophotometer and buffer control absorbance was subtracted from samples. Specific activity was expressed as ΔA_{400nm}/min/mg protein.

Reverse-Phase High Performance Liquid Chromatography

Crude venom samples were analyzed using reverse-phase high performance liquid chromatography (RP-HPLC). Methods were consistent for all samples despite samples being analyzed several years apart. Two milligrams crude venom was resuspended in 200 µL Millipore-filtered water. Samples were then centrifuged at 9,500 x g for 5 minutes and

filtered through a 0.45 µm syringe tip filter before injection onto a Jupiter 5 µm C_4 300Å 250 x 4.60 mm RP-HPLC column. Fractions were collected at a rate of 1 mL/min for 120 minutes and all 120 fractions were collected. Venoms were fractionated using a gradient of 0.1% trifluoroacetic acid in Millipore-filtered water (solution A) and 0.1% trifluoroacetic acid in 80% acetonitrile (solution B). Proteins were eluted with solution A at 95% and solution B at 5% from 0-5 minutes. Between 5-10 minutes, flow of B increased to 20%. From minutes 10-105, B ramped from 20% to 80%. Solution B continued to ramp to 100% between minutes 105-110. The gradient remained at 100% solution B for five minutes before returning to the starting gradient of 95% A and 5% B for the remainder of the run. Eluting proteins and peptides were then detected at 220 nm and 280 nm. Fractions corresponding to protein/peptide peaks were collected and placed in a -80 °C freezer overnight, then lyophilized for at least 12 hours.

Lethal Toxicity Assays

Venom toxicity methods were adapted from Mackessy (2008). Geckos used in lethal toxicity (LD_{50}) assays were obtained from Bushmaster Reptile (Boulder, CO, USA). Three adult *Hemidactylus frenatus* (3-4 g) were used at each dose level, and three venom samples for *C. pricei* (two venom samples for *C. tancitarensis*) were combined in order to obtain an average lethal toxicity measurement. Doses of 0.5, 1.0, and 5.0 µg venom/g body weight were administered initially, with dose adjustment as needed to determine LD_{50}. Lyophilized venom was reconstituted in 18.2 MΩ Millipore-filtered water to a concentration of 1.0 mg/mL. Doses appropriately adjusted to individual gecko mass were injected intraperitoneally anterior to the right hind leg using a 28G x ½ in.

needle and 0.5 mL syringe. A 24-hour time frame was used to determine lethal toxicity: if a gecko survived after 24 hours, that dose was considered non-fatal.

Statistical Analyses

Significance was calculated for enzymatic activities using a 2-way Analysis of Variance (ANOVA). A Tukey HSD test was completed post-hoc to determine differences between all combinations of means. P-values < 0.05 were considered statistically significant. *Crotalus transversus* was excluded from analysis due to availability of only one sample in the study. However, very limited number of samples were available for several locations, and due to violation of certain assumptions of ANOVA tests relating to sample size, statistical results should not be considered statistically rigorous.

Results

Gel Electrophoresis

Results of gel electrophoresis indicate that *C. tancitarensis* has a venom profile typical of type I venom. Overall venom profiles appear to be similar between neonate and adult *C. tancitarensis*, with a few key differences. Adults display an obvious PI metalloprotease band around 21 kDa and a double band around 14 kDa, indicating two compounds eluting in the phospholipase A_2 (PLA_2) range (Figure 3.2). Neonates lack both of these characteristics, with a very faint PI metalloprotease band and only a single band in the PLA_2 range (Figure 3.2, A). Overall, *C. tancitarensis* electrophoretic patterns are most similar to that of *C. transversus*, which also displays a PI metalloprotease band and double PLA_2 (Figure 3.2, B). All venom samples analyzed display a type I venom profile; the outgroups *C. triseriatus* and *C. pusillus* also exhibited toxins in the peptide myotoxin range (~5.0 kDa) (Figure 3.2, A).

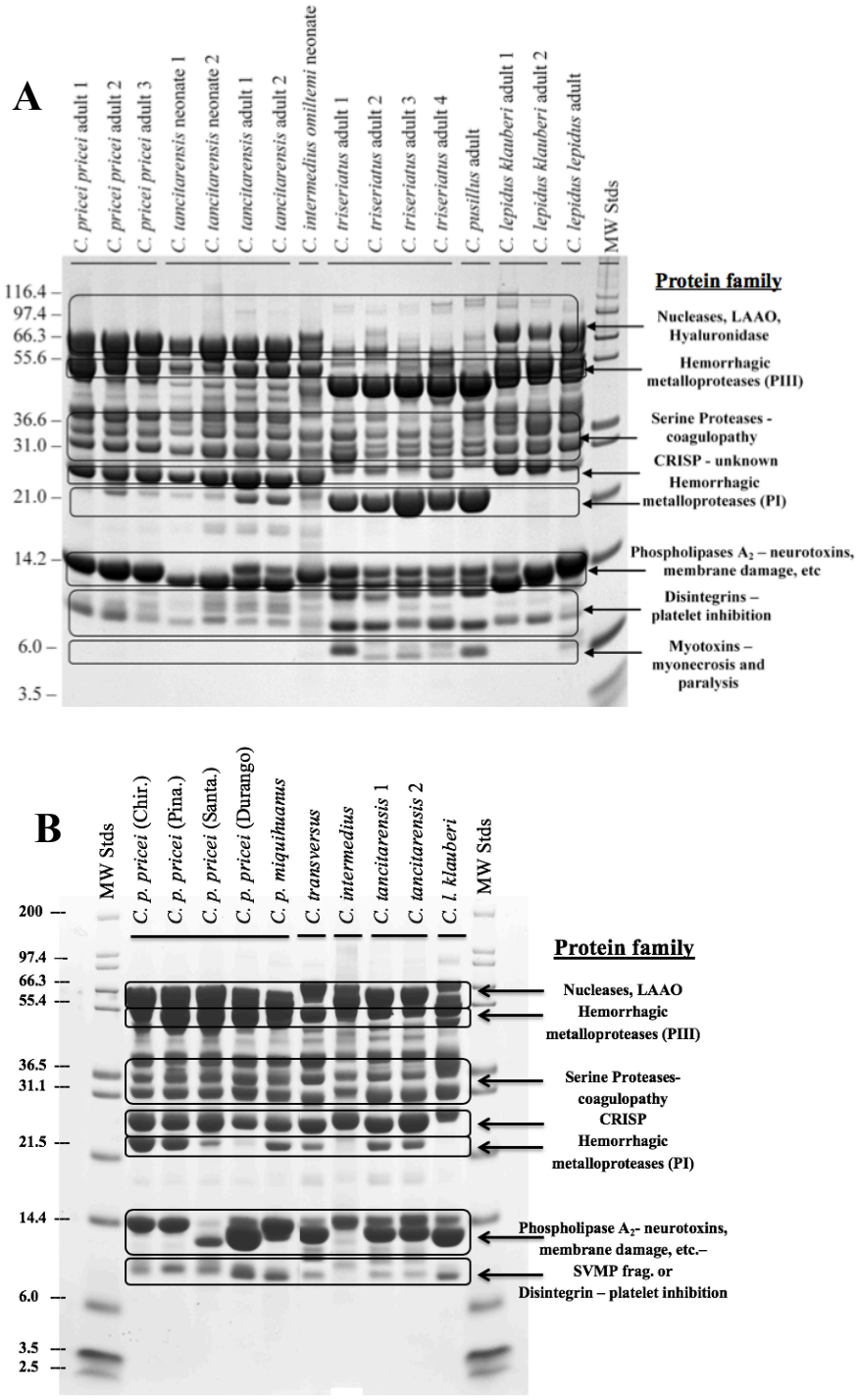

Figure 3.2. SDS-PAGE gel of various *Crotalus* species of central México compared to neonate and adult *C. tancitarensis* venoms (**A**) and *C. tancitarensis* adult venoms compared to other adults in the *Crotalus intermedius* group (**B**). *Crotalus p. pricei* were from various geographic locations: Chiricahua Mountains (Chir.), Pinaleño Mountains (Pina.), Santa Rita Mountains (Santa.), and Durango, México. Approximate molecular weight is displayed to the left and shown in kDa. Typical protein families, as determined by mass and previous experiments with purified toxins, are shown on the right.

Azocasein Proteolytic Assay

Azocasein metalloproteinase enzyme assay results showed a dichotomy between *C. tancitarensis* and *C. transversus* and the rest of the species analyzed. Both *C. tancitarensis* and *C. transversus* had low values of 0.34 and 0.32 $\Delta A_{342\ nm}$/min/mg respectively, while the other species analyzed showed activities of 0.95 $\Delta A_{342\ nm}$/min/mg or higher (Figure 3.3; Table 3.1). *Crotalus tancitarensis* metalloproteinase activity was significantly lower than *C. pricei, C. intermedius,* and *C. p. miquihuanus* ($p < 0.05$).

Thrombin-like and Kallikrein-like Assays

Enzyme assay results revealed several enzyme activity differences between *C. tancitarensis* and closely related species. *Crotalus tancitarensis* had noticeably lower thrombin-like and kallikrein-like serine protease activity (SVMP) than other species examined (Table 3.1 and Figure 3.3), but was only significantly different from *C. p. pricei* ($p < 0.05$). Specifically, kallikrein-like SVMP activity was relatively low, with a value of 47 nmol/min/mg. *Crotalus p. miquihuanus* crude venom showed the highest activity with a value of 5201 nmol/min/mg and was statistically different from *C. tancitarensis, C. p. pricei, C. intermedius,* and *C. triseriatus* ($p < 0.01$). The second lowest kallikrein-like SVMP value was *C. transversus* crude venom with a value of 112 nmol/min/mg.

Thrombin-like SVMP activities exhibited a similar trend, with *C. tancitarensis* venom showing the lowest value at 328 nmol/min/mg and *C. p. miquihuanus* with the highest value at 3236 nmol/min/mg (Table 3.1 and Figure 3.3). *Crotalus intermedius* had the second lowest thrombin-like SVMP activity with a value of 509 nmol/min/mg.

Crotalus p. miquihuanus was significantly different from *C. tancitarensis, C. p. pricei, C. intermedius,* and *C. triseriatus* ($p > 0.01$).

Phospholipase A_2 Assay I

Phospholipase A_2 assay (PLA_2) results indicated that *C. tancitarensis* had noticeably lower enzymatic PLA_2 activity of 34.6 nmol/min/mg compared to other species within the *Crotalus intermedius* group, but was only statistically significantly different from *C> miquihuanus* ($p < 0.01$) (Figure 3.3 and Table 3.2). However, the outgroup, *C. triseriatus,* had similar activity at 34.8 nmol/min/mg and was also significantly different from *C. miquihuanus* ($p < 0.01$). *Crotalus miquihuanus* had the highest PLA_2 activity with a value of 79.6 nmol/min/mg. The PLA_2 assay was not completed for neonate *C. tancitarensis* due to insufficient amounts of venom.

L-amino Acid Oxidase Assay

Enzyme activity for LAAO indicated that *C. tancitarensis* has moderate enzyme activity of 16.3 ∆A492nm/min/mg compared to other species in the *Crotalus intermedius* group (Figure 3.3, Table 3.2). *Crotalus transversus* exhibited the highest activity with 29.0 ∆A492nm/min/mg. The LAAO assay was not completed for *C. intermedius* or neonate *C. tancitarensis* due to insufficient amounts of venom. There were no significant differences in LAAO activities.

Phosphodiesterase Assay

Crotalus tancitarensis phosphodiesterase (PDE) activity yielded similar results to those of *C. transversus.* Both species had relatively low PDE activity (0.098 and 0.124 ΔA_{400nm}/min/mg respectively), though not as low as *C. p. pricei* (0.037) (Figure 3.3, Table 3.2). *Crotalus p. miquihuanus* had the highest activity at 0.232 ΔA_{400nm}/min/mg

and was significantly different from *C. p. pricei* ($p < 0.05$), though this is still relatively low compared to PDE specific activity of other *Crotalus* venoms (Mackessy, 2008). The PDE assay was not completed for *C. intermedius* or neonate *C. tancitarensis* due to insufficient amounts of venom.

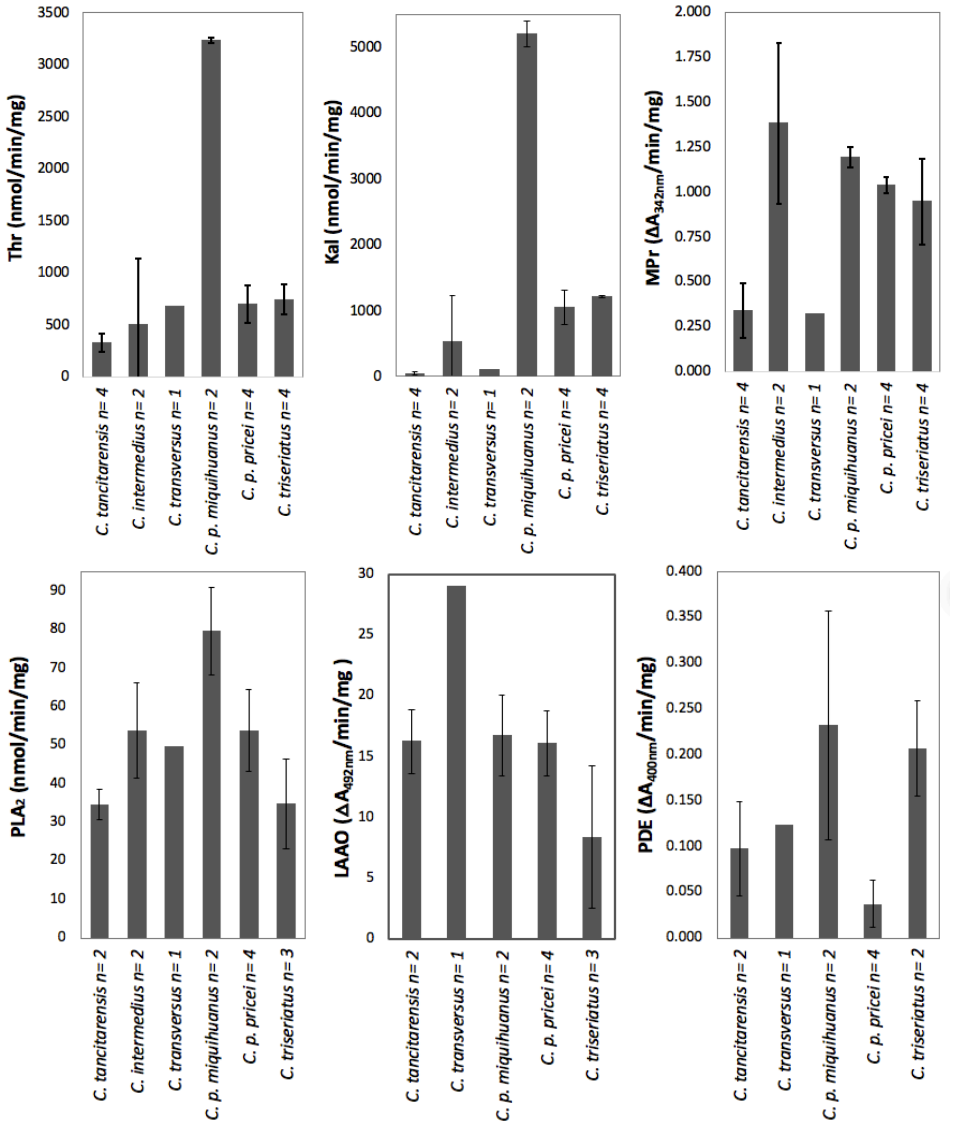

Figure 3.3. Average enzyme activities of mountain rattlesnakes. Abbreviations: thrombin-like (Thr), kallikrein-like (Kal), metalloproteinase (Azo), phospholipase A_2 (PLA_2), L-amino acid oxidase (LAAO), and phosphodiesterase (PDE).

Table 3.1. Enzyme activities of mountain rattlesnake venoms ($\bar{x}\pm$SD).

Species	Thr (nmol/min/mg)	Kal (nmol/min/mg)	MPr ($\Delta A_{342\ nm}$/min/mg)
C. tancitarensis n= 4	328 ± 87	47 ± 21	0.34 ± 0.15
C. intermedius n= 2	509 ± 630	536 ± 697	1.38 ± 0.45
C. transversus n=1	681	112	0.32
C. p. miquihuanus n= 2	3236 ± 29	5201 ± 196	1.19 ± 0.06
C. p. pricei n= 4	699 ± 182	1055 ± 261	1.04 ± 0.04
C. triseriatus n= 3	746 ± 147	1214 ± 18	0.95 ± 0.24

Abbreviations: thrombin-like (Thr), kallikrein-like (Kal), and metalloproteinase (MPr).

Table 3.2. Enzyme activities of mountain rattlesnake venoms ($\bar{x}\pm$SD).

