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An Analysis of Prey Resistance and Long-Term Temporal Changes in Venom Composition Within Rattlesnake Populations

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AN ANALYSIS OF PREY RESISTANCE AND LONG-TERM TEMPORAL CHANGES IN VENOM COMPOSITION WITHIN RATTLESNAKE POPULATIONS

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ABSTRACT

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Venoms are complex mixtures of toxic constituents used by venomous snakes to incapacitate prey, to defend against threats, and to aid in pre-digestion of prey items. Snake venoms vary based on a number of characteristics, including but not limited to individual identity, species, geographic range, and ontogeny. While much is understood about snake venom variability, relatively little is known as to how venom changes or is stable through time. This doctoral dissertation focuses on elucidating the nature and mechanism of compositional change in the venoms of two rattlesnake species, the Northern Pacific Rattlesnake (*Crotalus oreganus oreganus*) and the Desert Massasauga (*Sistrurus tergeminus edwardsii*). Investigating compositional change in snake venom, this dissertation has three major objectives:

- O1 To verify the quality of long-term stored samples for use in comparative analyses.
- O2 To compare the structure and function of snake venoms collected from the same geographic location at two, distant time points.
- O3 To detect and describe venom resistance in a rodent prey species of the rattlesnake *S. t. edwardsii* from Lincoln County, Colorado.

Tracking changes in populations over time requires the use of long-term stored samples, so the second chapter discusses the viability of using stored samples, evaluating whether degradation is a limiting factor. Venom samples collected ~35 years ago were examined to detect changes in protein content, identity and enzyme activity. Enzyme

assay data collected at the time of venom sample extraction were compared to data collected using the same samples and assay procedure in the present. SDS polyacrylamide gel electrophoresis (SDS-PAGE) and reverse phase high performance liquid chromatography (RP-HPLC) were used to detect features indicating degradation, such as lateral shifts in the position of stereotypical RP-HPLC peaks or the appearance of extra, unexpected SDS-PAGE bands that may result from proteolysis. Qualitative analyses did not reveal significant effects of degradation, but two enzyme activities were significantly lower for stored samples. While proteomic information was retained, some enzyme activity was lost after long-term storage, indicating that stored snake venom samples are viable sources of biological information, but require careful scrutiny of which feature are reliably retained over time. Special attention should be paid to the effects of degradation from storage in venom toxin quantity and quality.

The third chapter explores whether venom composition change is detectable after ~35 years in three populations of *C. o. oreganus*. Comparing samples from three populations in California collected ~35 years apart, changes in presence/absence of toxins by SDS-PAGE band analysis, in relative abundance of toxins by RP-HPLC peak area analysis, and in specific activities of six enzymes were evaluated. Evidence in the literature suggested that changes are possible, but it was hypothesized that there would be no major differences in venom composition between the two time points. Multivariate analyses revealed no significant changes in SDS-PAGE band presence/absence over ~35 years for any population. There was some evidence that enzyme activities differed significantly in one of three populations; however, activity values varied similarly between age group and geographic location, producing low support for ecologically

relevant differences between groups. There was a strong signal for separation of samples by their collection date in the HPLC peak area analysis; more recently collected samples all contained significantly more of a specific L-amino acid oxidase and snake venom metalloprotease compared to long-term stored samples. Multivariate approaches documented variation within and between samples; however, this variation appeared to be shared among samples, regardless of the date they were collected or their collection location. While rapid compositional change is hypothetically possible within such a short time period, the venoms of these populations have not diverged dramatically over the past four decades.

The fourth chapter addresses the hypothesis that diet plays a major role in driving snake venom evolution. Previous work in Lincoln County, Colorado, with *S. t. edwardsii* indicated that the venom of this rattlesnake species might differentially affect possible mammalian prey species. Initial assays evaluated whether some prey species are more or less resistant to the venom of *S. t. edwardsii* from Lincoln County, Colorado. It was determined that *Peromyscus maniculatus* resistance was restricted to *S. t. edwardsii* venom and did not confer resistance to a second sympatric rattlesnake, the Prairie Rattlesnake (*Crotalus viridis viridis*). Additionally, resistance was only found in *P. maniculatus* captured in Lincoln Co., indicating that this is a local adaptation. Finally, standard protein chemistry methods were used to attempt to isolate and describe a resistance molecule. Previous studies have described serum inhibitor molecules in other systems; however, mouse serum was not able to neutralize venom enzyme activity, and mass spectrometry identified a candidate resistance molecule as mouse serum albumin.

The final chapter summarizes the findings of the preceding chapters, and this dissertation presents a new trajectory in the study of natural toxins. The use of long-term stored snake venom samples is validated and comparing these samples against those collected from extant members of the same populations confirms that the continuum of a population's venom variation remains stable over several generations. The mechanism of this stability is not yet known, but there is increasing evidence that diet plays a role in genetic fixation of the types of toxins and combinations of toxins found in a given venom.

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To those of you I do not know and are still reading this, thank you for your time. I hope you find this dissertation enlightening and informative. My hope is that this document represents one of many pieces I will write in my professional career and that you will continue to follow me along these ever-processing lines of inquiry.

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CHAPTER I

SNAKE VENOM VARIATION AND RESISTANCE TO SNAKE VENOM IN PREY

Introduction

Venomous snakes employ their venoms primarily to capture prey (Mackessy, 2010b), but the mechanisms individual taxa use are diverse. Research continues to reveal new complexity that underlies venom's main trophic function. The charismatic forms and behaviors of venomous snakes, juxtaposed with their deadly chemical weapons, have made them a subject of interest from antiquity to the present (Klauber, 1956). As a threat to human health, snakebite envenomation continues to represent a still underappreciated health disparity worldwide (Chippaux, 2017). In recent years, researchers have also begun to develop an appreciation for venomous snakes as reservoirs of potential therapeutic molecules (e.g., King, 2011; Koh and Kini, 2012; Lewis and Garcia, 2003; Pal et al., 2002). As predators with specialized trophic weapons and strategies, venomous snakes have been of interest to the academic community as examples and models of ecological and evolutionary processes (Isbell, 2006; Lynch, 2007; Takeda et al., 2012). Venomous snakes have captured popular interest and imagination as well (Klauber, 1956; O'Shea, 2011). In all of these venues, our understanding of the natural history of these organisms has grown, especially in terms of the diversity and action of snake venoms (Lomonte and Calvete, 2017; Tasoulis and Ibister, 2017).

The impetus for improving our understanding of snake venom composition is motivated by several factors, including a lack of understanding of the often high levels of variation in snake venoms, in turn making treatment of medical cases difficult (Bush and Seidenburg, 1999; Hoggan et al., 2011; Weinstein, 2011). More extensive information about the breadth of venom diversity is necessary to be able to combat venom pathologies clinically (Ainsworth, et al. 2018; Hifumi et al., 2015; Theakston and Laing, 2014), because undocumented venom variation has led to public misunderstandings about the identity and danger of venomous snakes (Crew, 2014; Grenard, 2000). As human populations grow and push into untouched wilderness, and outdoor activities become more popular, the likelihood of venomous snake encounters, and subsequently envenomation cases, are expected to rise (Hayes and Mackessy, 2010). Lastly, recent pushes for statistical validity and availability of 'big data' highlight the need to sample more broadly to account for diversity and variability in venoms that cannot be accounted for by single or small sample sizes of snakes (Amrhein and Greenland, 2018; Benjamin et al., 2018; Leonelli, 2014; Marx, 2013; Trifonova et al., 2013). This literature review highlights what is known about snake venoms and their diversity. Rattlesnakes are the primary focus of this review, as two species are the object of this dissertation, but examples from other venomous snake taxa are included in an attempt to provide a broader view of venom compositional diversity. In addition to questions about venom composition, researchers have continued to pursue the elucidation of the origin of and factors leading to the evolution of venom composition. This review will also address one leading hypothesis, the influence of diet, to explain how snake venoms can vary through time and space.

Snake Venom and Venomous Snakes

Venoms are complex mixtures of toxic components used by snake predators to incapacitate prey (Mackessy, 2010b), aid in pre-digestion of prey (Mackessy, 1988; Thomas and Pough, 1979), and in defense (Bohlen and Julius, 2012; Panagides et al., 2017). Among snakes (suborder Serpentes), four main taxa produce venoms: the mole vipers (family Atractaspididae); the cobras, kraits, coral snakes, and other relatives (family Elapidae); certain members of the large, polyphyletic family Colubridae; and lastly, the group containing the taxa of primary interest for this dissertation, the vipers (family Viperidae).

These groups have in common specialized venom-producing tissues/organs and venom-delivering systems. In the case of the vipers, a primary venom gland containing a large lumen for venom storage is connected to an accessory gland before continuing via a secondary duct to the base of two large fangs that are fixed to the maxillae (Mackessy, 1991). The maxillae rotate on the prefontal and ectopterygoid bones, allowing the fangs to extend down from the roof of the mouth to penetrate prey tissues during an envenomation event, and then retract for storage while the mouth is closed. The deployment and retraction of fangs combined with a fast strike allows vipers to incapacitate their prey with a minimum amount of contact, thus avoiding any possible injury from struggling prey (Kardong, 1986; Klauber, 1956).

In the pitvipers, the subgroup of viperid snakes containing two genera of rattlesnakes (subfamily Crotalinae), *Crotalus* and *Sistrurus*, a heat-sensing pit organ adds extra accuracy to the strike (Chen et al., 2012; Chen et al., 2017; Kardong and Bels, 1998). Known as ambush or sit-and-wait predators, rattlesnakes stereotypically search

for locations where desired prey items pass and position themselves to strike at prey as it moves along these routes. Active foraging is forsaken for the chance to target passing prey, typically delivering enough venom to incapacitate the prey during the short period it passes in front of the ambush position. Some rattlesnakes may strike and hold onto prey that pass (Chiszar et al., 1986; Dullemeijer, 1961; Kardong, 1982), but most appear to allow prey to escape from the ambush location before later detecting and ingesting incapacitated prey by following the chemosensory signature induced by the so-called relocator proteins, disintegrins (Saviola et al., 2013).

General Composition of Snake Venoms

Snake venoms are complex mixtures that include toxic molecules, simply described as toxins, associated molecules that appear to preserve the integrity of the venom mixture, and other products from the secretion of the toxic mélange and cell death, such as cellular debris. Venom toxins stored in the lumen of the glands of venomous snakes are kept in a low pH environment controlled by the secretion of acid from mitochondria-rich cells (Mackessy and Baxter, 2006). In addition to a low pH environment, molecules such as citrate (Mackessy and Baxter, 2006; Odell et al., 1998) and peptide inhibitors (Grams et al., 1993; Munekiyo and Mackessy 2005) serve to preserve toxic molecules in an inactive state until released into the body of an envenomated target.

The overwhelming majority of molecules found in venoms are proteins and peptides. Enzyme and non-enzyme protein toxins, representing a limited number of protein families, are present (a maximum of approximately 30) across all venomous snake taxa (Calvete et al., 2007; Mackessy, 2010b; Tasoulis and Ibister, 2017). However,

while there are a limited number of protein families represented, variation within and between families gives rise to thousands of described toxins, with potentially many more yet unknown. The key to understanding the complexity and diversity of snake venoms is the fact that while members of a given toxin family share a common lineage and thus some shared structural features, the toxic actions of individual molecules (function, biological role) may vary greatly. For example, snake venom toxins in the phospholipase A_2 family have diverse functions, from disrupting neuromuscular junctions, to causing myotoxicity, to disrupting blood coagulation (Harris and Scott-Davey, 2013; Kini, 2003; Saikia et al., 2013). This diversity in pathological action, variability in the number of toxins present in a venom, and variability in the relative abundance of toxins in a venom create an inordinate number of combinations that make a specific venom (for catalogs of complete snake venom proteomes see Calvete, 2013a; Tasoulis and Ibister, 2017).

Current studies of snake venom diversity rely on a combination of proteomic descriptions of the identity and relative abundance of snake venom toxins and biochemical and pathological descriptions of the actions of snake venom toxins (Calvete et al., 2009; Chippaux, 1991; Lomonte and Calvete, 2017). The diversity of toxic actions of snake venoms necessitates a holistic description of both the form and function of toxins, as purely proteomic studies are yet unable to define unequivocally where and how toxins will act *in vivo*. For example, a number of prey-specific toxins/venoms are described where some taxa suffer lethal toxicity if envenomated and others experience no pathological consequences (Heyborne and Mackessy, 2013; Mackessy et al., 2006; Modahl and Mackessy, 2016; Modahl et al., 2018; Pawlak et al., 2006; Pawlak et al., 2009). Identification of individual toxins is possible through proteomic methods, but

understanding the specific (and sometimes selective) nature of their toxicity requires other analytical approaches. A wealth of descriptive work remains, and considering the possible diversity of venom phenotypes, available data only represent a small fraction of what exists in current and historic populations of venomous snakes. Because venoms are trophic weapons under the influence of evolutionary processes, one can describe some generalizable trends and characteristics of venoms within and between venomous snake taxa.

Snake Venom Variation

Venom Compositional ChangeWithin the Lifetime of Individual Snakes

A number of studies have identified variation in venom composition within the lifetimes of individual snakes. Considering that venoms are trophic weapons used for prey capture, it is cogent to note that shifts in venomous snake prey choice are well-documented from neonate to adult (Ernst and Ernst, 2012; Klauber, 1956). In many cases, a shift in prey base appears to be primarily associated with a shift in behavioral preference for prey items the individual is able to ingest. Snakes sharing periods of thermoregulatory optima with their preferred prey may also explain a shift in prey base. Larger-bodied adult individuals may be able to withstand cooler temperatures to ambush prey that are crepuscular or nocturnal, such as small rodents, whereas smaller juvenile individuals that are restricted to more diurnal thermoregulatory patterns are restricted to prey that are contemporaneously active, such as other ectotherms like lizards (Aubret and Shine, 2010; Shine and Sun, 2003).

Ontogenetic shifts in venom appear to track a shift in prey base in many venomous snake taxa; however, there are an equal number of cases where documented

ontogenetic shifts *do not* seem to match prey choice shifts. Whether this incongruence in theory and ecological reality results from a lack of natural history data or an inappropriate hypothetical generalization remains to be seen. Among the Elapidae, Modahl and Mackessy (2016) compared the venoms of juvenile and adult Monocled Cobras (*Naja kaouthia*) and identified shifts in the abundance of certain isoforms and the total activity of phospholipase A_2 toxins. Interestingly, the highly toxic 3-finger toxin α-cobratoxin appeared in both juvenile and adult venoms; in this case, detectable ontogenetic shifts may not correlate with changes in prey base, as this ubiquitous toxin was capable of incapacitating both mammalian and reptilian prey (Modahl and Mackessy, 2016). Ontogenetic shifts in venom correlating with shifts in prey base are documented in the Australian Brown Snakes (genus *Pseudonaja*) where researchers showed that the change from non-coagulant juvenile venoms to coagulopathic adult venoms were associated with switching from reptilian to mammalian prey (Cipriani et al., 2017; Jackson et al., 2016). No shift in prey preference was observed in species in the genus *Pseudonaja* lacking an ontogenetic shift in venom composition (Cipriani et al., 2016).