Species	PLA_2 (nmol/min/mg)	LAAO (ΔA_{492nm}/min/mg)	PDE (ΔA_{400nm}/min/mg)
C. tancitarensis n= 2	34.6 ± 3.8	16.3 ± 2.6	0.098 ± 0.052
C. intermedius n= 2	53.8 ± 12.4	--	--
C. transversus n= 1	49.8	29.0	0.124
C. p. miquihuanus n= 2	79.6 ± 11.4	16.8 ± 3.3	0.232 ± 0.124
C. p. pricei n= 4	53.8 ± 10.5	21.5 ± 3.6	0.037 ± 0.025
C. triseriatus n= 3	34.8 ± 11.7	8.4 ± 5.9	0.207 ± 0.052

Abbreviations: phospholipase A_2 (PLA_2), L-amino acid oxidase (LAAO), and phosphodiesterase (PDE).

Reverse-Phase High Performance Liquid Chromatography

Results revealed similar overall venom RP-HPLC profiles among the species examined (Figures 3.4-3.7). All species displayed a prominent peak between minutes 18-20 and a clustering of peaks of varying abundance between minutes 55-90. Overall, *C. tancitarensis* appeared to have a profile most similar to *C. transversus* (also see appendix, Figure C. 3), based on peak presence and peak height, particularly between minutes 55-90 where most proteins eluted and most variation between individuals venoms appears.

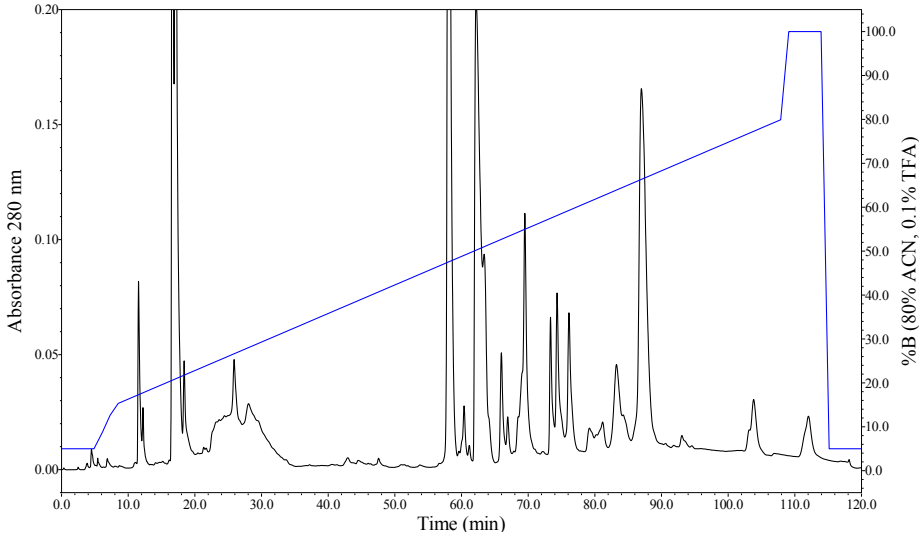

Figure 3.4. Reverse-phase HPLC chromatogram of *C. tancitarensis* 2 venom (2.0 mg). Elution gradient is indicated by the blue line, with concentrations displayed on the right side of the chromatogram.

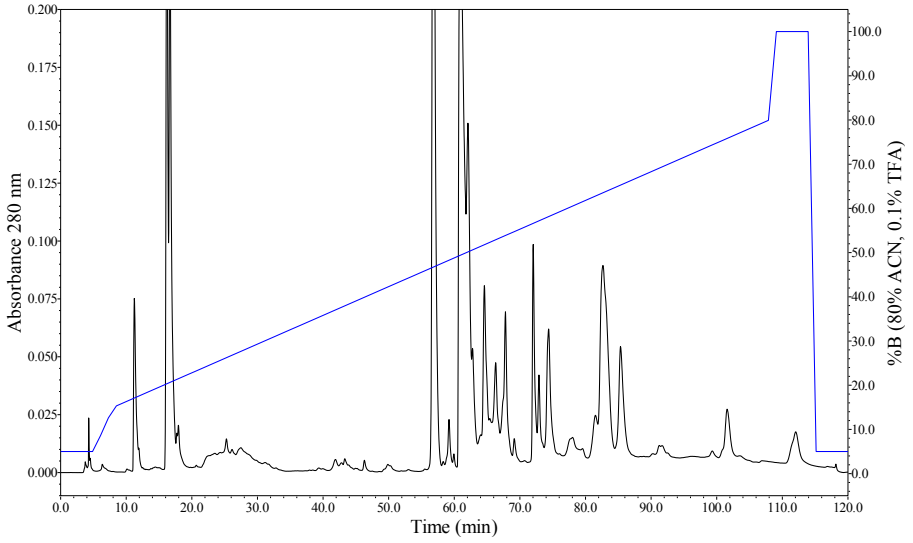

Figure 3.5. Reverse-phase HPLC chromatogram of crude *C. transversus* venom (2.0 mg). Elution gradient is indicated by the blue line, with concentrations displayed on the right side of the chromatogram.

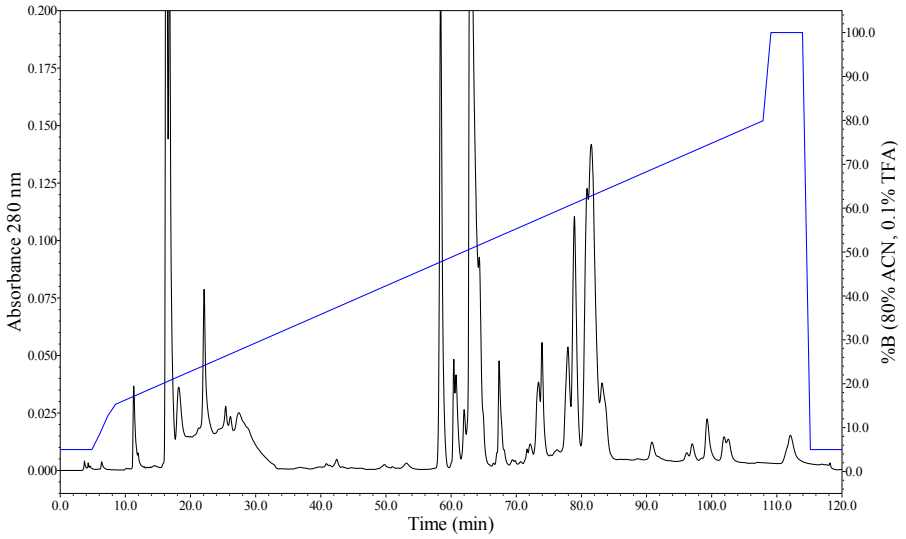

Figure 3.6. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. price pricei* venom. Elution gradient is indicated by the blue line, with concentrations displayed on the right side of the chromatogram.

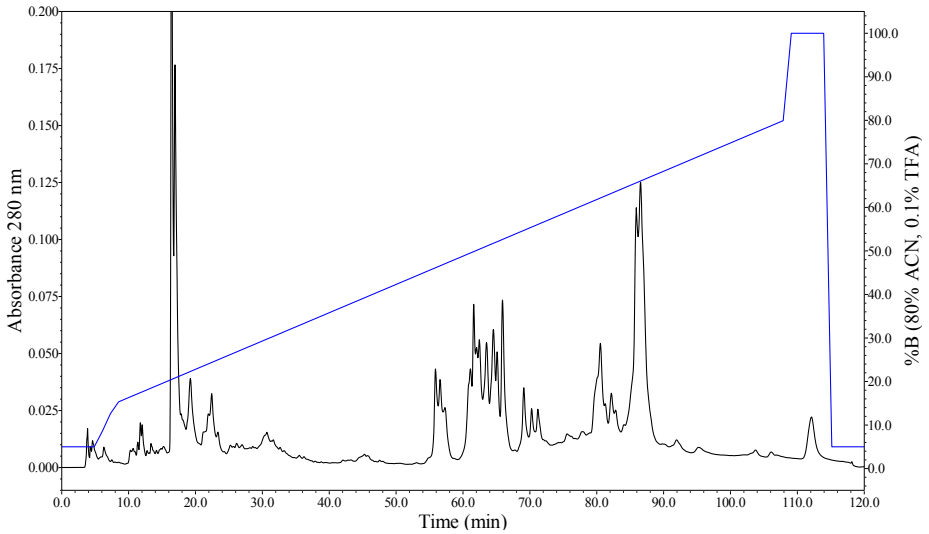

Figure 3.7 Reverse-phase HPLC chromatogram of crude *C. intermedius* venom (2.0 mg). Elution gradient is indicated by the blue line, with concentrations displayed on the right side of the chromatogram.

Lethal Toxicity Assays

Both *C. pricei* and *C. tancitarensis* displayed high toxicity towards geckos, with LD_{50} values below 1 μg/g (Figure 3.8). *Crotalus pricei* venom was slightly more toxic towards geckos than *C. tancitarensis,* though the difference (0.13 μg/g) is likely not

biologically significant. In comparison, the lethal dose of *C. p. pricei* venom when tested on Non-Swiss albino (NSA) mice is 1.25 µg/g (Mackessy, 2008). The amount of venom collected from *C. tancitarensis* was insufficient to complete LD_{50} assays on NSA mice. The level of toxicity toward mice exhibited by *C. p. pricei* is likely comparable in *C. tancitarensis*, because there is no evidence of neurotoxins in either venoms, based on RP-HPLC and gel electrophoresis.

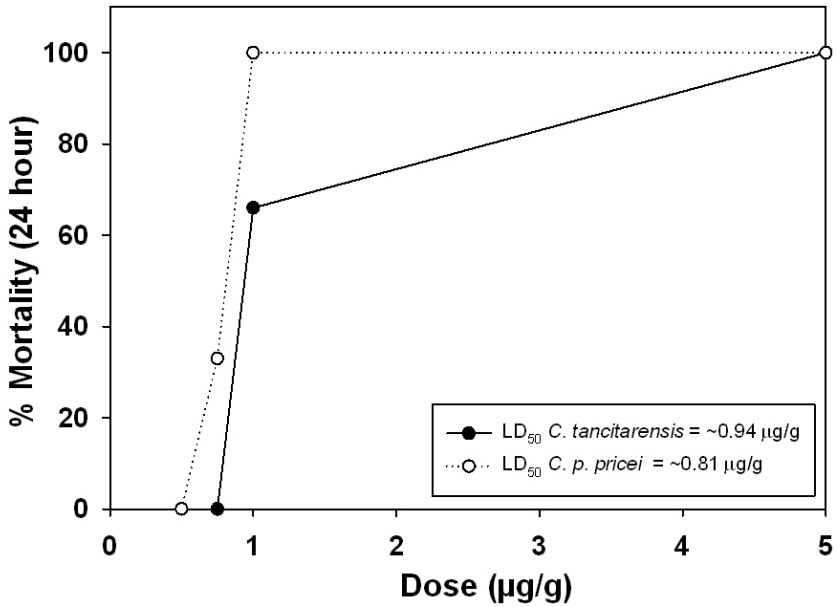

Figure 3.8. Lethal toxicity (µg/g) of adult *C. tancitarensis* venom and adult *C. p. pricei* venom toward *Hemidactylus frenatus*.

Discussion

Numerous studies have analyzed species diversification throughout the Mexican highlands (e.g., Bryson et al., 2011a, b; Coblentz and Riitters, 2005; Gottfried and Hodges, 2005; Mastretta-Yanes et al., 2015; Peterson and Navarro-Sigüenza, 1999), and these biodiversity hotspots are home to numerous endemic species, many of which are still being described. During the LGM (approximately 23,000 – 10,000 yr BP), Mexican highland habitat was connected via pine-oak corridors and likely allowed for genetic

connectivity between species now located in isolated mountain ranges (Arriaga et al, 2005; Metcalfe et al., 2000; Thompson and Anderson, 2000). Because of the isolated nature of these communities, non-vagile herpetofauna have differentiated substantially since the LGM (Blair and Sánchez-Ramírez, 2016; Bryson et al., 2011a, b). This and earlier periods of discontinuity and differentiation also gave rise to the diversity of rattlesnakes and the isolated high elevation populations present in the Americas today. Despite the lack of connectivity between populations of *Crotalus* in the Mexican highlands and Arizona, the venom of montane species analyzed in this study show relatively little differentiation.

The classification of *Crotalus tancitarensis* as a separate species was based on distinct morphological characteristics, scale patterns, and geographic isolation. This isolation has likely existed since the LGM. Along with these defining characteristics, *C. tancitarensis* has a venom profile relatively similar to species in the *Crotalus intermedius* group (particularly *C. transversus* and *C. p. pricei*), but with distinct differences. The results of this study support both hypotheses: *C. tancitarensis* venom is similar to other species in the *Crotalus intermedius* group based gel SDS-PAGE and RP-HPLC, and this venom displays characteristics of a type I venom. This venom can be considered a unique type I, containing moderate metalloprotease activity and lacking any apparent neurotoxins, but exhibiting high toxicity towards model prey (*Hemidactylus frenatus*). Overall, the venom profile of *C. tancitarensis* appears most similar to *C. transversus* based on RP-HPLC analysis, gel electrophoresis, and enzyme activity. Both species displayed relatively low SVSP (thrombin-like and kallikrein-like) and SVMP (azocasein) activities compared to the other members of the *Crotalus intermedius* group. Conversely,

C. p. pricei and *C. p. miquihuanus* venom samples displayed high SVSP and SVMP activity compared to other species of rattlesnakes (Chapter II; Mackessy, 2008). Additionally, Saviola et al. (2017) found that *C. lepidus* and *C. willardi,* Sky Island rattlesnakes with similar ecology to species within the *Crotalus intermedius* group, have relatively high SVSP and SVMP activity. Low metalloprotease and serine protease activity is relatively uncommon in species that exhibit no apparent neurotoxic compounds. These enzymatic proteins are responsible for the tissue degradation and disruption of hemostasis that aid in prey acquisition in species with type I venom (Mackessy, 2010). Enzymatic phospholipase A_2 activity was relatively consistent with PLA_2 activity levels seen with other small, high elevation rattlesnakes (Saviola et al., 2017).

Though lack of an apparent neurotoxin and moderately high SVMP activity suggests type I venom, *C. tancitarensis* also displays high toxicity. *Hemidactylus frenatus* acts as a model for reptilian prey in this study, although recent evidence suggests that natural prey may exhibit generalized toxicity tolerance towards high elevation *Crotalus* venoms (Grabowsky, unpublished). *Crotalus pricei* has been previously shown to be relatively toxic to Non-Swiss albino lab mice as well (LD_{50} = 1.25 μg/g; Mackessy, 2008). Though mammalian model LD_{50} assays were not completed for *C. tancitarensis,* the general consistency of enzymatic activity and venom profile with *C. p. pricei* suggest *C. tancitarensis* venom follows a similar pattern of toxicity towards mammals. Generally, very low SVMP activity level, coupled with high toxicity, indicates a venom profile consistent with a type II venom containing presynaptic neurotoxic PLA_2s or similar toxins. In comparison, *C. oreganus concolor* and *C. adamanteus*, species with very

different body sizes and life histories, have rather similar SVMP activities of 0.135 and 0.333 $\Delta A_{342\ nm}$/min/mg respectively, and while *C. adamanteus* venom is more consistent with type I, *C. o. concolor* venom shows high toxicity towards mammalian models and is considered type II (Mackessy, 2008; Margres et al., 2016; Smith and Mackessy, 2016). Based on these distinctions, the venom profiles of *C. tancitarensis*, *C. transversus* and other species examined in this study should be considered type I venoms.

The similarities of *C. tancitarensis* and *C. transversus* venom characteristics complements results of phylogenetic analysis based on mitochondrial DNA and dispersal-extinction-cladogenesis modeling described in Bryson et al. (2011). Based on a mixed-model Bayesian approach, *C. tancitarensis* and *C. transversus* create a monophyletic clade that likely diverged from *C. intermedius* during the Pliocene era, between 5.33 and 3.6 million years BP (Bryson et al., 2011; Cohen et al., 2018). Many of the major mountain ranges throughout México, including those within the Trans-Volcanic Belt, experienced major climatic and vegetation shifts during the Last Glacial Maximum (LGM) around 25,000 years BP (Bryson et al., 2011; McDonald, 1993; Metcalfe et al., 2000). During this time, the climate of the now xeric Central Méxican Plateau and other similarly warm dry regions was much cooler and wetter, allowing for movement of species adapted to montane or pine-oak ecosystems to move between ranges. *Crotalus tancitarensis* and *C. transversus* likely diverged much later than this, possibly during the LGM when *C. p. pricei* and *C. p. miquihuanus* diverged due to geographic isolation of the Sierra Madre Occidental and Sierra Madre Oriental (Bryson et al., 2011). This could explain the similarities between venom phenotypes expressed in *C.*

tancitarensis and *C. transversus* as opposed to the higher SVMP and SVSP enzymatic activities expressed in the rest of the *Crotalus intermedius* group.