Ontogenetic shifts are most widely described among members of the Viperidae. Early studies of juvenile venoms noted shifts in particular toxin classes, but did not address the ecological implications of such shifts (Bonilla et al., 1973; Fiero et al., 1972; Jimenez-Porras, 1964). Mackessy (1988) showed that shifts from reptilian to mammalian prey species in juvenile to adult Pacific Ratllesnakes (*Crotalus oreganus oreganus* and *C. o. helleri*) were associated with a change from highly toxic venoms with low metalloprotease activity to lower toxicity venoms with high metalloprotease activity. In the Midget Faded Rattlesnake (*Crotalus oreganus concolor*) ontogenetic changes were

observed but in a dissimilar pattern to *C. oreganus ssp.* (Mackessy et al., 2003); no increase in metalloprotease activity was observed, but peptide myotoxins and coagulopathic serine protease activity increased. There do not appear to be strong trends describing which venom toxin families change and how they change through the life of a viper; however, there appears to be ample evidence that venom ontogeny is to be expected across the family. The literature is rich in studies of other viper species where ontogenetic shifts in venom composition are documented including, but not limited to, Timber Rattlesnake (*Crotalus horridus*), Eastern Diamondback Rattlesnake (*Crotalus adamanteus*), Central American Rattlesnake (*Crotalus simus simus*), Palm-Pitvipers (genus *Bothriechis*), and Lanceheads (genus *Bothrops*) (Augusto-de-Oliveira et al., 2016; Alape-Girón et al., 2008; Guérico et al., 2006; Durban et al., 2013; Margres et al., 2015; Pla et al., 2017; Rokyta et al., 2017; Saldarriaga et al., 2003; Wray et al., 2015; Zelanis et al., 2010; Zelanis et al., 2011).

Published accounts describing the relative stability or flux of adult venoms are sparse. Two groups have tested for seasonal shifts in adult venom composition. Gubenšek et al. (1974) found that two protein gel bands previously identified as lethal toxins (Sket et al., 1973) were present in venom of Common Sand Adders (*Vipera ammodytes*) extracted in the summer and disappeared from the gel analysis in venom extracted in the winter. Gregory-Dwyer et al. (1986) tracked monthly-extracted venom samples from three rattlesnake species (genus *Crotalus*) and found no changes in isoelectric focusing patterns during a 20-month period. While the issue of seasonality is not well studied, observations of venoms from populations of marked and recaptured rattlesnakes in Weld and Lincoln Counties, Colorado, match the latter observation of

venom composition remaining stable throughout the lifetime of a snake (Mackessy, personal communication; McCabe, unpublished observation).

Some researchers have investigated the effects of captivity and handling of snakes on their venom composition. Claunch et al. (2017) compared the venom composition of captive Pacific Rattlesnakes (*Crotalus oreganus ssp*.) exposed to a novel stressor against control animals left in peace, and no significant differences between the groups were found. Williams and White (1992) tracked venom activity of a single captive Common Brown Snake (*Pseudonaja textilis*) over 12 months, finding some activities to decrease during the summer months and others to increase during this period; however, whether this effect is repeatable or is tied to captive care was not addressed. Studies of the effects of long-term captivity showed mixed results where some taxa appear to experience slight shifts in venom protein composition (Freitas-de-Sousa et al., 2015), while others did not experience shifts in composition (McCleary et al., 2016). Gibbs et al. (2011) showed minor changes in venom composition when captive Dusky Pygmy Rattlesnakes (*Crotalus miliarius barbouri*), especially adults, were fed different types of prey over 26 months. Venom composition appeared to be phenotypically responsive to changes in diet, and the authors assumed that the venom changed to match the physiology of the new prey item, to facilitate prey capture or increase digestive efficiency. In general, there appears to be minor evidence that venom composition may change over the lifetime of a venomous snake, but the literature is scant in describing the nature and mechanisms of these changes.

Venom Compositional Differences Within and Between Species of Venomous Snakes

Technological advances in the study of proteomics have led to an exponential increase in complete descriptions of venoms that allow for analysis within and between taxa (Calvete, 2017; Calvete et al., 2007). The recent work of Tasoulis and Ibister (2017) to catalog and analyze trends in venom proteomes constructed in the past decade supports the observation that snake venoms are relatively homologous, composed of a limited group of toxin families. They note that while their cumulative analysis identified 63 toxin families, greater than half of these are present in less than 5% of the taxa analyzed. Previous research has documented trends that appear to separate snakes at higher order taxonomic designations, such as the lack of disintegrins in elapid venoms (Calvete, 2013b; Moura-da-Silva et al., 1996) or apparent absence of three-finger toxins in most viperid venoms (Aird et al., 2013; Sanz et al., 2006). The venoms of the rattlesnakes (genera *Crotalus* and *Sistrurus*) appear to follow one of two trends, either containing high concentrations of P-I and/or P-III metalloproteases and being relatively less toxic (Type I) or higher toxicity venoms with little to no metalloproteases present (Type II) (Mackessy, 2008). However, in general, venom composition is not a useful indicator of taxonomic identity (Calvete et al., 2009; Johnson, 1968; Serrano et al., 2005; Soto et al., 1988; Straight et al., 1992). In addition to the overlap in shared toxin families and pathologies, venomous snake populations may have high levels of venom variability within species.

Population characteristics are known to drive variation in snake venoms within species. A connection between sex and venom composition is shown in some taxa but

not others. Mebs and Kornalik (1984) showed that among siblings from a litter of Eastern Diamondback Rattlesnakes (*Crotalus adamanteus*), females had an additional basic toxin that males lacked. Another within-litter study of Jararaca venom (*Borthrops jararaca*) found sex-based differences in the abundance and activities of certain toxins (Menezes et al., 2006; Pimenta et al., 2007). Because these studies were conducted in single litters of snakes, it is impossible to parse whether sex-based differences are generalizable to these species as a whole or if there is some other lineage-based explanation. Recent work in hybrid rattlesnakes showed that some toxic activities in the venom of hybrid offspring corresponded to their same-sex parent, while others were intermediate between the parents' activities (Smith and Mackessy, 2016). Sex-based differences in venom composition may be apparent between siblings because they are linked genetically, but more information is needed to confirm this hypothesis. Studies investigating intersexual venom differences in unrelated individuals most often indicate that there are no detectable intersexual differences (Chippaux, 1991; Mackessy, 2010b). Tan et al. (2017) showed that although the Temple Pit Viper (*Tropidolaemus wagleri*) has sexual dimorphism, venom composition does not vary between sexes. There are of course exceptions; Daltry et al. (1996) showed that female Malayan Pit Viper (*Calloselasma rhodostoma*) venoms contained an extra band in their isoelectric profiles.

Studies identifying differences between geographic populations of the same species are more common. Variability in the presence/absence of the subunits of the potent heterodimeric phospholipase A_2, Mojave Toxin, from the medically important Mojave Rattlesnake (*Crotalus scutulatus scutulatus*) have been extensively described in populations across the southwestern United States and Mexico (Glenn and Straight, 1978;

Glenn et al., 1983; Borja et al., 2014; Borja et al., 2018; Huang et al., 1992; Rael et al., 1993; Sunagar et al., 2014; Wilkinson et al., 1991). Dagda et al. (2013) investigated variation in metalloprotease activity in *C. s. scutulatus* and differentiated four separate regions of composition based on a combination of biochemical and genomic data. Borja et al. (2018) further showed that populations with venoms containing Mojave Toxin were extremely toxic and had low hemorrhagic and proteolytic activity (Type I) and that populations with venoms lacking Mojave Toxin were relatively less toxic and had high levels of hemorrhagic and proteolytic activity (Type II). Interestingly, populations situated in between those with a clear Type I/II phenotype showed an intergrade phenotype where venoms contained some Mojave Toxin and had moderate hemorrhagic and proteolytic activities. Studies in other taxa have documented shifts in the relative abundance and activity of a variety of toxins/toxin families based on geographic location (Forstner et al., 1997; Margres et al., 2015; McCleary et al., 2016; Nuñez et al., 2009; Shashidharamurthy et al., 2002; Straight et al., 1992).

There is some evidence that variation between geographic locations has appeared because of the influence of the geographic landscape itself or other factors such as population dynamics or other historical population traits like founder effects. Creer et al. (2003) tested for phylogenetic signal in the diversity of phospholipase A_2 isoforms and in venom-wide profiles of island populations of Stejneger's Pit Viper (*Trimeresurus stejnegeri*) finding that variation between tested populations could not be explained by neutral evolutionary processes; they surmised that selective pressures like diet or founder effects were more important for explaining venom variability. Thorpe et al. (1995)

similarly correlated venom bands with ecological factors, finding that several bands were associated with geographic distance, while others were connected to features of local diet.

What may come as no surprise is that venom variability within and between venomous snake species appears to be the rule and not the exception. Unfortunately, a true appreciation of this natural diversity does not appear widely in the literature since a focus on the power of whole venom proteome description is dominated by studies that describe the venom characteristics of only one to five individuals (e.g. Gibbs et al. 2013a; Kohlhoff et al., 2012; Vejayan et al., 2014). In other words, snake venom toxinology appears to have a great amount of breadth in attempting to catalog a variety of species, but is lacking in depth of information about the diversity of venom composition within individual species. However, a recent push for integrative approaches that track venom from genotype to phenotype to elucidate mechanisms of venom expression and fluctuation (Durban et al., 2013; Gibbs et al., 2018; Lomonte and Calvete 2017; Modahl and Mackessy, 2016) promises to bridge this gap in our understanding. One major hypothesis to explain compositional variation in snake venoms is the connection between diet and venom, and the interactions of venomous snake predators and their prey that may drive venom evolution. The remainder of this chapter addresses what is understood of the interplay between trophic adaptation and ecological dynamics.

Evolution of Resistance to Toxins in Prey of Venomous Snakes

Venoms are simple to complex mixtures of toxic components that are conveyed through specialized delivery systems to subdue prey (Mackessy, 2002; Mackessy, 2010b), and possibly to aid in pre-digestion of prey tissues (Mackessy, 1988; Pough and Groves 1983). For prey species on the defensive side of the predator/prey dyad, becoming a meal

greatly decreases lifetime fitness, and predictably many forms of predator evasion have been documented. This section discusses the nature of chemical defenses against predator venoms, often described as venom resistance, that have arisen in response to the selective pressure imposed by the chemical weapons of predators. For the purpose of this discussion, venom resistance is defined as the endogenous chemical/physiological capacity of a prey species to prevent or hinder the pathologic consequences of envenomation by a predator species. By this definition, in the absence of resistance mechanisms, venoms are pathological to prey species. This venom antagonism is in contrast to cases where a predator's venom has no bioactive effect on one or a group of potential prey species, but may be lethal to other species or groups of species (i.e., prey-specific venoms, Heyborne and Mackessy, 2013; Mackessy and Saviola, 2016; Pawlak et al., 2006; Pawlak et al., 2009). Venoms represent complex molecular weapons to defend against, and venom resistance is assumed to be conferred by venom-resistant molecules or mechanisms that are able to neutralize partially or fully the negative effects of a venom and its toxic constituents. Successful resistance should allow prey species to evade capture and digestion. There is evidence that in some cases, chemical neutralization of venomous components may not be sufficient to allow prey species to escape predator behaviors that enable prey capture, regardless of the effectiveness of venoms. However, behavioral responses that allow prey species to evade predators, or allow predators to successfully capture prey species, independent of the role of venom, will not be discussed. In general, the present discussion focuses on the chemical arms race between venomous animals and their resistant prey.

Instances of resistance are discussed in relation to the venoms they are able to neutralize. Each section provides information regarding efficacy of resistance, mechanism(s), phylogenetic breadth of resistance, phylogeographic distribution of resistance, as well as other relevant information about the nature of the predator/prey pairs in question. The discussion here centers on chemical arms races between venomous predators and resistant prey; that is, the focus remains only on cases where snakes act as predators in trophic interactions and not cases where predators of venomous snakes have resistance against snake prey. Following the predator-specific sections is a concluding discussion of our current understanding of prey resistance to natural toxins, future directions for resistance research, and possible applications of resistance systems for practical and theoretical purposes.

Coevolution of Predator Venoms and Prey Resistance

When considering prey resistance, the underlying issue is whether a coevolutionary response to the selective pressure of predator venom exists within the system. Venoms, as derived trophic adaptations, are expected to experience selection pressure from mechanisms that allow prey species to evade predation. The appearance of resistance molecules in response to the derivation of new snake venom toxicities is expected to follow Dawkins and Krebs' (1979) model for an arms race between two taxa in an antagonistic coevolutionary relationship. A predator develops a chemical weapon, venom, which is used to subdue a prey species. As predators capitalize on susceptible individuals, the diversity of the prey population becomes limited to those individuals who are able to evade predation. These remaining individuals may persist because of phenomena such as behavioral modifications, changes in microspatial distribution, or the

appearance of a chemical mechanism that inhibits the toxic action of the predator's venom, namely resistance. This resistance phenotype is expected to increase over time as the snake predator becomes increasingly incapable of incapacitating prey with the new resistance phenotype. Variation in predator and prey phenotypes are expected to follow each other through time in a frequency-dependent manner that creates new resistances to new toxicities and vice versa.

This scenario, of the development and maintenance of resistance in prey, requires a number of assumptions. First, predator/prey pairs are expected to associate with each other for stable periods of time. By definition, predators and prey should respond in sequential and reciprocal manners as the opposing partner develops a new offensive or defensive strategy. Van Valen (1973) described this stable reciprocity in his postulation of the Red Queen hypothesis. Much as the Red Queen in Lewis Carroll's *Through the Looking Glass* tells Alice that to stay in one place she must keep running, Van Valen hypothesized that for either predator or prey to 'stay in one place' (i.e., persist through evolutionary time), they must continue to evolve. By extension, if one of the predator/prey pair was unable to continue to respond to a newly derived trait in the other partner, they would soon become extinct, assuming intense predation pressures on the susceptible prey phenotype, or starvation in predators with insufficiently toxic venoms. Extant predator/prey pairs should demonstrate some balance between the relative abundance of resistant and susceptible individuals, and relative abundance of generally toxic to increasingly non-toxic individuals, keeping in mind that this balance may be skewed towards one partner or the other at any given time point.

In addition to stable reciprocity, the timeline of coevolutionary relationships is expected to develop over longer rather than shorter time scales. When investigating the frequency and mechanism of resistance, it may be that the newly evolved resistance or toxicity is at such low abundance that detection of this functionality is nearly impossible. In the real time of academic research, the turnover of enough generations of predator or prey species to produce a new functionality may be too slow for any given researcher to describe in a lifetime. Additionally, whether novel toxicity or resistance is diversifying, or is being selected against, may depend on the historic length of predator/prey associations. Sunagar and Moran (2015) compared the rate of diversification of a variety of toxin groups against the relative age of a number of venomous species' lineages. These authors proposed a 'two-speed' mode of venom evolution, where more recent lineages of venomous predators, such as cone snails and venomous snakes, show increased diversifying selection, and older lineages, such as cnidarians and spiders, appear to be under increased levels of purifying selection. The authors proposed that diversifying selection for venomous predators would be associated with prey base or niche expansion; however, it is possible that diversifying selection may allow for maintenance of a stable relationship with current prey species because novel resistance and toxicity appear as a result of stable reciprocity. Venom evolution does in snakes does not require changes in the identity of their prey, just changes in the venom tolerance of current prey. In any case, younger or older lineages are not fixed in a selective regime and may experience a switch from purifying to diversifying selection and vice versa. Thus, it appears that the age of the lineage in question may increase the likelihood that resistance is a prominent feature of prey populations or that the toxicity of the predator

may have an advantage over prey defenses, again making resistance more difficult to detect.