While overall enzymatic activity in this study is based on combined averages of adult and neonate *C. tancitarensis* venom, ontogenetic shifts in venom composition are common and should be taken into consideration when evaluating venom compositional trends (Mackessy, 1985, 1988, 2008, 2018). Results of gel electrophoresis indicate neonate *C. tancitarensis* have lower metalloprotease activity based on faint bands in the PI metalloprotease range around 21 kDa (Figure 3.6 A). However, only neonate 1 had lower activity (0.112 $\Delta A_{342\ nm}$/min/mg), while neonate 2 and both adults all had higher activities. Another distinct difference between neonates and adults appeared around the PLA_2 band range based on electrophoretic analysis (Figure 3.6 A). Both adult venoms exhibited two clear PLA_2 bands, while neonates showed only one band. Unfortunately, there was not enough neonate venom material to complete PLA_2 enzyme assays, so only results of adults are shown in Table 2. In this case, ontogenetic shifts in composition of *C. tancitarensis* venom cannot be determined definitively, but based on electrophoretic data, some shifts in activity levels are expected.

Based on the above analyses, *C. tancitarensis* and other species within the *Crotalus intermedius* group appear to have venom phenotypes characteristic of type I venoms, with *Crotalus tancitarensis* most similar to *C. transversus,* consistent with close relationships hypothesized on the basis of mitochondrial DNA analysis. Small sample sizes for each group analyzed make it difficult to state that the trends observed are typical for entire populations, but given the highly localized distribution of *C. tancitarensis*, the descriptions presented here are likely representative for this species.

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CHAPTER IV

NICHE MODELING OF A LIZARD SPECIALIST, *CROTALUS PRICEI PRICEI* (TWIN-SPOTTED RATTLESNAKE) AND ITS NATURAL PREY, *SCELOPORUS JARROVII* (YARROW'S SPINY LIZARD)

Abstract

Crotalus pricei pricei (Western Twin-spotted Rattlesnake) is a small species restricted to the sky islands of Arizona and México. This species is protected in Arizona and is threatened by climate change, human activity, and illegal collection, but little is known about the spatial ecology of these snakes. The goal of this study is to determine resource selection of *C. p. pricei* by creating a species distribution model and explore parameters influencing this aspect of its natural history. *Sceloporus jarrovii* (Yarrow's Spiny Lizard) probability estimates derived from the resource selection probability function (RSPF) were included in the model for *C. p. pricei* to account for effect of prey selection on current range of *C. pricei*. Results indicate that presence predictions (RSPF) for *S. jarrovii* positively correlate with presence of *C. p. pricei.* Probability of use for both species is highest primarily at high elevations in Sky Island habitat, indicating migration between mountain ranges is unlikely. These results illuminate the importance of high elevation conditions and could influence conservation plans of high elevation reptiles, particularly in the American Southwest.

Introduction

Crotalus pricei pricei (Western Twin-spotted Rattlesnake) is a diminutive species that is found only in the Sky Islands of Arizona and México. Though this species is protected in Arizona and is threatened by climate change, human activity, and illegal collection (Kupfer et al., 2005; Prival and Schroff, 2012), few studies have focused on spatial ecology of these snakes. *Crotalus p. pricei* has a restricted diet, specializing on lizards in the genus *Sceloporus*. An ongoing, 17+ year study by Prival and Schroff (2012) has found that approximately 68% of *C. p. pricei's* diet consists of *Sceloporus sp.*, with all samples that were identified to species level being *Sceloporus jarrovii* (Yarrow's spiny Lizard).

Variation in snake venom composition is likely driven by adaptive evolution and can lead to development of prey-specific toxicity in species with specialized diets (Arbuckle et al., 2017; Barlow et al., 2009; Gibbs and Mackessy, 2009; Pawlak, et al., 2006, 2009). Coevolutionary patterns have been observed between venomous snakes and their prey, typically in areas of high predator and prey abundance or with specialist species (Barlow et al., 2009; Biardi et al., 2006; Poran et al., 1987). Toxin resistance has also been observed in prey as an adaptation to counter attacks from venomous animals (Arbuckle et al., 2017). Poran et al. (1987) explored ground squirrel resistance to *Crotalus oreganus* venom and found that they are resistant to toxins in areas of high predator density. In these snake-rich regions, *C. oreganus*, California Ground squirrels (*Otospermophilus beecheyi*) comprise approximately 69% of the diet. In the Chiricahua Mountains, *S. jarrovii* makes up approximately 67.6% of *C. p. pricei* diet, indicating that predator-prey dynamics could be an important driver of resource selection in this system

(Prival and Schroff, 2012). Since the ecology of these two species appears to be intertwined, driving forces need to be further studied to determine extent of the relationship.

Crotalus p. pricei is typically restricted to habitats between elevations of 1860 and 3050 meters (Prival and Schroff, 2012), while *S jarrovii* can tolerate a wider range of elevations as low as 1370 m up to 3550 m (Jones and Lovich, 2009). Sky Island ecosystems are characterized by high elevation Madrean pine-oak ecosystems surrounded by low elevation deserts, xeric shrubland, and grassland (Arriaga et al., 2005). Given the geographic locations and isolated nature of Sky Islands, they are particularly vulnerable to vegetation shifts due to climate change. An estimated temperature increase of about 0.89 °C occurred during the twentieth century, with approximately half of the measurable increase in global surface temperature between 1951 and 2012 attributed to anthropogenic influences (IPCC, 2013). As an ectotherm reliant on environmental conditions for thermoregulation, *C. p. pricei* is particularly vulnerable to rapid climate change. This warming trend could promote detrimental repercussions for ectotherms, since physiological adaptation cannot keep up. Climate-based models for reptile species have become more common in recent years, and results show that there is a clear correlation between temperature increase and either significant range shift or reduction in range size, depending on the species (Barrows 2011; Boyle et al., 2016; Ceia-Hasse et al., 2014; Davis et al., 2015; Douglas et al., 2016; Jarnevich et al., 2018; Lawing and Polly 2011; van Riper et al., 2014). This provides further evidence that niche models of high elevation reptiles such as *C. p. pricei* must be created in order to understand the current extent and predicted outcomes of climatic shifts.

An upward shift in elevational range due to climate change could further isolate *C. p. pricei* populations (within and between mountain ranges). Resulting genetic isolation can occur within and across Sky Islands as a consequence of disjunct ranges. Since *C. p. pricei* is restricted to higher elevations, movement between mountain ranges and subsequent gene transfer is effectively impossible (Lomolino et al., 1989). For specialized ectotherms of low vagility, reliant on thermoregulation via environmental (primarily solar) energy sources, movement up or down the steep elevation gradients and across inhospitable environments to access other appropriate Sky Island habitat is simply not possible.

Mitochondrial genomic analysis has shown that the high elevation rattlesnakes of the Méxican highlands (including *C. triseriatus, C. aquilus, C. lepidus, C. pusillus, C. ravus, C. intermedius, C. transversus, C. tancitarensis,* and *C. pricei*) diverged during the Pleistocene period (Bryson et al., 2011a; 2011b). Assuming that *C. pricei* currently has morphology and physiological needs similar to what it had during Sky Island isolation, the last possible gene flow events between *C. pricei* populations (including both *C. p. pricei* and *C. p. miquihuanus* subspecies) occurred approximately 23,000 – 10,000 yr BP, during the Last Glacial Maximum (LGM) (Bryson et al. 2011a; Metcalfe et al., 2000), when Méxican highland habitat was likely linked via ephemeral pine-oak woodlands. Additionally, dispersal from the Sierra Madre Occidental range to the Sky Islands (AZ) could have only occurred prior to the end of the LGM via these corridors.

For many types of modeling, presence-absence data is needed to construct an accurate representation of species distribution or habitat use based on habitat characteristics. Resource selection functions (RSF) and resource selection probability

functions (RSPF) are multivariate functions often used to model the probability of resource use for populations or species where no true absence data is present. These models are generally referred to as presence-use models. These methods quantify preference based on biologically relevant habitat variables by comparing known species locations and randomly selected availability points based on known habitat preference (Aarts et al., 2012). Species probability of occurrence can be estimated by modeling the logistic form of the RSPF or the exponential form of the RSF (Manly et al., 2002). Recently, RSF have been used to model probability of use and are considered more intuitive (McDonald et al., 2013), but this method does not work with all datasets and should be evaluated on a case-to-case basis depending on sampling methods (Manly et al., 2002; McDonald et al., 2013). Though these modeling methods have become more relevant to modeling reptile species distribution, little work has been done with mountain rattlesnakes (but see Hatten et al., 2016).

One study spatially investigated Pleistocene environmental impacts on the phylogeny of *C. pricei* and close relatives using Maxent software (Bryson et al., 2011). No prior studies investigating the spatial relationship between *C. pricei* and *S. jarrovii* have been completed. Considering that the diet of *C. pricei* is composed mainly of *S. jarrovii, S. jarrovii* predicted presence is expected to be an important factor in *C. pricei* presence. Because both species primarily occur at higher elevations, lower temperatures are likely correlated with presence as well. Accurate understanding of spatial relationships is crucial when determining the need for, development of, and success probability of conservation efforts, which are likely to become necessary for *C. pricei* in the near future. This study aims to characterize the factors contributing to distribution

overlap of *C. p. pricei* and its primary prey source, *Sceloporus jarrovii* (Yarrow's Spiny Lizard) and provide predictive occurrence maps in Arizona. Results of this study provide empirical evidence outlining habitat requirements for both species, and offer insight into climatic factors that may inhibit species persistence.

Hypotheses

Two hypotheses have been developed to address the knowledge gaps outlined and add to the empirical evidence regarding *C. p. pricei* distribution and predator-prey interactions in Southeastern Arizona.

H_1 As a lizard specialist, *C. p. pricei* distribution will show close concordance with range of preferred prey, *S. jarrovii.*

H_0 *Crotalus p. pricei* range will not completely overlap that of *S. jarrovii*.

Prediction *S. jarrovii* will have a greater range of possible habitats than *C. p. pricei* once environmental constraints are applied to a presence-only distribution model. The known range of *S. jarrovii* exceeds that of *C. p. pricei,* so this model should predict greater habitat availability.

H_2 The resource selection probability function (RSPF) produced for *C. p. pricei* will largely be explained by the predicted range for *S. jarrovii,* based on the RSPF (probability of use) produced for *S. jarrovii.*

H_0 The predicted range for *S. jarrovii* will not be a strong predictor for the distribution of *C. p. pricei.*

Prediction Since *C. p. pricei* relies so heavily on *S. jarrovii* for dietary purposes, it is expected that the predicted occurrence (RSPF) of *S. jarrovii* is a strong predictor for the occurrence of *C. p. pricei* throughout Arizona.

Methods

Data Sources and Spatiotemporal Extent

Crotalus p. pricei and *S. jarrovii* locality data was obtained from several credible sources including VertNet (portal.vertnet.org/), Arctos Collection Management

(arctos.database.museum), GBIF (Global Biodiversity Information Facility; www.gbif.org), and the Heritage Data Management System (HDMS; Arizona Game and Fish Department) (Figure 4.1). Data was truncated to localities within Arizona state boundaries and observations recorded between 1970 and 2017, with the assumption that more recent entries are more accurate. All locality data was screened for spatial errors and projected to NAD 1983 UTM Zone 12 before use in analyses. Spatial filtering (Phillips et al., 2009; Veloz, 2009) was applied to *C. p. pricei* locations in order to reduce sampling bias in areas that are prone to frequent resampling, particularly the area in which a long term study site exists (Prival and Schroff, 2012), and 80 % of the resulting points were used as training data in model development..

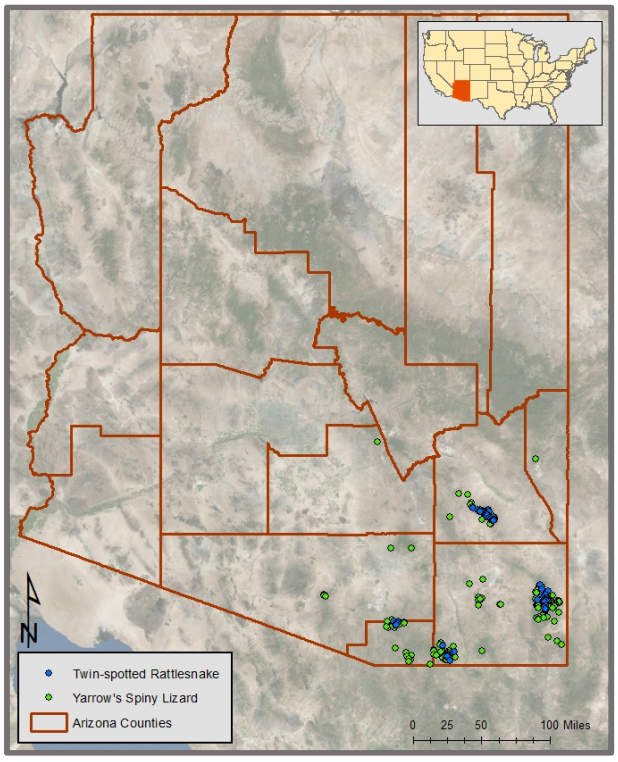

Figure 4.1. *Crotalus pricei* (Twin-spotted Rattlesnake) and *Sceloporus jarrovii*(Yarrow's Spiny Lizard) observation points used in RSF and RSPF models. Data includes observations from 1955-2017 obtained from VertNet.org (VertNet), Arctos.org (Arctos Collection Management), and GBIF (Global Biodiversity Information Facility).

Covariate selection was based on apparent biologically relevant characteristics of known *C. p. pricei* and *S. jarrovii* habitat (Table 4.1). *Crotalus p. pricei* is known to occur above ~1800 m and primarily inhabit montane pine-oak forest with access to southeast facing talus-rich slopes (Prival and Schroff, 2012; Campbell and Lamar, 2004).

ArcGIS (Version 10.5.1) was used to organize and apply spatial data to species observation and availability points. Availability points were randomly derived from within the area theoretically accessible to each species to account for all possible locations within the extent of species physiological limits and spatially balanced to limit clustering of points. Availability (or pseudo-absence) points can be generated on a variety of methods (Jarnevich et al., 2017). Because true absence data was not available for this dataset, availability points were extracted from within the range of each species, truncated by elevation since this is suggested to be a defining feature in habitat of both species (Campbell and Lamar, 2004; Jones and Lovich, 2009; Northrup et al., 2013; Prival and Schroff, 2012). Number of availability points was determined using modified methods outlined in Northrup et al. (2013), but modified by using availability points within the elevation range of each species given that this study represents an initial analysis of *S. jarrovii* and *C. pricei* resource selection.

Covariates used in data analysis were derived from outside sources and applied to species point observations downloaded from online databases. All covariates selected for analysis were predicted to be biologically relevant for both *C. p. pricei* and *S. jarrovii* habitat selection by examining known ecological constraints based on previously published natural history information (Prival and Schroff, 2012; Jones and Lovich, 2009) (Table 4.1). Due to the stratified nature of vegetation communities in the Sky Islands and

climatic variation characteristic of the sharp elevation gradient, vegetation types, temperature and precipitation variables were considered of biological importance to both species. Proportion vegetation type and tree canopy cover percentage were derived using ArcGIS with files downloaded from LANDFIRE (USDA and USDOI; www.landfire.gov), bioclimatic variables were accessed through WorldClim.org, and elevation data was downloaded from The National Map (USGS; nationalmap.gov). Before using files in analysis, file format and projected coordinate system were standardized to NAD83 UTM Zone 12. All modeling and covariate evaluation was completed in program R (Version 1.1.383) with packages 'car' (Fox and Weisberg, 2011), 'pscl' (Jackman, 2017), and 'raster' (Hijmans, and van Etten, 2012).

Table 4.1. Covariate descriptions.

Covariate	Description	Resolution
TreeMean	Mean proportion of tree cover within $90m^2$ neighborhood	30 m
ShrubMean	Mean proportion of shrub cover within $90m^2$ neighborhood	30 m
HerbMean	Mean proportion of herbaceous cover within $90m^2$ neighborhood	30 m
Percent Tree Cover (TreeCov)	Percentage of area covered by tree canopy	30 m
Elev (Elevation) above mean sea level	Measured in meters	30 m
Annual mean temperature (Bio1)	Measured in Celsius	1 km
Max. temperature of warmest month (Bio5)	Measured in Celsius	1 km
Min. temperature of coldest month (Bio6)	Measured in Celsius	1 km
Temperature annual range (Bio7)	Measured in Celsius, maximum temperature-minimum temperature	1 km
Mean temperature of warmest quarter (Bio10)	Measured in Celsius	1 km
Mean temperature of coldest quarter (Bio11)	Measured in Celsius	1 km
Annual precipitation (Bio12)	Measured in millimeters	1 km
S. jarrovii RSPF (*C. p. pricei* dataset only)	Resource selection probability function for developed from final *S. jarrovii model*	30 m

Vegetation type data (Tree Mean, Shrub Mean, Herb Mean) downloaded from LANDFIRE (USDA and USDOI). Tree Canopy Cover was downloaded from National Land Cover Database (NLCD). Seven bioclimatic variables were accessed through WorldClim.org and include bio1, bio7, bio10, bio11, bio12. Elevation data was downloaded from The National Map (USGS).

Covariate Selection and Data Exploration

Variance inflation factors (VIF) and Pearson's Correlation Coefficients were used to evaluate covariates for multicollinearity. Covariates with VIF values above 1 were considered for elimination, but biological relevance was also taken into consideration when eliminating covariates. Pearson's Correlation Coefficients above 0.7 (strongly correlated) were eliminated and those with values between 0.5 and 0.7 (moderately correlated) were considered for elimination based on biological significance or lack thereof (Rodgers and Nicewander, 1988). VIF values of over 10 are considered highly correlated and were also considered for elimination depending on biological relevance (Neter et. al., 1990).

Summary statistics and model fit statistics were compared across single covariate generalized linear models (GLM) using a binomial distribution to determine strength of predictor variables and predictor variables to include in model development. Coefficients (β), 95% confidence intervals (CI), and standard errors (SE) were compared across covariates to determine those most likely to explain *S. jarrovii* and *C. p. pricei* presence. Covariates with smaller coefficients, larger SEs, and CIs overlapping zero were considered for elimination. Model fit statistics were compared across all GLMs, specifically Akaike's Information Criteria (AIC), ΔAIC, McFadden's R^2, and log-likelihood (LL) values. All covariates not eliminated were used in final model development. Frequency histograms were used to analyze visually the range of used and availability points for covariates selected for development of final model.

Model Selection

After summary statistics were calculated for each single covariate binomial GLM, a global logistic regression resource selection probability function (RSPF) was developed to estimate the response variable (*C. p. pricei* use and *S. jarrovii* use) across all predictor variables selected as ecologically relevant and lacking multicollinearity. Model development reflects methods described in Manly et al. (2002). Models were developed using a forward stepwise model selection process to determine which combination of predictor variables best describe both *C. p. pricei* and *S. jarrovii* site selection and create the most parsimonious model. The information theoretic theorem was used as a basis for model evaluation (Akaike, 1973). Final model selection was evaluated by comparing model fit statistics (AIC, ΔAIC, McFadden's R^2, and LL) across all models. Models with a ΔAIC of >2 were considered significantly different (Anderson and Burnham, 2002). RSPF model output (prediction values) undergo a monotonic transformation from probabilities to log of odds ratio that range from negative infinity to positive infinity. Because prediction values (log of odds ratio) are directly proportional to probability, prediction values from RSPF were interpreted as probability of use based on predictor variables included in the final model.

Model Evaluation

To test model accuracy and predictability, model evaluation was performed following methods outlined in Fielding and Bell (1997). A receiver operating characteristic (ROC) curve was developed to assess predictive power of the final models, and area under the curve (AUC) was used as a predictability metric. This value (AUC) is equivalent to the concordance index of the model, or the probability that predictions of

the model and outcomes are concordant. 20% of the original locality points for each species were withheld as an independent dataset and used in model evaluation.

Results

Data Sources

Data selection resulted in 198 *C. p. pricei* observations points, 301 *S. jarrovii* observation points, and a total of 7,000 available background points used in model development. Spatial autocorrelation was present in the data points selected for *C. p. pricei* due to sampling bias within an area used as a long-term study site for *C. p. pricei* (Prival and Schroff, 2012). This issue was addressed by eliminating "duplicate" data points within 30 m of each other. Twelve covariates were selected to use in model development.

Covariate Selection and Data Exploration

Sceloporus jarrovii. After covariates were evaluated for fit and multicollinearity (Table 4.2), the covariates retained for *S. jarrovii* model development were proportion of shrub vegetation, proportion of tree vegetation, percent tree cover, proportion of herbaceous vegetation, elevation, minimum temperature of coldest month, mean temperature of coldest month, and annual precipitation (Figure 4.2). Generally, VIF values of over 10 are considered highly correlated (Neter et. al., 1990), though one covariate with a value >10 (mean temp. of coldest quarter) was retained based on low Pearson's Correlation Coefficients, moderately high beta estimate, and biological relevance.

Density histograms and univariate model coefficient estimates for covariates used in the final RSF model show a positive use correlation with annual precipitation (ß estimate of 0.012), percent tree cover (ß= 0.09), proportion tree vegetation (ß= 3.1), but a negative correlation for proportion of shrub vegetation (ß= -3.3)(Table 4.2; Figure 4.2).

Table 4.2. Single predicator model statistics for *S. jarrovii*.

Univariate Model	ß	Std. Error	Lower 95% CI	Upper 95% CI	R^2	AIC	ΔAIC	AIC Rank
TreeCov	**0.089**	**0.004**	**0.08**	**0.10**	**0.235**	**1923**	**0**	**1**
Bio5	-0.431	0.020	-0.47	-0.39	0.226	1947	24	2
Bio10	-0.548	0.025	-0.60	-0.50	0.224	1952	29	3
TreeMean	**3.114**	**0.177**	**2.78**	**3.47**	**0.182**	**2056**	**133**	**3**
Bio12	**0.012**	**0.001**	**0.01**	**0.01**	**0.171**	**2084**	**161**	**4**
ShrubMean	**-3.325**	**0.219**	**-3.77**	**-2.91**	**0.164**	**2101**	**178**	**5**
Bio7	-0.431	0.023	-0.48	-0.39	0.164	2102	179	6
Bio1	-0.507	0.026	-0.56	-0.46	0.160	2111	188	7
Elev	**0.003**	**0.000**	**0.00**	**0.00**	**0.159**	**2115**	**192**	**8**
Bio11	**-0.498**	**0.029**	**-0.55**	**-0.44**	**0.120**	**2213**	**290**	**9**
Bio6	**-0.251**	**0.029**	**-0.31**	**-0.19**	**0.029**	**2439**	**516**	**10**
HerbMean	**-1.260**	**0.460**	**-2.27**	**-0.45**	**0.004**	**2503**	**580**	**11**

Abbreviations: Elevation (Elev), tree canopy cover (TreeCov), annual mean temperature (Bio1), max. temperature of warmest month (Bio5), min. temperature of coldest month (Bio6), temperature annual range (Bio7), mean temperature of warmest quarter (Bio10), mean temperature of coldest quarter (Bio11), and annual precipitation (Bio12).

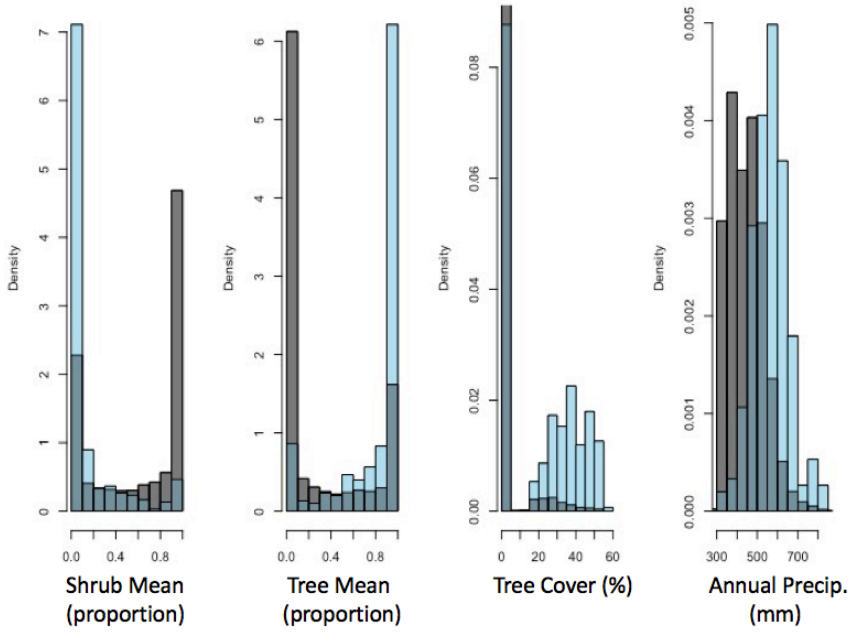

Figure 4.2. Density histograms displaying range of final predictor variables for *S. jarrovii*. Available is displayed in grey, used is displayed in light blue, and overlap is displayed in grey/blue.

Crotalus pricei pricei. After evaluation of covariates for the *C. p. pricei* dataset (Table 4.3), those retained for model development included elevation, proportion of shrub vegetation, percent tree cover, annual precipitation, mean temperature of coldest quarter, temperature annual range, and *S. jarrovii* probability of use (RSPF) (Figure 4.3). Elevation was moderately to highly correlated with annual precipitation (0.74) and mean temperature of coldest quarter (0.76), but was retained due to lowest individual GLM AIC value (best ranked by AIC), moderate VIF value (7), and biological relevance.

Frequency histograms and coefficient estimates for covariates used in the final RSF model show a positive use correlation with annual precipitation (ß= 0.02), percent tree cover (ß= 0.06), mean temperature of coldest quarter (ß= 1.3), elevation (ß= 0.007), *S. jarrovii* RSPF (ß= 0.005), but a negative correlation for proportion of shrub vegetation (ß= -3.2) and temperature annual range (ß= -0.8) (Figure 4.3). Given that none of the remaining covariates included in both the *S. jarrovii* and *C. p. p pricei* model

development had 95% CIs overlapping zero, we can safely assume they exemplify robust predictors and have at least a minor correlation with species use.

The top single predictor variable GLM for *S. jarrovii* was percent tree cover. This covariate displayed a low SE (0.003), but a small coefficient estimate (ß= 0.09). The top single predictor variable GLM for *C. p. pricei* was elevation with a low SE (0.0004), low 95% CI (0.007 - 0.008), and small positive coefficient estimate (ß= 0.007).

Table 4.3. Single predicator model fit statistics for *C. p. pricei*.

Univariate Model	ß	Std. Error	Lower 95% CI	Upper 95% CI	R^2	AIC	∆AIC	AIC Rank
Elev	**0.007**	**0.000**	**0.007**	**0.008**	**0.646**	**647**	**0**	**1**
Bio5	-0.834	0.040	-0.916	-0.759	0.611	709	62	2
Bio10	-1.107	0.055	-1.219	-1.005	0.587	754	107	3
Bio1	-1.187	0.059	-1.308	-1.077	0.541	836	190	3
Bio11	**1.269**	**0.067**	**-1.405**	**-1.143**	**0.439**	**1022**	**375**	**4**
Bio12	**0.022**	**0.001**	**0.020**	**0.024**	**0.429**	**1039**	**392**	**5**
Bio7	**-0.840**	**0.042**	**-0.925**	**-0.760**	**0.391**	**1108**	**462**	**6**
Bio6	-0.550	0.037	0.624	-0.477	0.140	1563	916	7
TreeCov	**0.059**	**0.004**	**0.052**	**0.067**	**0.109**	**1621**	**974**	**8**
ShrubMean	**-3.219**	**0.324**	**-3.899**	**-2.625**	**0.107**	**1623**	**977**	**9**
SCJA_RSPF	**0.005**	**0.000**	**0.004**	**0.006**	**0.079**	**1674**	**1028**	**10**
TreeMean	**1.563**	**0.185**	**1.208**	**1.935**	**0.045**	**1735**	**1089**	**11**

Abbreviations: Elevation (Elev), tree canopy cover (TreeCov), annual mean temperature (Bio1), max. temperature of warmest month (Bio5), min. temperature of coldest month (Bio6), temperature annual range (Bio7), mean temperature of warmest quarter (Bio10), mean temperature of coldest quarter (Bio11), and annual precipitation (Bio12).

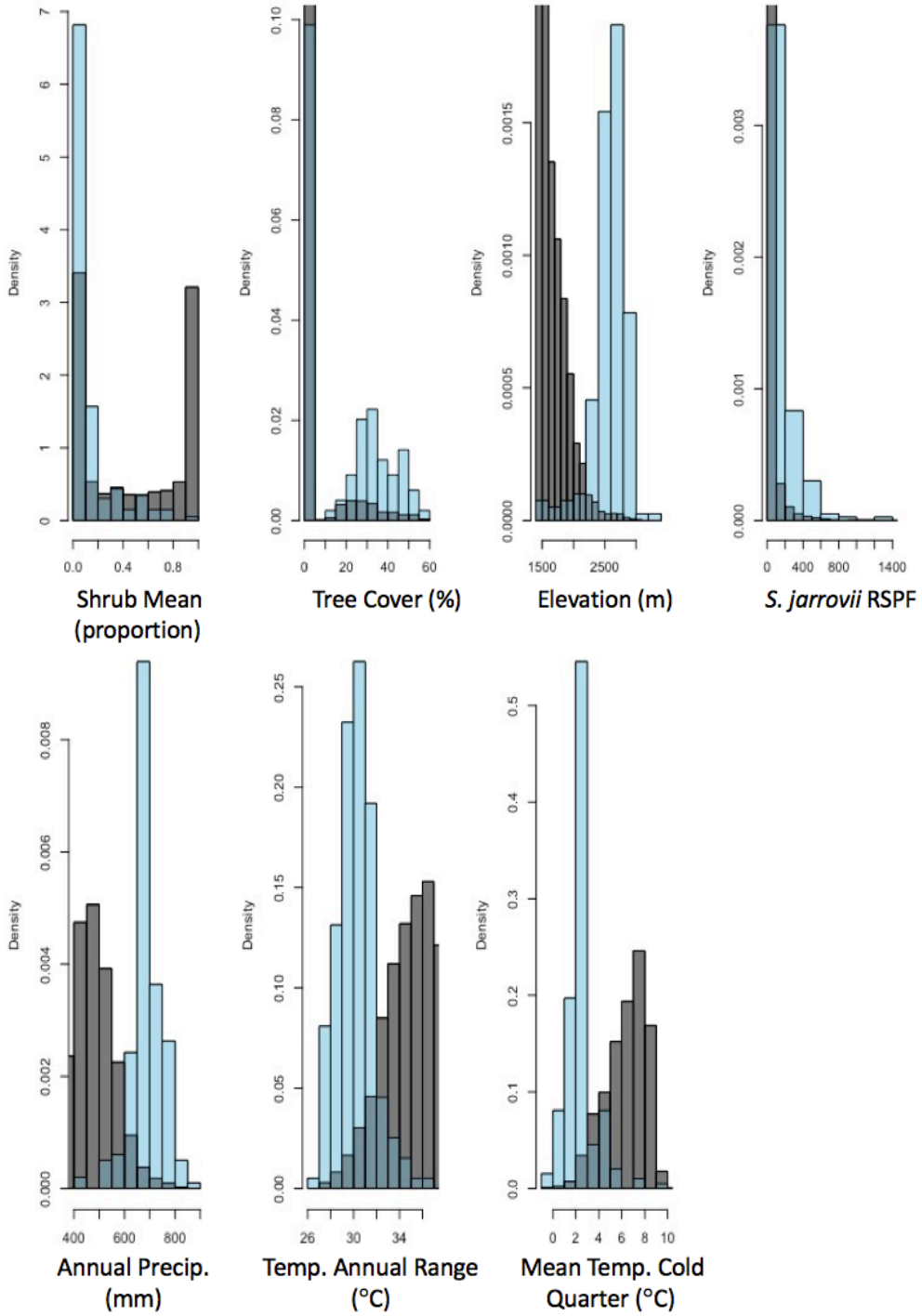

Figure 4.3. Density histograms displaying range of final predictor variables for *C. p. pricei*. Available displayed in grey, used displayed in light blue, and overlap in displayed in grey/blue.

Model Selection

The GLM including percent tree cover, proportion tree vegetation, annual precipitation, and proportion shrub vegetation ranked as the best-fit model for *S. jarrovii,* after evaluating all model fit statistics with a pseudo R^2 value of 0.299 (Table 4.4). The pseudo R^2 value did not increase between the second best model and top model based on AIC rank, indicating that including the elevation variable did not significantly increase model fit. The top GLM for *C. p. pricei* included all covariates retained after evaluation of multicollinearity and correlation, with a pseudo R^2 of 0.68 and a ∆AIC of 24.77 between the first and second best fit models (Table 4.5). For prediction purposes, the *S. jarrovii* RSPF was excluded from the *C. p. pricei* model due to potential for covariate conflation and inaccurate predictability for *C. p. pricei* use based on shared covariates in the final models (annual precipitation and percent tree cover).

Using the *S. jarrovii* RPSF as a single covariate in the *C. p. pricei* model resulted in slight model fit with an R^2 value of 0.08 and positive correlation with *C. p. pricei* use (ß= 0.005).

Table 4.4. Model evaluation statistics for *S. jarrovii*.

Model	Predictor Variables	R^2	Log-likelihood	AIC	∆AIC	AIC Rank
4	**TreeCov, TreeMean, Bio12, ShrubMean**	**0.299**	**-880**	**1769**	**0**	**1**
5	TreeCov, TreeMean, Bio12, ShrubMean, Elev	0.299	-879	1770	1	2
Global (1)	TreeCov, Elev, Bio6, Bio11, Bio12, HerbMean, ShrubMean, TreeMean	0.2	-877	1772	3	3
3	TreeCov, TreeMean, Bio12	0.294	-885	1778	9	4
2	TreeCov, TreeMean	0.282	-900	1806	37	5

Bold text indicates the best model based on evaluation statistics. Abbreviations: Elevation (Elev), tree canopy cover (TreeCov), min. temperature of coldest month (Bio6), temperature of coldest quarter (Bio11), and annual precipitation (Bio12).