It is cogent to note that while a chemical arms race scenario is presently a "best guess factor" as the driving force for biochemical diversification of venoms over evolutionary time, numerous cases of prey-specific toxicities and venom resistances are documented in the literature (Barlow et al., 2009; Heyborne and Mackessy, 2013; Mackessy et al., 2006; Modahl and Mackessy, 2016; Modahl et al., 2018; Pawlak et al., 2006; Pawlak et al., 2009, which lends support to a coevolutionary relationship between toxicity and resistance. In support of the chemical arms race scenario, research into the relationships between venomous snakes and their resistant prey have served as a test case. Current information about a diversity of resistant prey is prefaced by a discussion of theoretical and methodological approaches to evaluating the importance of coevolutionary processes in the development of resistance.

Resistance to Snake Venoms

Natural resistance to predator venoms is best described in prey species of venomous snakes, particularly mammals. The impetus for this wealth of knowledge comes from the attempt by snake venom researchers to elucidate the merits of the hypothesis that diet has served as a major selective pressure shaping snake venom composition. Over the past several decades, researchers have demonstrated that venom composition may vary across geographic space and ontogenetically (see Mackessy, 2010b) and has been purported to vary with diet (e.g., Gibbs and Mackessy, 2009; Sanz et al., 2006). The more recent championing of diet as a major driver for venom compositional change is born out of an institutional debate over the origin of venom; i.e.,

whether venom is the product of neutral or selective processes over evolutionary time. Near the end of the twentieth century, the issue of the origin of snake venoms as the product of neutral or selective processes became a major theoretical divide between venomous snake biologists. Scientists such as Dietrich Mebs (2001) and Mahmood Sasa (1999) argued that because snakes delivered venom in such large quantities, many times more than was sufficient to incapacitate prey, venom must not have arisen from selective processes and was 'overkill.' Considering the discrepancy between the minimum amount of venom required for prey capture and the actual amount delivered, they argued that venom components were too metabolically costly to be used in such large quantities. Additionally, they noted that the individual components of venom were so toxic across a variety of possible prey species that there did not appear to be selection for specific toxicities. To these authors, venom arose out of neutral evolutionary processes that allowed for the sequestration and concentration of modified somatic molecules into what we observe today as the components of snake venom.

This neutral view was quickly challenged by research showing that the notion of overkill was unlikely. Hayes et al. (2002) demonstrated that venomous snakes had control over the amount of venom released in striking a prey item. The amount of venom delivered was more than absolutely necessary to subdue prey items, but control over venom delivery indicated that there was a functional role for allowing large volumes to be expressed in snakebite envenomation. Saviola et al. (2013) demonstrated that, at least in venomous snakes from the family Viperidae (vipers, pitvipers, and other solenoglyph venomous snakes), the large bolus of venom was required in order to deliver a particular molecule in high enough concentration to allow the snakes to recover their envenomated

prey item. Viperid snakes often use a sit-and-wait ambush strategy and strike prey as they cross the snake's path; prey that has fled the ambush site and succumbed to the effects of the venom is then recovered, often at some distance to the ambush site. The process of prey relocation may be challenging because prey may escape in any direction in three-dimensional space, and thus a relocator molecule is needed to track the envenomated prey item effectively. At this point, an arms race hypothesis was explored to explain the evolution of the complex phenotype of snake venom and associated delivery systems.

A number of prey species groups show resistance to snake venoms, and a wide variety of evidence has been used to corroborate a chemical arms race scenario. Each species group will be treated separately, and data have been compiled on the prevalence and mechanism of resistance. Any study attempting to uncover coevolutionary relationships between species pairs faces the challenge of using extant and historical evidence to infer reciprocity across evolutionary time. A number of approaches are often used and synthesized to confirm coevolution (Futuyma and Slatkin 1983). In the case of resistance/toxicity systems, the demonstration of resistance through standard toxicity assays is required. Anecdotal evidence for prey ability to avoid predation may not be explained by chemical resistance; resistance must be confirmed through direct challenges with physiologically and biologically relevant doses of venom. As novel phenotypes should appear in a single individual or small population of individuals and radiate out in the direction of gene flow, locality of both predator and prey must be taken into account. A record of the geographic distribution of populations with resistance or susceptibility may further allow for spatial correlation with the range of the venomous partner species.

Thus, a biogeographic account of current resistance may be constructed. Longitudinal documentation of the biogeography of a particular resistance mechanism may offer some insight into the rate of change in the dynamics of resistance and toxicity for a given species pair. To date, it does not appear that this type of long view has been established for any system involving snakes, and even if one could be constructed, if reciprocal responses occur over evolutionary time, this may preclude any detection of active flux in the relationship between toxicity and resistance within the lifetime of a given researcher.

Following initial screening for resistance, mechanistic descriptions are often elucidated that demonstrate the direct ability of prey physiologies to negate the pathologic effects of venoms. As mentioned earlier, prey species are challenged by the (often) complex phenotype of predator venom, and their responses may range from a wholesale attempt to neutralize the diversity of toxins in a venom to mechanisms that attack a limited number of toxins. Finally, some attempt must be made to connect species pairs in evolutionary time and demonstrate stepwise evolutionary change. This correlation through time is the most difficult line of evidence to obtain as current technologies limit these types of studies to phylogenetic comparisons between predator and prey species complexes (Filipiak et al., 2016; Page, 2002; Suchan and Alvarez, 2015). Correlation between the divergence of predator and prey clades would seem to indicate reciprocal evolutionary divergence; however, correlational analyses are limited in their ability to confirm causality between the coevolution of toxicity and resistance and speciation or divergence in predator and prey taxa. It is also possible that some common biotic or abiotic pressure, unrelated to potential coevolutionary scenarios, caused cladogenesis in both predator and prey species, and resistance is secondarily derived.

Resistance to snake α–neurotoxins. A resistance mechanism that has been confirmed across a diversity of mammalian predators and prey is the ability to tolerate snake α-neurotoxins, acetylcholine receptor (AChR) agonists. Ovadia and Kochva (1977) demonstrated that mongoose sera challenged with venoms from snakes in the family Elapidae (cobras, kraits, and other opisthoglyphous snakes) was able to neutralize the effects of the venom. Later research uncovered that this resistance to elapid venoms is directed against α-neurotoxins that make up a significant portion of the total venom protein. Barchan et al. (1992) sequenced the mongoose AChR and detected a number of non-synonymous mutations in the ligand binding site of the AChR. Hypothesized structures for these mutations indicate a conformational change in the ligand binding site that prevents α-neurotoxins from binding while still allowing acetylcholine (Ach) to bind its receptor. Later work (Asher et al. 1998) further demonstrated that the mongoose's resistant AChR prevented α-neurotoxins from binding while still allowing ACh to bind with higher affinity than non-resistant type AChR found in rats. This elevated binding affinity indicated that mongoose AChR was able to prevent complete binding of α-neurotoxins while allowing ACh to bind with little steric or concentration-dependent competitive hindrance from α-neurotoxins that had inundated synaptic junctions. A slight conformational change was sufficient to create near complete resistance to α-neurotoxins.