Table 4.5. Model evaluation statistics for *C. p. pricei*.

Model	Predictor Variables	R^2	Log-likelihood	AIC	ΔAIC	AIC Rank
Global (1)	Elev, TreeCov, ShrubMean, Bio12, Bio7, Bio11, *S. jarrovii* RSPF	0.682	-289	593	0	1
4	**Elev, Bio11, Bio12, Bio7**	**0.665**	**-304**	**617**	**24**	**2**
5	Elev, Bio11, Bio12, Bio7, TreeCov	0.666	-303	618	25	3
6	Elev, Bio11, Bio12, Bio7, TreeCov	0.666	-303	619	26	4
3	Elev, Bio11, Bio12	0.658	-310	629	35	5
2	Elev, Bio11	0.648	-319	645	51	6

Bold text indicates the best model based on evaluation statistics. Abbreviations: Elevation (Elev), tree canopy cover (TreeCov), annual mean temperature (Bio1), max. temperature of warmest month (Bio5), min. temperature of coldest month (Bio6), temperature annual range (Bio7), mean temperature of warmest quarter (Bio10), mean temperature of coldest quarter (Bio11), and annual precipitation (Bio12).

Resource Selection Probability Function

The RSPF for *S. jarrovii* shows a relatively restricted distribution for southeastern Arizona (Figure 4.4). The final model for *S. jarrovii* indicates that 29.9% of the variation seen in *S. jarrovii* use points can be explained by percent tree cover, proportion tree vegetation, annual precipitation, and proportion shrub vegetation (Table 4.6; Figure 4.4). *Crotalus p. pricei* probability of use appears to be slightly more restricted than *S. jarrovii* (Figure 4.5). Probability of use is geographically as expected, with high elevation Sky Island regions showing most likely occurrence of both species and encompassing known distribution of species. The top *C. p. pricei* model included the *S. jarrovii* RSPF, though this was not accepted as the final model due to predictor variable conflation. The final model for *C. p. pricei* included elevation, annual precipitation, temperature annual range, and mean temperature of coldest quarter and had an R^2 value of 0.665 (Table 4.7; Figure 4.5). This indicates that 66.5% of the variation seen in *C. p. pricei* use points are explained by the covariates included in the final model.

Table 4.6. Final multivariate RSPF model statistics for *S. jarrovii*.

Predictor Variable	ß	Std. Error
Annual precipitation (Bio12)	0.004431	0.001
ShrubMean	-1.306796	0.368
Percent tree cover (TreeCov)	0.054267	0.004
TreeMean	0.542765	0.330

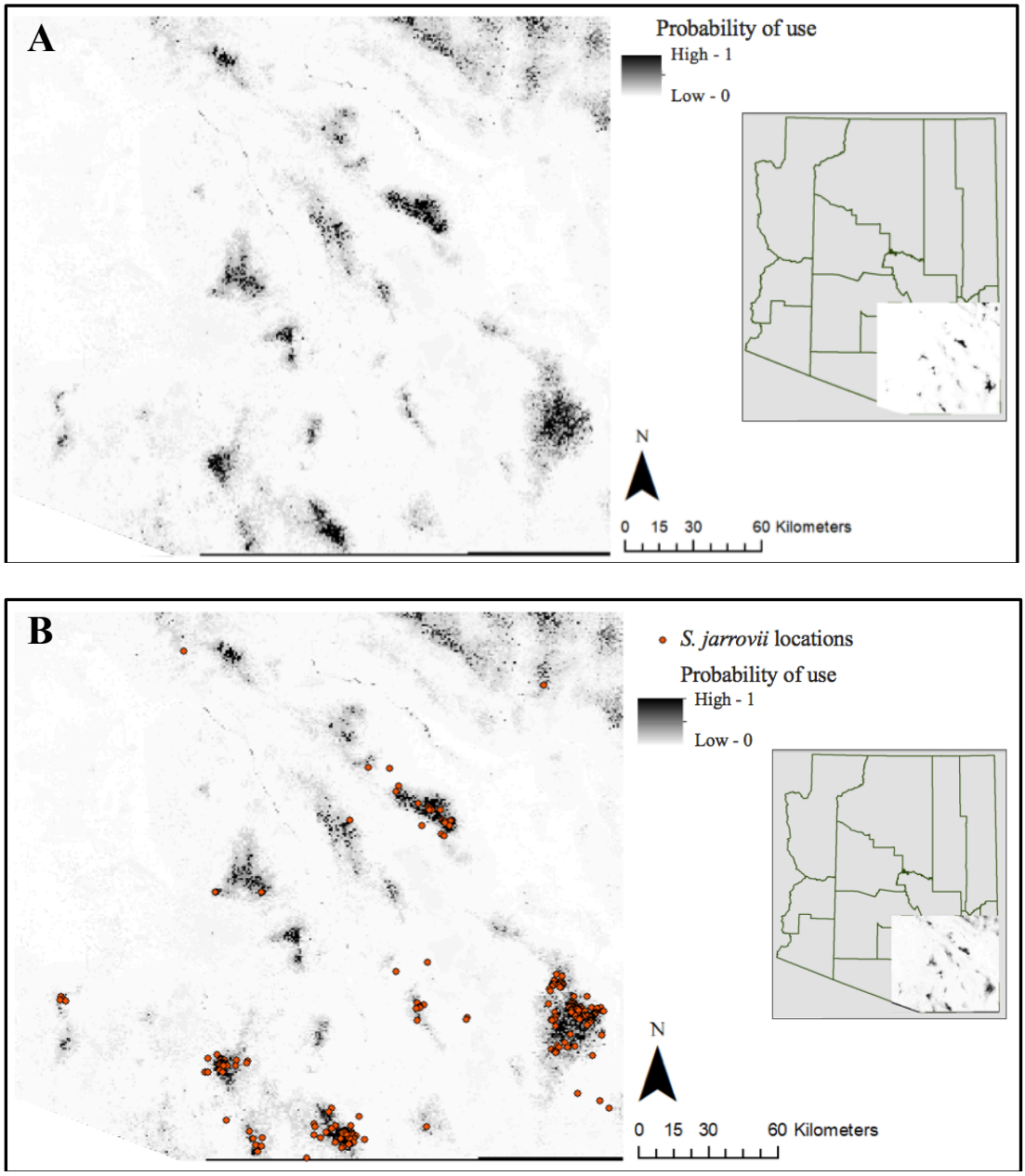

Figure 4.4. RSPF model predictions for *S. jarrovii* probability of use in southeastern Arizona without documented *S. jarrovi* locations (**A**) and with locations overlain in red (**B**). *Sceloporus jarrovii* locations from 1970-2017 and obtained from VertNet.org, Arctos.org, and GBIF.org.

Table 4.7. Final multivariate RSPF model statistics for *C. p. pricei*.

Predictor Variable	ß	Std. Error
Mean temp. of coldest quarter (Bio11)	-0.139	0.162
Temperature annual range (Bio7)	-0.647	0.184
Annual precipitation (Bio12)	-0.024	0.005
Elevation	0.009	0.001

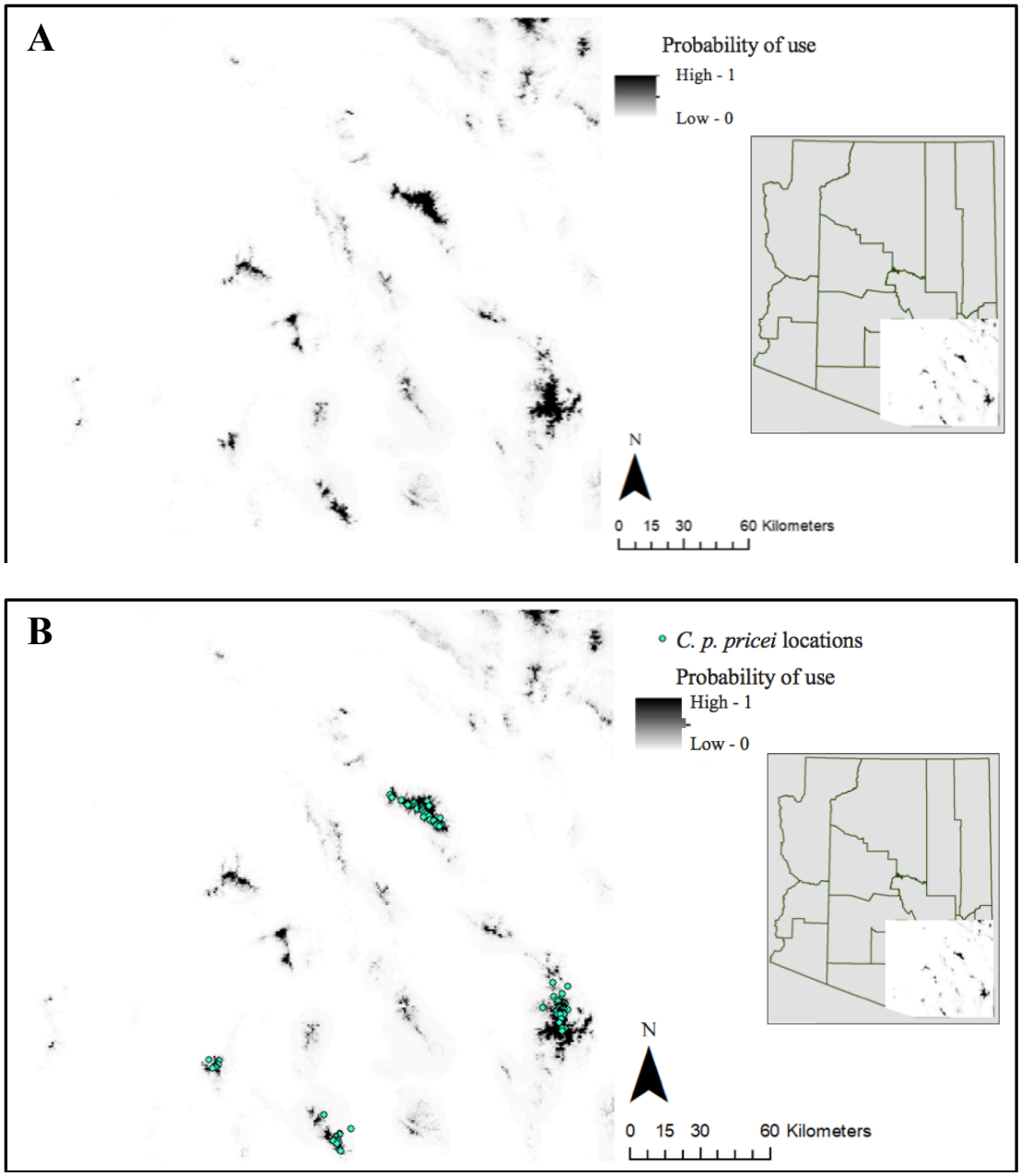

Figure 4.5. RSPF model predictions for *C. p. pricei* probability of use in southeastern Arizona without documented *C. p. pricei* locations (**A**) and with locations overlain in blue (**B**). *Crotalus p. pricei* locations from 1970-2017 and obtained from VertNet.org, Arctos.org, GBIF.org, and the HDMS (Arizona Game and Fish Department).

Model Evaluation

Twenty percent of the use localities were withheld from each of the species datasets; 50 locality points were withheld from the *C. p. pricei* dataset and 75 points were withheld from the *S. jarrovii* dataset. The ROC plots showed a concordance index (AUC) of 0.856 for the *C. p. pricei* RSPF and concordance index of 0.891 for the *S. jarrovii* RSPF. This means that the *C. p. pricei* model correctly classified data points (used and available) 85.6% of the time, while the *S. jarrovii* model classified data points 89.1% of the time.

Discussion

The correlation between species occurrence and selection of habitat is crucial to understanding the ecology of a species. Niche modeling of high elevation species has become more prominent in recent years and has illuminated the various effects that accelerated climate change and other human-induced disturbances has on these species (Davis et al., 2015; Jeffress et al., 2013; Lawler et al., 2009; Parmesan, 2006). Climate projection models completed at a large geographic scale estimate an upwards or poleward shift in species range due to climatic effects during this century. Central American highlands and other topographically prominent features, such as Sky Islands, are the most likely to experience substantial environmental changes (Jeffress et al., 2013; Lawler et al., 2009), with one study predicting an average of 90% turnover in species composition in these areas over the next 100 years (Lawler et al., 2009). *Crotalus p. pricei* and other herpetofauna endemic to Sky Island habitats are constrained by these high elevation mountains, and their occurrence is predicted by environmental variables consistent with colder and wetter montane habitat that is threatened by climate change.

Results indicate that *S. jarrovii* and *C. p. pricei* select for similar habitat characteristics, but specific variables affect this selection to different extents. Elevation is a stronger predictor for *C. p. pricei*, with a larger R^2 value and better model rank than other univariate predictor variables (Table 4.3). This result is expected given that the known elevation range for *C. p. pricei* extends well above 2700 m (Prival and Schroff 2013). Based on the dataset analyzed, the elevation range for *C. p. pricei* is between 1500-3200 m and the range for *S. jarrovii* is between 1200-3100 m, consistent with model results. Expected outcomes of the relationship between annual precipitation and species presence observations were supported; both species display positive correlation with increase in precipitation, consistent with Sky Island habitat (Arriaga et al., 2005; Kupfer et al., 2005). Species presence also showed positive correlation with colder temperatures based on ß estimates of univariate temperature models (Tables 4.2, 4.3). *Crotalus willardi obscurus* (New Mexico Ridge-nosed Rattlesnake), another Sky Island endemic, was similarly correlated with colder temperatures based on niche modeling completed in Maxent (Davis et al., 2015). This apparent reliance on cooler climate could pose a problem if predicted environmental shifts occur in the Sky Islands. Projections estimate that vegetation gradients throughout Sky Islands will shift up in elevation with the estimated 3° Celsius increase and 25% precipitation increase predicted over the next 100 years (IPCC, 2013; Kupfer et al. 2005). Both *C. p. pricei* and *S. jarrovii* show positive correlation with mean proportion of tree cover (ß= 1.563 and ß= 3.114 respectively) and percent tree canopy cover (ß= 0.059 and ß= 0.089 respectively) (Table 4.2, 4.3). Because the majority of Sky Island areas occur below 3000 m (Arriaga et al.

2005), there is little room for *C. p. pricei* and *S. jarrovii* ranges to expand upwards to avoid these detrimental climatic shifts.

It is apparent that *S. jarrovii* predicted presence has an effect on *C. p. pricei* presence, but not to the extent that was expected, contrary to hypothesis 1 (which stated that predicted presence of *C. p. pricei* would largely be explained by the *S. jarrovii* model output). The ß estimate for the univariate *S. jarrovii* RSPF was 0.005, indicating presence of *C. p. pricei* is slightly positively correlated with *S. jarrovii* probability of use. The correlation between predator and prey occurrence was expected due to the frequent occurrence of *S. jarrovii* scales in *C. p. pricei* fecal samples, similarity in known distribution for both species (Jones and Lovich, 2009; Prival and Schroff 2013) and similar response to covariates used for modeling. Furthermore, preliminary analysis completed at University of Northern Colorado indicates that *S. jarrovii* may demonstrate adaptive tolerance to *Crotalus* venom, but not specifically to *C. p. pricei* venom (see Chapter II). Together, these findings suggest a close predator-prey relationship, mirrored by results of the *C. p. pricei* model evaluated using only the *S. jarrovii* RSPF that supports a positive correlation between *S. jarrovii* predicted presence and *C. p. pricei* presence. Likewise, this co-occurrence suggests that *S. jarrovii* and *C. p. pricei* have adapted to similar physiological constraints consistent with life in pine-oak ecosystems at higher elevations.

Due to strong predictive power of both annual precipitation and annual mean temperature (Table 4.4, 4.3), and inability of these species to disperse due to geographic isolation, projected climatic shifts are expected to be detrimental to both species. The Sky Islands represent a uniquely stratified environment composed of ecosystems that change

with increase in elevation and subsequent decrease in temperature (Arriaga et al., 2005). Consequently, because reptile species restricted to Sky Islands seem to be restricted by the same temperature and precipitation constraints as vegetation in these ecotones (Arriaga et al., 2005), vegetation health in these high elevation systems could be used as a proxy for reptile persistence.

Consistent with predicted results, *C. p. pricei* showed a more narrow predicted distribution than *S. jarrovii* (Figures 4.4, 4.5). However, this very narrow estimation is likely due to predictive power of the covariates used in the *C. p pricei* model (Table 4.7), process of availability point selection, and sampling bias.

Based on the conservative estimation of high probability areas for *C. p. pricei,* we can make a few assumptions about the habitat and modeling capabilities. Prediction errors are typically placed in three general categories: biotic errors due to exclusion of biologically relevant predictor variables, algorithmic errors due to limitations of the model algorithm used, and inconsistency in the data gathering processes (primarily sampling bias) (Fielding and Bell, 1997). Unfortunately, lack of empirical ecological data for both *S. jarrovii* and *C. p. pricei* may have resulted in inadvertent exclusion of relevant predictor variables. Another limitation of developing RSPF models for species with little ecological background knowledge is appropriate selection of background regions from which availability points are selected and then identifying the influence of these points on prediction values. Given the variation in prediction results of *S. jarrovii* and *C. p. pricei,* it is likely that the background region truncated by elevation (from which *C. p. pricei* availability points were sampled) was too broad (Figures 4.5 and 4.6). This variation in availability likely resulted in over-prediction based on elevation, evident in probability of

use for *C. p. pricei* (Figure 4.6) and the inflated pseudo R^2 values seen with a majority of models developed (Tables 4.3 and 4.5). Additionally, inherent limitations of presence-only studies include sampling bias, geospatial inaccuracies, and lack of a consistent sampling method (Phillips et al. 2009). Sampling bias is common when examining presence-only (use/available) data and often results in spatial autocorrelation and inflates model accuracy (Kramer-Schadt et al., 2013). This often results in type I errors (false positives) and misleading covariate estimates (Kramer-Schadt et al., 2013; Kühn, 2007). Evaluation of the *C. p. pricei* model yielded a relatively high concordance index (0.86), meaning that 86% of the use/available points were classified correctly and few false positives occurred. However, covariate inflation is apparent when considering the high R^2 (0.66) value of the final *C. p. pricei* model and similarly high R^2 (0.65) value of some covariates such as elevation. Given that there are a few well-known and readily accessible *C. p. pricei* populations and the areas with frequent resampling of locality points was only addressed by limiting data points near the long-term study area (Prival and Schroff, 2012), sampling bias likely influenced results. Phillips et al. (2009) suggested that sampling biases be addressed by applying similar sampling bias to the availability points selected, though this was not possible with the datasets used in this study due to the extent of sampling bias differing between Sky Island regions. Additionally, the sampling bias evident in the *C. p. pricei* dataset can only be assumed and the extent of bias is hard to decipher.

Even based on the narrow predictions of the *C. p. pricei* RSPF, migration between the four Sky Island mountain ranges with known populations (Figure 4.1) would be extremely unlikely (Figure 4.5). The combination of a potential decrease in habitat due to

upward shift in vegetation and genetic isolation due to inability to cross between Sky Islands will likely lead to reduced population numbers, increasing the potential for bottleneck and genetic drift effects. The results of this study indicate that the climatic variables and vegetation features associated with high elevation systems are all important to persistence of both *C. p. pricei* and *S. jarrovii* (Table 4.5, 4.7), and they should be taken into consideration during development of conservation plans for these unique Sky Island species.

Conclusion

Regardless of modeling limitations, areas with high probability of occurrence could be good habitat for *S. jarrovii* and *C. p. pricei* and should be surveyed to ascertain that no novel populations inhabit these areas. Evidence collected suggests that there are several habitat characteristics consistent with high elevation ecosystems that both *S. jarrovii* and *C. p. pricei* require for persistence. Furthermore, predicted values from the RSPF for *S. jarrovii* can be used to predict locations of *C. p. pricei* to a certain extent. Using this information, wildlife managers can access areas with predicted *C. p. pricei* occupation and evaluate population sizes and densities. By evaluating *C. p. pricei* and *S. jarrovii* populations using habitat predictions developed in this study, a modeling framework has been created to aid in future niche modeling of high elevation herpetofauna. Further modeling and output evaluation should be completed to understand better resource selection for these species and determination of specific conservation needs.

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CHAPTER V

CONCLUSIONS

Mountain rattlesnakes have received little research attention apart from preliminary phylogenetic classification. While these studies are vital for defining important genetic, geographic and morphological characteristics, important questions regarding venom composition and habitat requirements still need to be answered. Venom toxin information is required to identify novel toxins used by snakes to incapacitate prey; additionally, this information has snakebite treatment and medicinal applications. Detailed natural history and defined ecological parameters are needed to understand the basic biology of these specialized animals, as well as to develop conservation plans, likely to become much more pressing needs in the near future due to climate change. The studies outlined in this thesis aimed to add to the empirical evidence regarding the venom of little-known rattlesnake species in the *Crotalus intermedius* group, niche selection of *C. p. pricei,* and predator-prey relationship between *C. p. pricei* and its natural prey.

Because mountain rattlesnakes of México and the United States are so speciose it is likely that their venoms contain a wide variety of toxins, and few of these toxins have been isolated and analyzed to identify specific physiological pathways involved following envenomation. Recently, multiple toxins isolated from venomous reptiles have been investigated for use in medical treatments. Contortrostatin, a disintegrin isolated from Copperhead venom, has shown promising application in treatment of numerous

conditions including herpes simplex virus, breast cancer, and prostate cancer (Hubbard et al., 2012; Lin et al., 2010; Swenson et al., 2004). Epifibatide is a hemotoxin isolated from Pygmy Rattlesnake (*Sistrurus miliarus barbouri*) that inhibits platelet aggregation and is used to treat non-ST-segment elevation acute coronary syndrome (Liu et al., 2009). Many other toxins are currently being tested for pharmaceutical applications or have properties potentially applicable to biomedicine (Yau Sang et al., 2016; Mackessy, 2010).

Additionally, snakebite has been designated a neglected tropical disease by the World Health Organization (WHO) due to lack of effective treatments worldwide. WHO estimates between 81,000 to 138,000 deaths globally occur each year due to snakebite. WHO considers over 200 snake species to be of medical importance, and unfortunately, many of these venomous species are common in countries with minimal access to antivenom in the rural tropics. Unfortunately, lack of empirical evidence regarding mountain rattlesnake venom leaves many questions regarding snakebite and medicinal applications unanswered.

Summary of Chapter II

Chapters II (and III) investigated the venom phenotypes of rattlesnakes in the *Crotalus intermedius* group in hopes of expanding the current knowledge of mountain rattlesnake venom composition. *Crotalus pricei* venom represents a classic type I venom, with high metalloprotease activity, though the previously reported relatively high lethal toxicity in mice (1.25 μg/mL) makes this species one of the more toxic type I venom snakes (Mackessy, 2010). Though most *C. pricei* venom samples used in Chapter II show similar profiles based on RP-HPLC chromatography and SDS-PAGE gel electrophoresis, there were distinct differences in PLA_2 toxins present in *C. p. miquihuanus* and *C. p.*

pricei from Durango. The atypical PLA_2 toxin peak in these samples appeared just after the typical elution times of neurotoxic PLA_2 toxins such as Mojave toxin and crotoxin and deserve further analysis to determine exact identity, activity and pathology (Mackessy, 2008; Smith and Mackessy, 2016). Furthermore, despite *S. jarrovii* comprising the majority of *C. p. pricei* diet, *S. jarrovii* does not appear to display specific resistance to *C. p. pricei* venom, but shows a generalized resistance to *Crotalus* venoms, with LD_{50} values ranging from 6.9 µg/mL for sympatric populations with *C. p. pricei* to 7.9 µg/mL for *C. l. klauberi* venom. Both species co-occur with *S. jarrovii* and include this lizard in their diet.

Summary of Chapter III

Chapter III investigated the venom composition of little-known mountain rattlesnakes and their venom phenotype relationships to each other in the context of known phylogenetic information. Very little has been published regarding *C. tancitarensis* since Alvarado-Díaz and Campbell (2004) first described the species, but based on the extremely limited distribution of this species (one mountain - Cerro Tancítaro), as much ecological, venomic, and genetic information should be collected as possible to determine species status and potentially develop conservation plans. *Crotalus tancitarensis* displays a type I venom profile, with moderate metalloprotease activity and HPLC venom profiles similar to classic type I venom snakes (Mackessy, 2008). Based on biochemical assays, RP-HPLC, and gel electrophoresis, *C. tancitarensis* shows venom composition most similar to that of *C. transversus*. This pattern reflects preliminary genetic analysis completed on the *Crotalus intermedius* group; *C. tancitarensis* and *C. transversus* are nested together and likely diverged sometime during the LGM (~25,000

years BP) after diverging from *C. intermedius* during the Pliocene era (~5.33 and 3.6 million years BP) (Bryson et al., 2010; McDonald, 1993; Cohen et al., 2018).

Summary of Chapter IV

Chapter IV described the habitat characteristics associated with *C. p. pricei* presence and developed a multivariate species distribution model estimating values proportional to probability of presence for both *C. p. pricei* and its main prey species, *S. jarrovii.* Elevation was the strongest single predictor variable for *C. p. pricei* ($R^2 = 0.65$) while percent tree cover was the strongest predictor for *S. jarrovii* ($R^2 = 0.24$). When predicted probability of *S. jarrovii* presence (RSPF for *S. jarrovii*) was used as a univariate predictor for presence of *C. p. pricei,* the model was not well supported compared to other single covariate models, with a R^2 value of 0.08 and AIC rank of 11/13. *Crotalus p. pricei* exhibited positive association with both annual precipitation and mean temperature of coldest quarter, parameters correlated with Sky Island habitats. Given the importance of mean temperature of coldest quarter for *C. p. pricei* and annual precipitation and percent tree cover for both species RSPF, precipitation changes and temperature increase consequences of climate change could significantly alter distribution of both species. However, spatial autocorrelation was prevalent in the *C. p. pricei* dataset and this likely led to inflated model accuracy, particularly for the covariates typical for the regions of extensive survey.

Based on the RSPF models created for both *C. p. pricei* and *S. jarrovii,* climatic variables and vegetation associated with high elevation Sky Island habitat is important for persistence of both species. This pattern likely extends to mountain rattlesnakes of

México, including *C. tancitarensis.* Given that *C. tancitarensis* has such a limited known distribution, evaluation of habitat and population numbers should be prioritized.

Conclusions

Based on all evaluations outlined in this thesis, mountain rattlesnakes display unique inter- and intraspecific venomic, habitat selection, and evolutionary patterns. These unique characteristics make these species ideal for modeling dispersal, divergence, and geographic venom phenotype patterns of rattlesnakes and other organisms endemic to Sky Island ecosystems. Further studies should be conducted to estimate better the factors affecting habitat selection in all species of the *Crotalus intermedius* group and determine if toxins specific to these distinctive species occur within their venoms.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS

UNIVERSITY of NORTHERN COLORADO

IACUC Memorandum

To: Steve Mackessy

From: Laura Martin, Director of Compliance and Operations

CC: IACUC Files

Date: 1/24/2018

Re: IACUC Protocol Approval 1302D-SM-S-16

The UNC IACUC has completed a final review of your protocol "Analysis of Venoms from Viperid Snakes- Biochemical Composition, Activities".

The committee's review was based on the requirements of the Government Principles, the Public Health Policy, the USDA Animal Welfare Act and Regulations, and the Guide for the Care and Use of Laboratory Animals, as well as university policies and procedures related to the care and use of live vertebrate animals at the University of Northern Colorado.

Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. **This protocol has been assigned the following number 1302D-SM-S-16.**

The next annual review will be due before **May 25, 2018**.

IACUC Memorandum

To: Dr. Stephen Mackessy and Dr. Todd Castoe

From: Laura Martin, Director of Compliance and Operations

CC: IACUC Files

Date: March 3, 2017

Re: IACUC Protocol 1701D-SM-S-20 Approval

The University of Northern Colorado IACUC has completed a final review of your protocol, *Systematics, Introgression, and Adaptation in Western Rattlesnakes: a Model System for Studying Gene Flow, Selection, and Speciation. Analysis of Venoms from Viperid Snakes - Biochemical Composition and Activities*. The protocol review was based on the requirements of Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the Public Health Policy on Humane Care and Use of Laboratory Animals; and the USDA Animal Welfare Act and Regulations. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. This protocol has been assigned the following number 1701D-SM-S-20.

The next annual review will be due before March 3, 2018.

Sincerely,

Laura Martin, Director of Compliance and Operations

UNIVERSITY of NORTHERN COLORADO

IACUC Memorandum

To: Steve Mackessy

From: Laura Martin, Director of Compliance and Operations

CC: IACUC Files

Date: 9/30/2016

Re: IACUC Protocol Approval 1302D-SM-S-16

The UNC IACUC has completed a final review of your protocol "Analysis of Venoms from Viperid Snakes- Biochemical Composition, Activities".

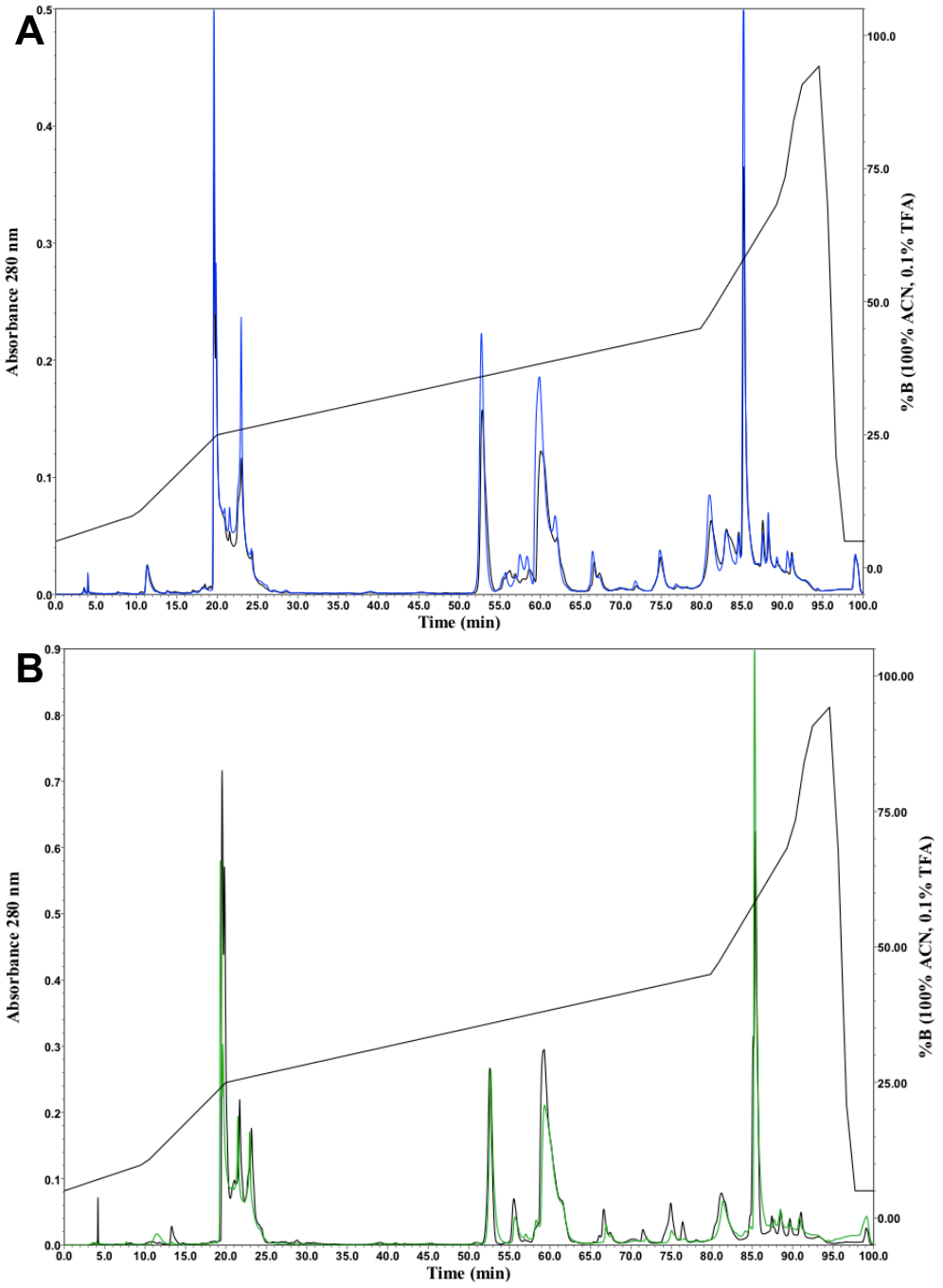
The committee's review was based on the requirements of the Government Principles, the Public Health Policy, the USDA Animal Welfare Act and Regulations, and the Guide for the Care and Use of Laboratory Animals, as well as university policies and procedures related to the care and use of live vertebrate animals at the University of Northern Colorado.

Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. **This protocol has been assigned the following number 1302D-SM-S-16.**

The next annual review will be due before **May 25, 2017**.