In addition to mongooses, similar conformational changes in acetylcholine receptors have been documented in other prey species of venomous snakes such as, the Javelin Sand Boa (*Eryx jaculus*), the Dice Snake (*Natrix tessellata*), and the European Hedgehog (*Erinaceus europaeus*), as well as in venomous snakes such as the Chinese

Cobra (*Naja atra*) (Barchan et al., 1992; Neumann et al., 1989). Resistance in *N. atra* is most likely protection against autoenvenomation; however, it is possible that this resistance allows evasion from cannibalism or predation by other sympatric elapid snakes. The example of *E. europaeus* provides an additional mammalian example of resistance to α-neurotoxins, but perhaps the most intriguing example of resistance is the case of the two non-venomous snakes (*E. jaculus* and *N. tessellata*). Considering the ongoing debate among snake venom toxinologists about the ultimate origin of snake venom proteins and the delivery apparatus (e.g., Fry et al., 2012), the appearance of α-neurotoxin resistance across more basal snake taxa begs the question of whether resistance is intrinsic to snake physiology or has appeared independently several times throughout the radiation of the snakes. In any case, a better understanding of the molecular origin of snake resistance to snake venoms could indicate a coevolutionary predator-prey situation if the hypothesis that resistant, non-venomous snakes were once or are currently preyed upon by venomous snakes is supported.

Resistance in woodrats (Genus *Neotoma*). As a followup study to anecdotal evidence of resistance in Southern Plains Woodrats (*Neotoma micropus*), Perez et al. (1978) challenged woodrats with venom from the Western Diamondback Rattlesnake (*Crotalus atrox*), showing that these rodents had greatly elevated tolerance to the venom compared to a laboratory mouse control. Perez et al. (1979) further showed that this resistance mechanism was able to significantly decrease the hemorrhagic effects of *C. atrox* venom for *N. micropus*. De Wit (1982) screened a second *Neotoma* species, the Eastern Woodrat (*Neotoma floridana*) with the venom from Osage Copperhead (*Agkistrodon contortrix phaeogaster*) and detected a similar resistance to hemorrhagic

toxins. It appeared that venom resistance was shared across the genus. Using electron microscopy, Huang and Perez (1982) further showed that *N. micropus* suffered little hemorrhage or muscle damage following envenomation. Some mitochondrial and myofibril damage were detected, but it appeared that resistance also prevented myotoxic pathologies, especially in comparison to laboratory mouse controls. A candidate anti-hemorrhagic resistance molecule was purified and partially described by Garcia and Perez (1984). This single, non-enzymatic resistance molecule was able to bind and neutralize *C. atrox* toxins. Binding was shown to be non-polyvalent, and the authors concluded that this candidate molecule was not an immunoglobulin. Unfortunately, it does not appear that further descriptive work has been completed on this resistance molecule, and no biogeographic or further phylogenetic information is available regarding the distribution and prevalence of this resistance mechanism in *Neotoma*.

Resistance of ground squirrels (Genus:*Otospermophilus*) to snake venom metalloproteases. Another well-described example of snake venom resistance are endogenous Snake Venom Metalloprotease Inhibitors (SVMPIs), best documented in a number of squirrel species in the genus *Otospermophilus* (formerly *Spermophilus*). Biardi and Coss (2011) showed that Rock Squirrel (*Otospermophilus variegatus*) serum was able to neutralize the pathological effects of venom from two species of rattlesnake, the Western Diamondback Rattlesnake (*Crotalus atrox*) and Prairie Rattlesnake (*Crotalus viridis viridis*), that were sympatric to assayed squirrel populations. Challenges with venom from an allopatric species of rattlesnake, the Northern Pacific Rattlesnake (*Crotalus oreganus oreganus*) were not successfully neutralized. Interestingly, the venom used in these experiments was commercially purchased; however, even without a

confirmation of matching locality between predator and prey samples tested, there still appeared to be an inhibitory effect against individuals from a sympatric predator species. In the same year another team (Biardi et al., 201l) published a description of an SVMPI isolated from California Ground Squirrel (*Otospermophilus beecheyi*)serum. This molecule was able to prevent tissue damage and hemorrhage normally expected from envenomation by the sympatric *C. o. oreganus*. Further, resistance was positively correlated with the proximity of rattlesnake populations to resistant *O. beecheyi* populations; that is, resistance was ineffective against distant populations of *C. o. oreganus*, indicating that resistance is geographically localized and requires predation (or at least offensive) pressure from the colocalized rattlesnake population to select for resistance. The authors recognized that while other mammals do not have similar SVMPI's that serve as resistance molecules, there appears to be convergence of defenses against hemorrhagic toxins, a hallmark of many viperid snake venoms. Future work in mammalian resistance to viperid venoms will confirm or reject convergence to defenses against hemorrhagic toxin classes of snake predators.

Resistance to snake venoms in the opossums (Family Didelphidae). A final group of prey items with described resistance to venomous snake predators are the Opossums (Mammalia: Didelphidae). Jansa and Voss (2011) reported an increased number of non-synonymous changes in gene sequences of a hemostatic protein, von Willebrand Factor (vWF), in opossums known to prey on venomous snakes. These researchers found that these non-synonymous changes are associated with binding sites for C-type lectin-like proteins found in some viperid snake venoms; changes to these regions were inferred to decrease binding affinity with these toxins. These data do not

indicate that opossums preyed upon by venomous snakes have similar resistance, but later work (Voss, 2013) found that a number of opossum species could be confirmed as venomous snake prey, and that their relationships to known, resistant species of opossums makes it plausible that they would also likely show changes to vWF. However, beyond these types of phylogenetic correlations, evidence for resistance against venom challenges is not available and physiological data would be required to verify that resistance to C-type lectin-like proteins is sufficient to allow for evasion from predation by venomous snakes.

Correlational Evidence for Resistance /Toxicity Coevolution in Venomous Snakes

The extent of information regarding resistance to snake venoms varies depending on the species group of interest and may include as little as an initial confirmation of resistance to a full description of the resistance mechanism. In relatively few cases, functional information can be paired with evolutionary analyses to test the underlying assumptions of a chemical arms race. Barlow et al. (2009) investigated a potential coevolutionary relationship between venom specificity toward scorpion prey in four species groups of the genus *Echis* (Saw-scaled Vipers). They used a Bayesian Inference method to plot a phylogeny of these four groups and compared the relative amounts of scorpion versus rodent prey found in the stomach contents of museum specimens, as well as toxicity assays (LD_{50}) toward scorpions (*Scorpio maurus*), to species relationships. Venoms of species groups with the highest amounts of scorpions in their diet were the most toxic against scorpion prey, while the *E. coloratus* group, rodent specialists, showed the lowest toxicity. Relative abundance of a particular type of prey scaled with the relative toxicity of the venom; for example, the *E. ocellatus* group had an intermediate

amount of dietary scorpions and showed an intermediate toxicity toward live scorpion prey. The implication of this increased toxicity toward preferred prey group was that *Echis* venom has undergone selection favoring increased toxicity toward a preferred prey type. While Barlow et al. (2009) did not test for scorpion resistance, the demonstration of prey specificity that follows the best resolution of *Echis* phylogenetic relationships indicated a positive selective pressure for enhanced toxicity, perhaps driven by prior prey resistance mechanisms. For example, a common ancestor to *Echis* may have retained toxicity toward scorpions while sympatric Rodentia developed resistance, to the point that only *Echis* phenotypes that could shift to non-rodent prey were able to persist. Secondary diversification of the venom toxins may have restored high toxicity toward rodent prey, favoring a shift in those lineages to specializing on rodents. The availability of non-scorpion taxa, preference towards these taxa (how often they attempt to prey on them), and the relative resistance or susceptibility of these taxa would be needed to corroborate reciprocal selectivity of venom and resistance.

In the case of opossums, anti-hemorrhagic toxicity has been correlated with phylogenetic comparisons of predator and prey species. Voss and Jansa (2012) compared South American opossums and vipers, revealing that species of opossums that were too large as adults to be ingested by vipers showed no resistance to venom. Non-resistance in larger prey taxa was interpreted as the result of non-predation; that venomous snakes had no behavioral inclination to attempt to prey on these overly large meals and thus no selective pressure to develop resistance was present. Verifying the assumption of reciprocity between predator and prey, resistance may arise or be maintained only in prey lineages that are likely targets of venomous snake predators.

Explanations of a Limited Literature on Natural Resistance

Assuming that random mutations that allow prey to tolerate snake venoms have appeared, prey resistance should persist because of the presence of a predator, yet available information is limited. Reaffirming the likelihood that predation pressures, particularly the trophic adaptation of venom, should affect the stability of resistance in prey, several explanations for a lack of information on resistance emerge. First, a dearth of reported resistance may result from variable and insufficient research effort: the simplest explanation would be that little effort has been made to screen candidate resistant prey. Even in the most well-described resistance systems, resistance to venomous snakes, mammalian resistance dominates the literature, despite abundant natural history accounts of venomous snakes consuming non-mammalian prey (Mackessy and Saviola 2016). Second, while some effort may have been made to investigate predator/prey interactions, the documentation of local specificity in some of the prey resistance systems discussed suggests that analyses may not detect resistance because of mismatches between the localities of predator and prey that are tested. The maintenance of resistance in a population of prey species may be dependent on the selective force of a particular venom profile that in turn is delimited by the overlapping ranges of local populations of predator and prey. Thus, assaying for resistance using a venom from outside of assayed individuals' local area may lead to the false conclusion that resistance is not present in a prey species or population. Third, beyond mismatching of predator/prey populations, small sample sizes also may allow resistant prey to be overlooked. Under a Red Queen dynamic, the frequency of resistance is expected to cycle through periodic minima. Low-frequency resistance phenotypes would be increasingly

harder to detect by random sampling. Lastly, while it is likely that resistance is more common than a limited literature indicates, it is also likely that resistance is not present at all, and the dearth of information accurately reflects this biological reality. All in all, future investigations in these least described predator/prey systems and continuing investigations in known resistance systems must consider that limitations in research design and effort may not capture the evolutionary processes driving reciprocal flux between resistance and toxicity.

Another explanation for limited information on prey resistance is the possibility that these predators do not exert enough predation pressure to cause selection for prey resistance. Simply, prey resistance may not ever appear in a significant portion of a prey population, despite the logic of coevolution under a chemical arms race hypothesis, because venomous species are not a strong enough selective force to maintain random mutations that confer resistance in prey. If predators move from specialist to more generalist diets over time, selection of novel toxicities may be favored and therefore reciprocal resistance may not appear. Initial development of toxicity against a limited number of prey species may allow predators to capitalize later on a wider range of related prey species with similar physiologies. With a wider prey base, predators would be able to take advantage of other food sources in the event that resistance does appear in some prey individuals. Therefore, if selective pressure from venomous predators is negligible, and the appearance of resistance alleles in a population only happens as a result of random mutation, the fixation of prey resistance in the population is unlikely, because these rare resistance alleles risk early extinction due to their low abundance. Finally, over time, overcoming the toxic action of venom by prey may prove insurmountable, and

our present-day analysis would detect venom toxicity to a variety of locally-available prey, but no or extremely small numbers of resistance mechanisms in prey. Alternatively, predation by venomous snakes may not be sufficient to maintain resistance mechanisms that have arisen in prey populations. Van Valen's (1973) theoretical framework expects stable reciprocity between predator and prey such that the dominant defensive or offensive strategy of one partner acts to stabilize the response of the other partner until such a time that a defensive/offensive innovation is able to shift the balance to a new equilibrium. If the partners become dissociated, it is possible that prey resistance may be lost over time without the stabilizing influence of predator toxicity.

The present discussion only considers chemical resistance to predators' venoms, but other strategies may evolve in response to the selective pressure of venom toxicity. Behavioral modifications that allow further generations of prey to persist in an area, may subvert the predation pressures of venomous animals and bypass chemically-based coevolutionary processes. For example, in one of the better described toxicity/resistance dyads (between Pacific Rattlesnakes and Ground Squirrels), several behaviors that prevent predation are documented. Certain populations of squirrels are known to tail-flag to signal their awareness of a nearby predator, resulting in the retreat of the approaching rattlesnake (Putman and Clark, 2014); others bombard approaching rattlesnakes with substrate to motivate predator retreat (Goldthwaite et al., 1990), and some rub themselves against shed skins of local rattlesnakes to mask individual scent and evade chemosensory detection (Clucas et al., 2008). While these populations may also have chemical defenses against predator venoms, behavioral modifications that disrupt predatory episodes exist as well, demonstrating that other prey species may not require physiological resistance

mechanisms if behavioral modifications are sufficient to elude detection and/or envenomation.

Future Directions in the Study of Toxin Resistance

The diversity and efficacy of prey resistance appears to be shaped by the selective pressure of predator toxicity as predicted by chemical arms race hypotheses. However, the fact that only a handful of well-described resistance systems exist in the literature demonstrates the need for further investigations into the diversity and extent of prey resistance. Future directions in the study of natural resistance to venoms must include screens for resistant prey species, using *in vitro* or *in vivo* assays to identify the capacity of prey species to avoid the normally pathological consequences of envenomation. Development of a well-supported alternative to LD_{50} determinations is crucial to reduce the number of native prey animals needed to demonstrate resistance and increase throughput, but at present there is no sufficient model to replace whole animal toxicity tests, particularly for unknown systems. Special attention should be paid to the interaction of local populations of predators and prey versus the effects of predator venoms on non-local populations of (possible) prey (e.g. Smiley-Walters et al., 2018). Further, the prevalence of resistance mechanisms that appear specific to local predators indicates that the development and propagation of resistance genotypes could be modeled to predict or detect the appearance of new resistance mechanisms or to track the spread of resistance mechanisms through prey populations across large landscapes that connect multiple populations. The detection of local resistance also may indicate that current information about the relative abundance of resistance in a given prey species is underestimated; multiple pairwise comparisons between local predator and prey

populations would be required across a significant portion of their sympatric range to document resistance or susceptibility unequivocally. Understanding that evolutionary processes are adequate but not necessarily ideal, reciprocal step-wise modifications to either toxicity or resistance mechanisms are expected to be the norm in coevolutionary systems, rather than wholesale changes to composition. The recent use of genome/transcriptome/proteome comparisons (i.e., Cardoso et al., 2010; Gibbs et al., 2009) could shed light on underlying trends in molecular evolution: how often do resistance genotypes change, how often do novel genotypes appear, and what resistance mechanisms are likely to experience the strongest selection?

Beyond research opportunities focusing on the evolutionary history and development of prey resistance, a better understanding of resistance mechanisms may provide a source for future biomedical innovation. Currently, clinical treatment, both medical and veterinary, of envenomation by venomous species commonly relies on the use of antivenom therapeutics and complementary treatment regimens to combat systemic pathologies such as hypofibrinogenemia, thrombocytopenia, myotoxicity, neurotoxicity and many other symptoms (Chippaux and Goyffon, 1998; Diaz, 2004; Rhoads, 2007). The incidence of envenomation by spiders, scorpions, and snakes are of particular concern considering their common occurrence, dramatic impacts to global health, and significant financial impacts to health systems. In an attempt to improve treatment, the World Health Organization ([WHO], 2007) deemed envenomation by snakes and scorpions to be a neglected public health issue and has suggested strategies to develop better antivenom therapeutics. While improvement of existing antivenom therapeutics promises to increase the efficacy of envenomation treatment, the addition of

venom resistance molecules to treatment protocols may further improve clinical outcomes. Resistance molecule therapeutics are not intended to replace antivenom therapies but instead work synergistically with existing treatment protocols to combat venom toxicities. As proof of concept, two classes of anti-snake venom compounds derived from resistant prey species have been cited as promising candidates for drug discovery. Thwin et al. (2010) provide a summative review of a number of these molecules, including a group of phospholipase A_2 inhibitors (PLIs) derived from venomous snake blood sera (Viperidae, Elapidae). The biological roles of these molecules is to prevent complications from auto-envenomation or envenomation by other sympatric (intra- and interspecific) venomous snakes. Hypothetically, clinicians could administer the appropriate antivenom to combat broad spectrum effects of envenomation and additionally employ a derived PLI in cases where patients present with envenomations from snakes with PLA_2-rich venoms. Treatment schedules that incorporate such molecules could be better tailored to individual patient needs to improve the efficacy of medical intervention and patient health outcomes.