APPENDIX B

SUPPORTING DATA FOR CHAPTER II


A
Absorbance 280 nm
%B (100% ACN, 0.1% TFA)
Time (min)
B
Absorbance 280 nm
%B (100% ACN, 0.1% TFA)
Time (min)

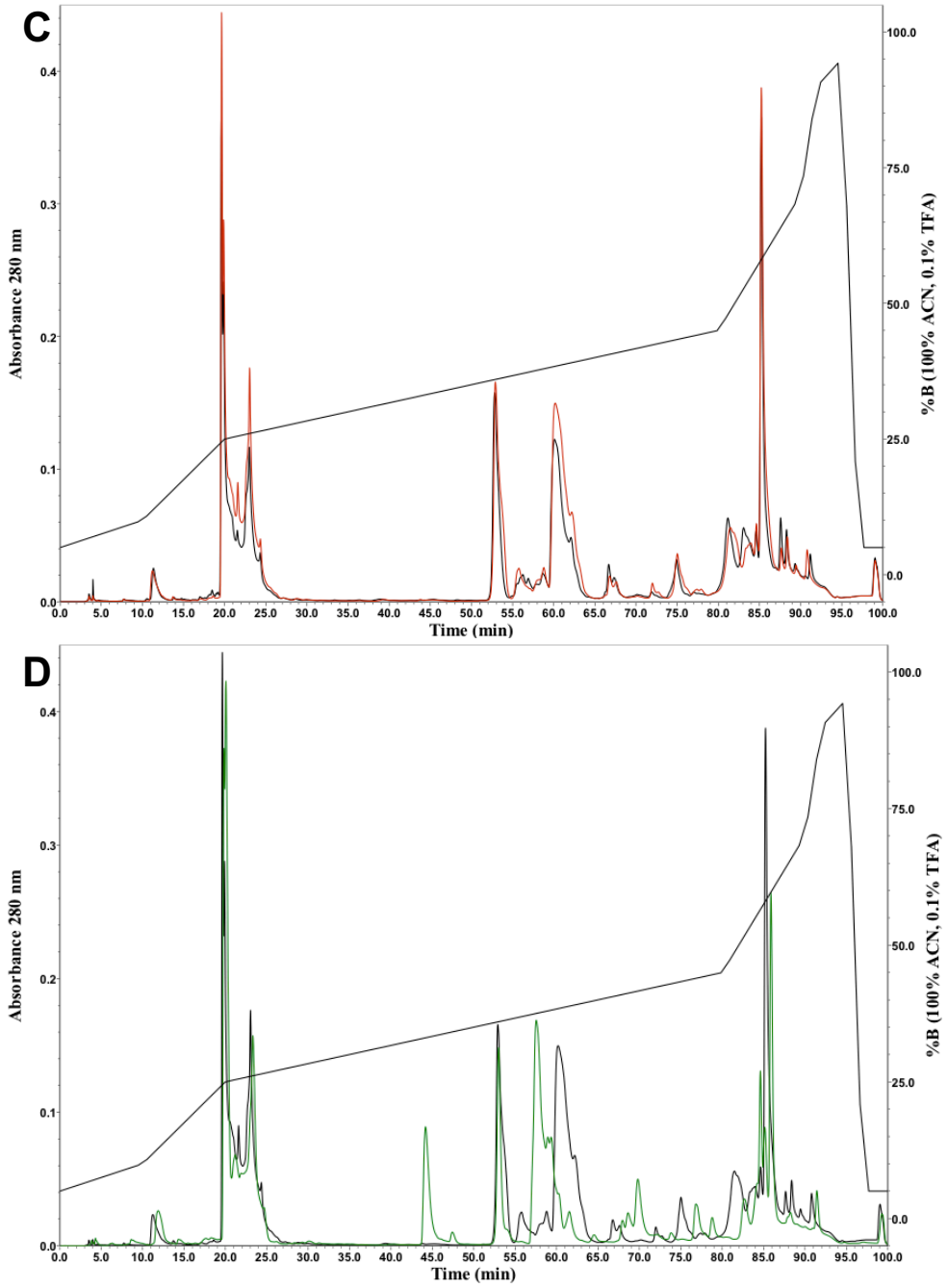


Figure B.1. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from (A) an overlay of two Pinaleño Mountain *C. p. pricei*, (B) an overlay of two Chiricahua Mountain *C. p. pricei*, (C) an overlay of a Pinaleño Mountain *C. p. pricei* and Chiricahua Mountain *C. p. pricei*, and (D) an overlay of a Durango *C. p. pricei* (green) and a Chiricahua Mountain *C. p. pricei* (black).

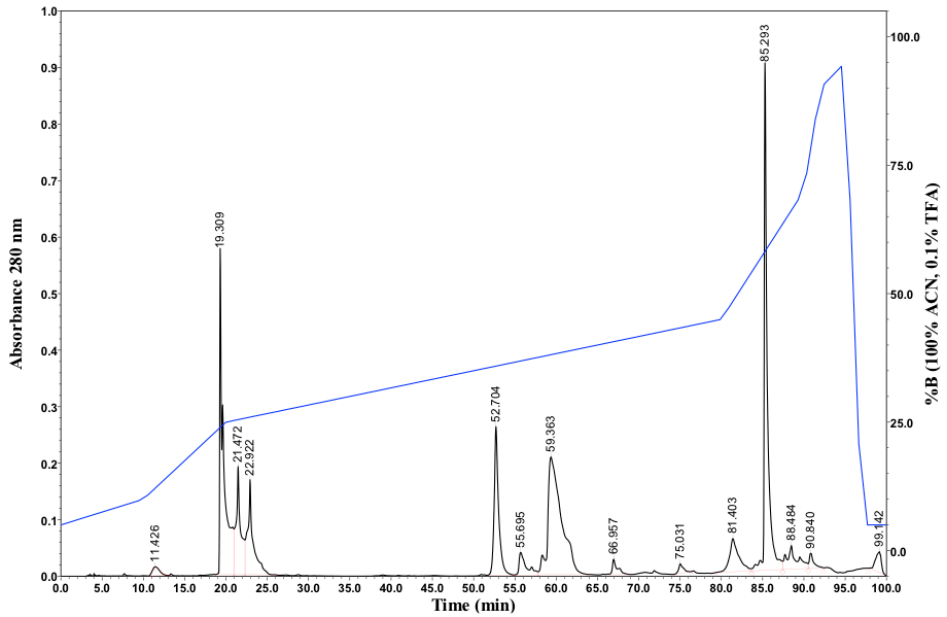


Figure B.2. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from the Chiricahuas (Onion Saddle, snake 483).

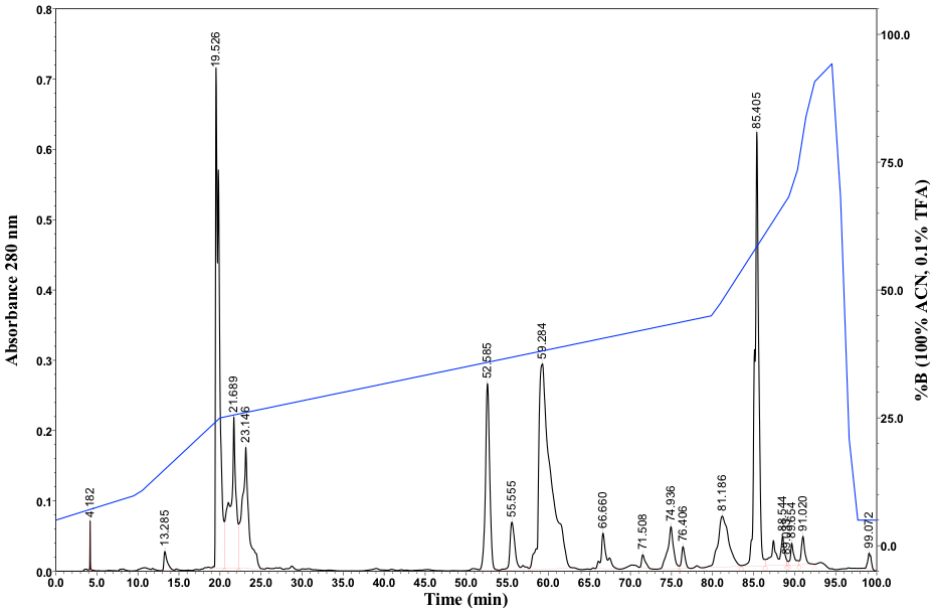


Figure B.3. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from the Chiricahuas (Onion Saddle, snake 482).

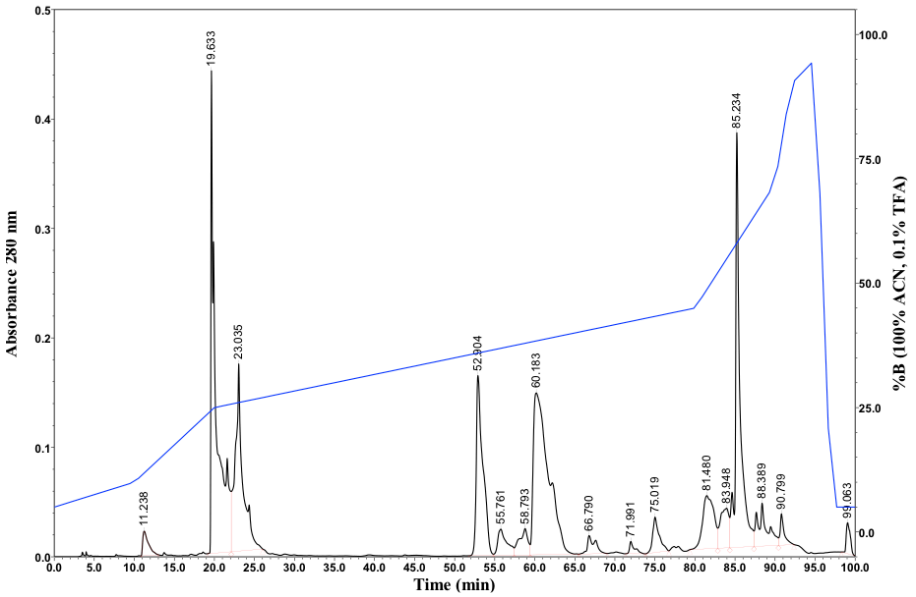


Figure B.4. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from the Chiricahuas (Onion Saddle, snake 503).

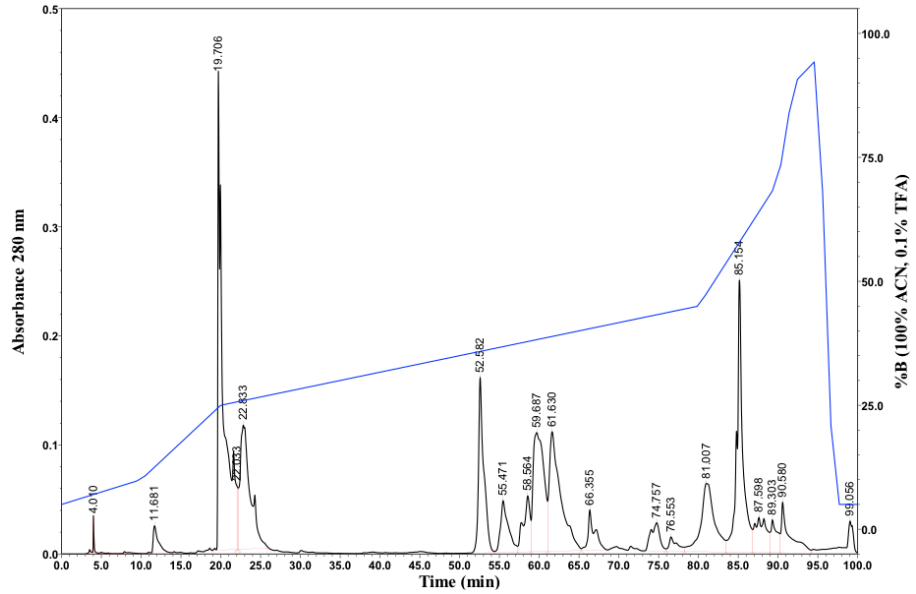


Figure B.5. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from the Chiricahuas (Onion Saddle, snake 493).

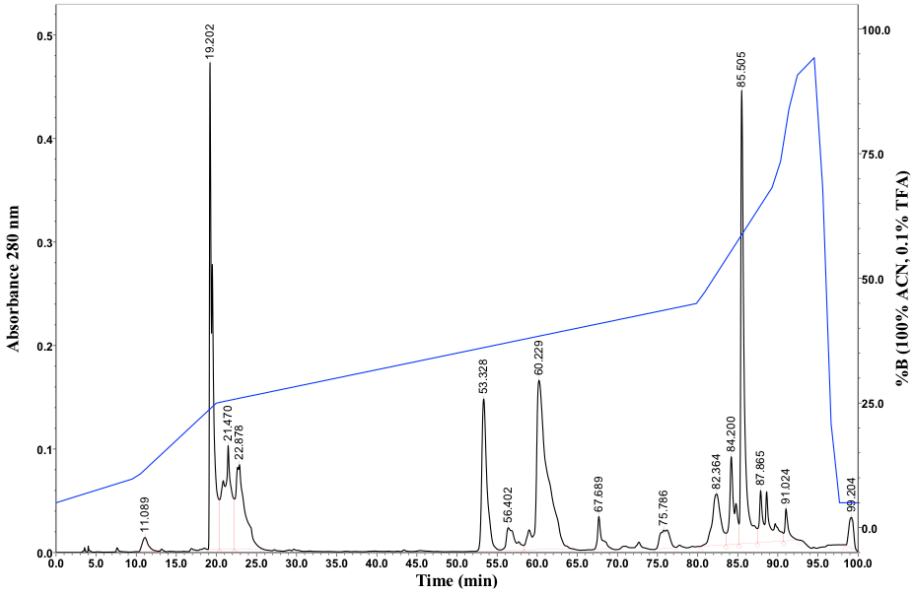


Figure B.6. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from the Chiricahuas (Onion Saddle, snake 42).

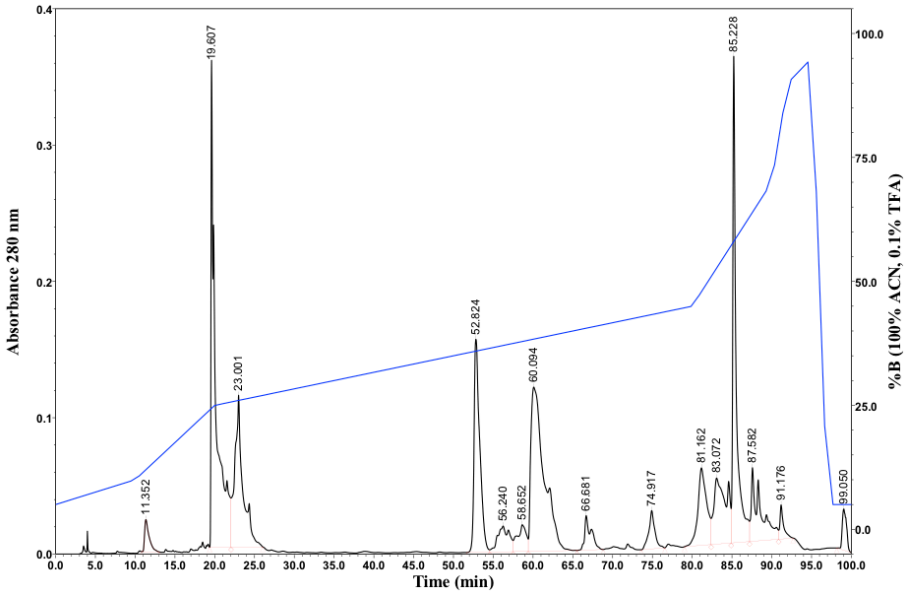


Figure B.7. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from the Pinaleño (snake 502).

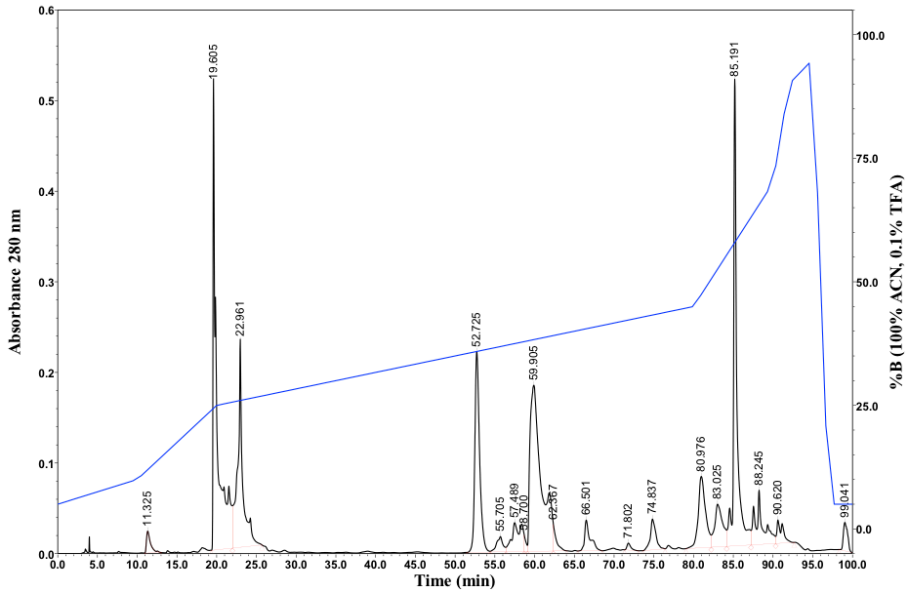


Figure B.8. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from the Pinaleño (snake 504).

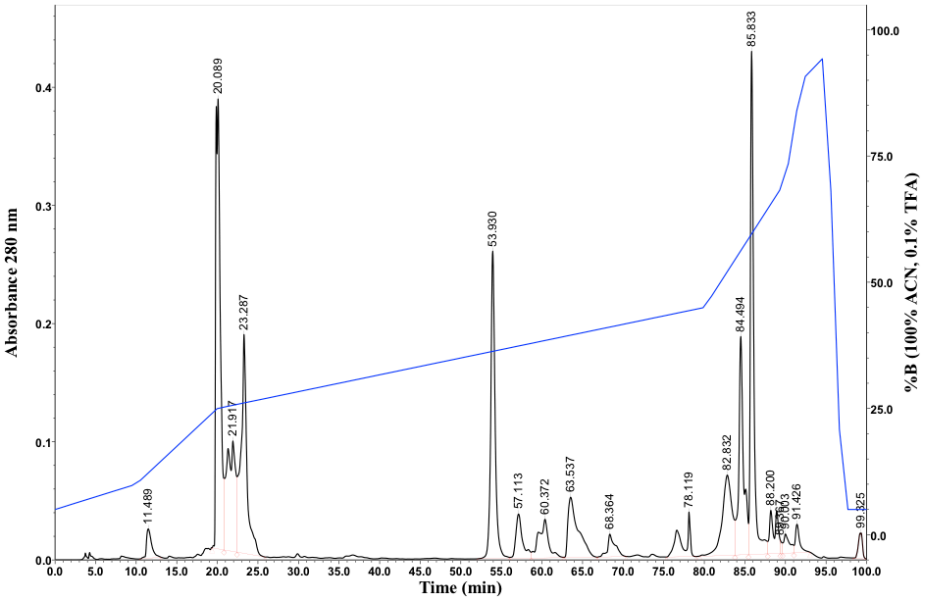


Figure B.9. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from the Santa Ritas (female).

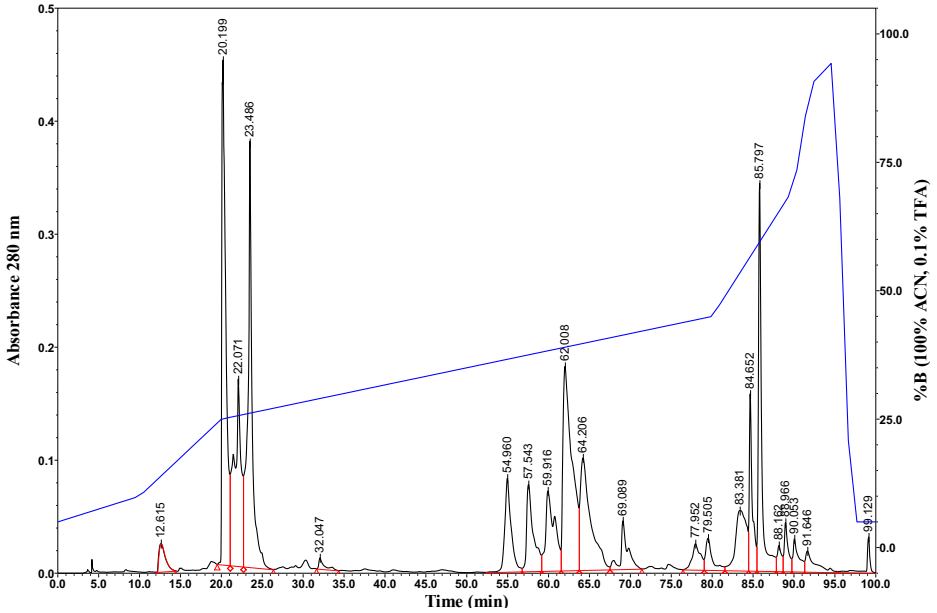


Figure B.10. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from the Santa Ritas (male).

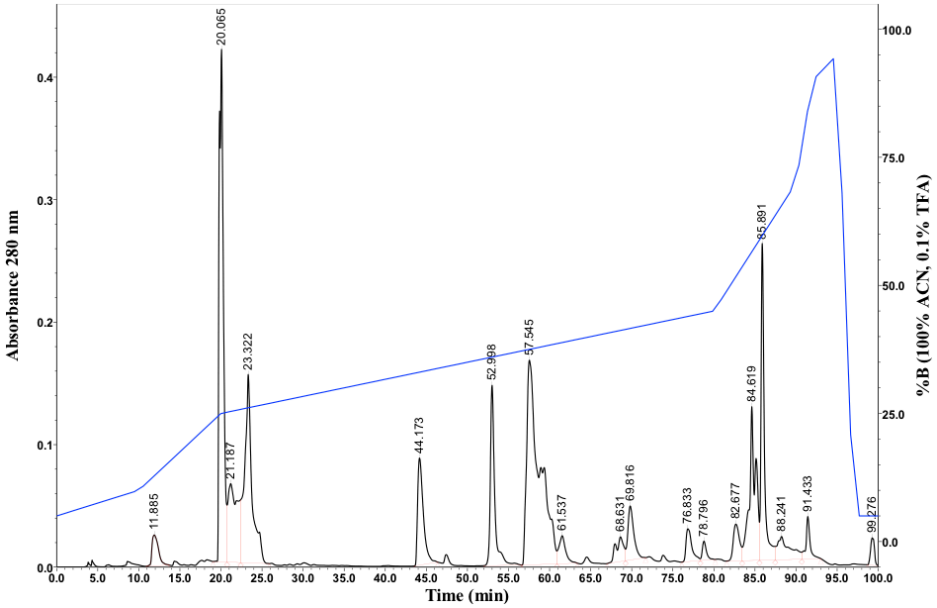


Figure B.11. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. pricei* venom from Durango, México (#3, extracted from captive specimen at the Chiricahua Desert Museum).

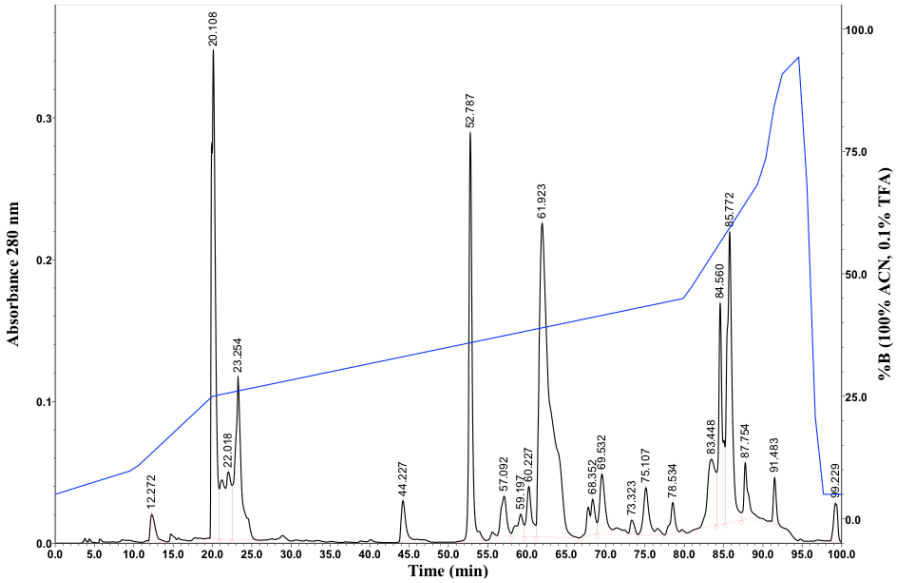


Figure B.12. Reverse-phase HPLC chromatogram of 2.0 mg crude *C. p. miquihuanus* venom from México.

APPENDIX C

SUPPORTING DATA FOR CHAPTER III

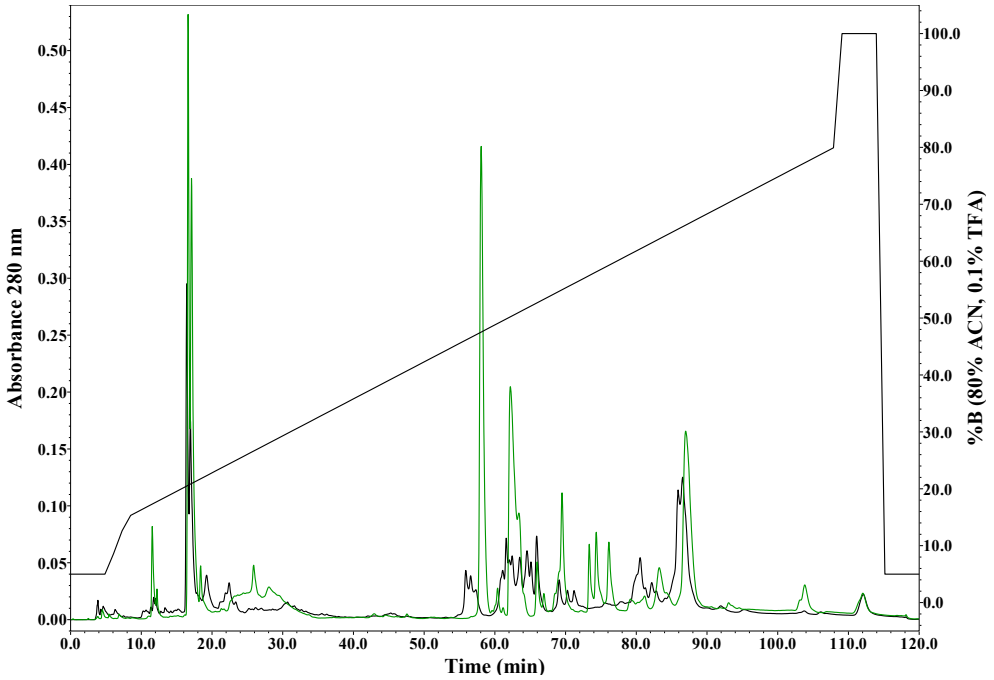


Figure C.1. Reverse-phase HPLC chromatogram and peak elution times of 2.0 mg crude *C. tancitarensis* venom (green) overlaid with 2.0 mg crude *C. intermedius* venom (black).

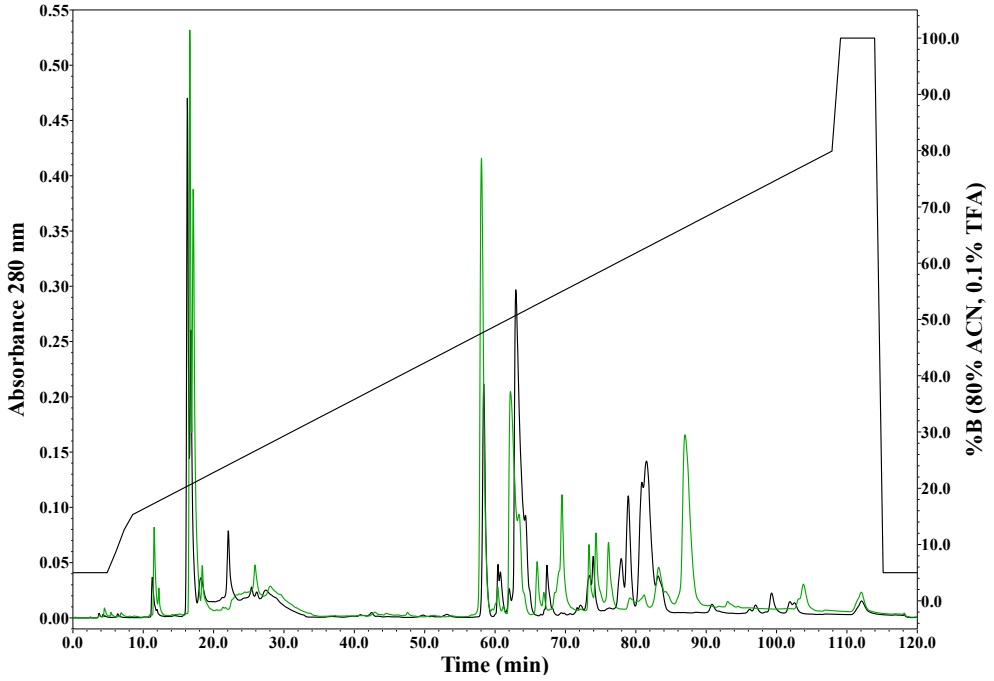


Figure C.2. Reverse-phase HPLC chromatogram and peak elution times of 2.0 mg crude *C. tancitarensis* venom (green) overlaid with 2.0 mg crude *C. pricei pricei* venom (black).

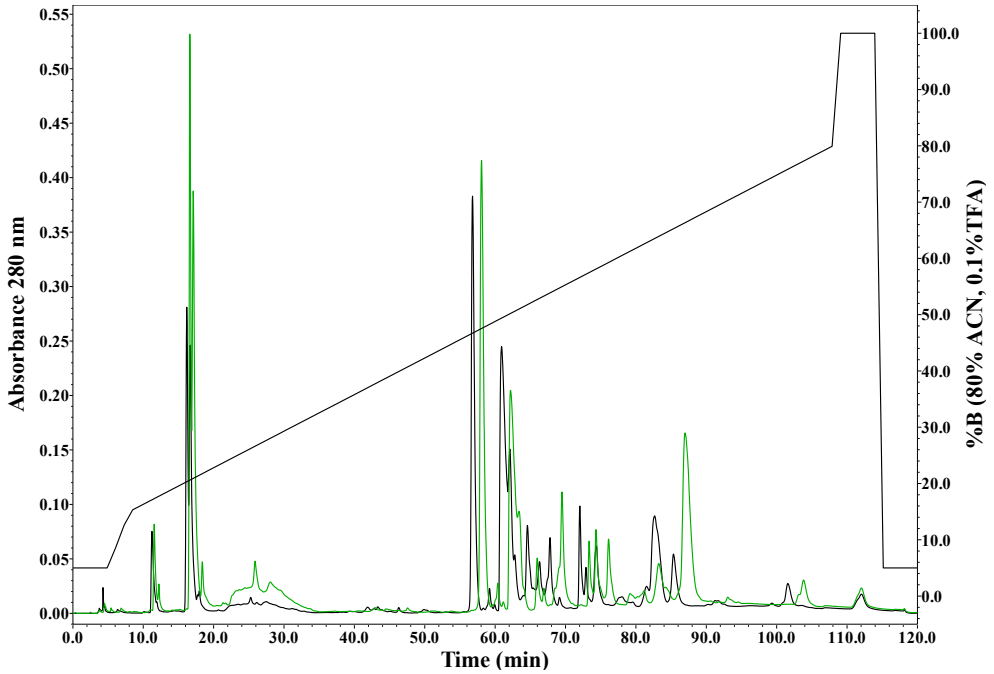


Figure C.3. Reverse-phase HPLC chromatogram and peak elution times of 2.0 mg crude *C. tancitarensis* venom (green) overlaid with 2.0 mg crude *C. transversus* venom (black).

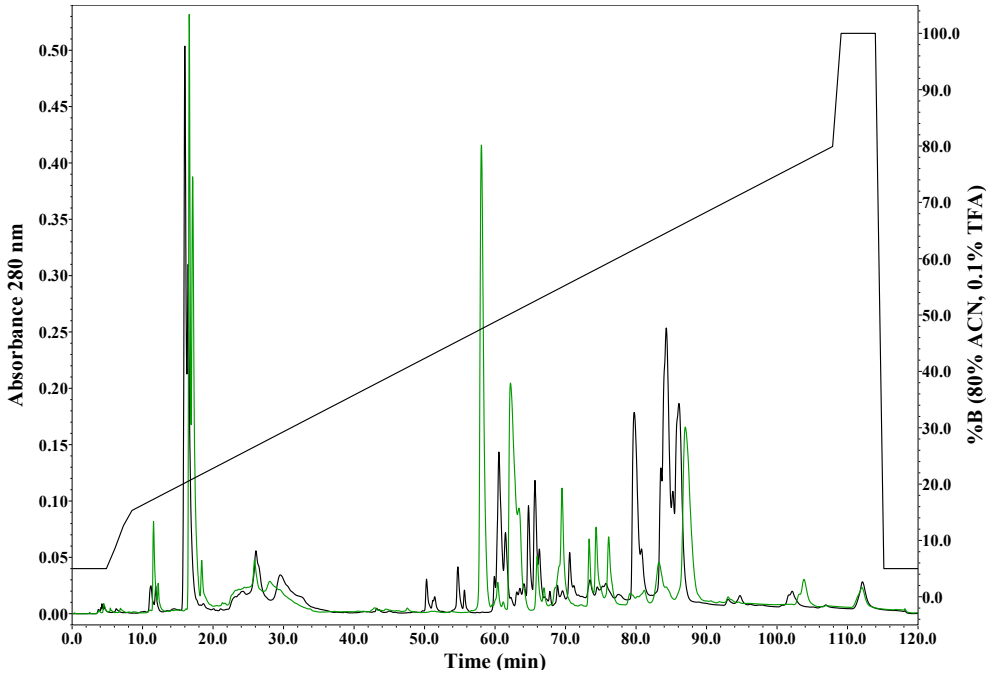


Figure C.4. Reverse-phase HPLC chromatogram and peak elution times of 2.0 mg crude *C. tancitarensis* venom (green) overlaid with 2.0 mg crude *C. triseriatus* venom (black).