In addition to PLIs, another promising class of resistance molecules for drug development are snake venom metalloprotease inhibitors (SVMPIs). As mentioned earlier, SVMPIs have been isolated from a wide range of mammalian prey species of snake predators. Especially in the Americas, SVMPIs promise an excellent addition to combat the hematologic pathologies experienced in a large number of snakebite envenomations (owing to a higher proportion of venomous taxa with snake venom metalloprotease-rich venoms; Mackessy, 2010a). Metalloproteases have been described as ‘gateway toxins’ (Biardi et al., 2011) because they break down structural elements

within tissues, potentially increasing the rate that other toxic components of the venom may infiltrate and access the bloodstream. Biardi et al. (2011) postulated that the therapeutic use of an SVMPI would limit access of venom components by destroying the ability of the venom to spread from the envenomation site. The biochemical functions of metalloproteases (hemorrhage, tissue destruction) would be blocked, and spread of venom would be attenuated. The hope is that this temporary neutralization of one part of the venom and subsequent sequestration of other toxins would allow antivenom therapeutics time to propagate to and neutralize the locally envenomated tissue. In short, resistance molecules such as PLIs and SVMPIs are expected to shorten treatment regimens by increasing immediate efficacy of antivenom therapeutics.

In conclusion, understanding of the prevalence and mechanisms of prey resistance to natural toxins remains limited to a small number of predator/prey systems. However, the prediction that prey species in tightly coupled predator/prey relationships may develop reciprocal chemical armaments against predator toxins motivates a continued effort to discover and describe resistance. Future studies should focus on assessing not only the mechanistic nature of resistance, but also the demography of resistance in natural populations of prey. Dedication to interdisciplinary approaches that couple molecular and ecological information will exponentially increase what is understood of the interactions between venomous predators and their resistant prey.

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CHAPTER II

ASSESSING THE BIOCHEMICAL AND PROTEOMIC INTEGRITY OF *CROTALUS OREGANUS* SSP. VENOM SAMPLES STORED FOR ~35 YEARS

Introduction

The natural activities of snake venom toxins have been exploited for their potential as medical therapeutics and continue to represent a wealthy repository of future therapeutic development (e.g., Perumal Samy et al., 2017; Vyas et al., 2010; Waheed et al., 2017). Snake venoms are also excellent model systems for exploring molecular evolution (Brust et al., 2013; Casewell et al., 2011; Doley et al., 2009; Reyes-Velasco et al., 2015), predator-prey coevolution (Holding et al., 2016; Jansa and Voss, 2011; McCabe and Mackessy, 2016), and comparative toxicology (Chippaux, 2017; Tasoulis and Ibister, 2017). Unfortunately, access to particular species of venomous snakes and their venoms may be restricted as the ability to capture and extract these animals is difficult due to limited funding, concern for the conservation of populations or species, and geopolitical boundaries. Stored venom samples have therefore become increasingly important as both a means of accessing now inaccessible snakes and they may act as possible representatives of historic populations. The use of stored samples leads to questions of whether the samples are viable as historical representatives: is the proteomic identity of the constituent toxins discernable? Is the activity/toxicity of the venom retained? Can the sample(s) be compared against freshly collected venom samples?

There appears to be a pervasive assumption among venom researchers that the quality of stored venom samples is retained stably through time and that comparisons between long-term stored and freshly obtained venom samples are valid. In the present study we reproduced original assay methodologies to compare the activity of snake venom samples as assayed originally and after ~35 years of storage.

Snake venoms appear to be intrinsically stable as biological samples because of their chemical composition. Snake venoms are complex mixtures of toxic components, the majority of which are proteins and peptides (Mackessy 2010b). Of the many venom protein families that are described, many contain toxins that are proteolytic (Calvete, 2013; Tasoulis and Ibister, 2017). However, at least three groups of venomous snakes (Families Elapidae, Viperidae, and Atractaspididae) are able to retain these bioactive substances in the lumen of specialized glands without suffering pathological consequences of the venom (Gans and Kochva, 1965; Mackessy and Baxter, 2006). A low pH in the lumen of the venom gland appears to maintain venom proteins in a quiescent state until they are deployed into the body of an envenomated target (Mackessy and Baxter, 2006). Organic acids, such as citrate, likely act as buffers that help to maintain venom protein integrity at slightly acid pH (Mackessy and Baxter, 2006) and may participate in other biochemical inhibitory interactions (Odell et al., 1998). Other molecules found in the venom mélange, such as small tripeptides, also serve to prevent venom enzymes from functioning while stored in the venom gland lumina (Grams et al., 1996; Munekiyo and Mackessy, 2005). Once secreted from serous tissues in the venom gland, most venom components are expected to remain relatively unchanged (Mackessy and Baxter, 2006). Recent research reveals that some molecular rearrangement may be

seen, as some zymogen toxins may require further posttranslational modification after being excreted into the lumen of the venom gland (Portes-Junior et al., 2014), so some enzymes may remain active even under the storage conditions of the venom gland itself.

Intrinsic stability can be maintained through standard laboratory handling practices to ensure bioactivity and toxin identity is preserved until the samples can be analyzed. In the laboratory, snake venom samples are most often stored in a dried form, commonly lyophilized. The general concern with any highly proteinaceous sample is the degradation of structure and activities in oxygen-rich and aqueous environments. Loss of higher order structure, especially tertiary structure, can occur through events such as the spontaneous racemization of side chain groups in amino acids, deamination events in the presence of certain sugars, or rearrangement of structurally important covalent linkages in the protein (Adelman, 1985; Ahern and Klibanov, 1988; Harding, 1985; Seis, 1986). To avoid this, desiccation and lyophilization (freeze-drying) are most frequently used to prepare snake venoms, followed by storage in low humidity and low temperature conditions (Chippaux, 1991).

Researchers have conducted studies of stored venom samples throughout the past century with mixed consensus on the stability of samples. Schottler (1951) tested the lethal doses of desiccated venoms (five genera from the families Elapidae and Viperidae) following 9-13 years of storage, finding that some venoms were relatively unchanged after storage, whereas others had become significantly less toxic. Schottler (1951) noted that decreases in activity were greatest in venoms with the highest hemorrhagic activity. Hemorrhage caused by snake venoms is often mediated by enzymatic toxins, such as snake venom metalloproteases, thus the decrease in toxicity of these venoms may be

explained by the loss of integrity of structural elements required for toxic enzyme action. Russell (1960) compared LD_{50} toxicity values for venoms from five rattlesnake species (Family Viperidae) stored 26 years and detected little to no differences in the toxicity. In both studies, researchers compared lethal dose values they collected against values collected in the past on the same sets of samples. Jesupret et al. (2014) used a battery of proteomic techniques, a coagulopathy assay, a neurotoxicity assay, and a snake venom detection kit to compare 52 samples of stored venoms against samples collected in the present from the same species (five genera from the family Elapidae) in approximately the same geographic locations. Most of the stored samples were collected approximately 50 years before the study, and one was collected 80 years before the study. If the stored samples were not exposed to air, there were negligible differences between the proteomes of stored and 'fresh' venom samples. Unlike the two aforementioned studies, Jesupret et al. (2014) compared the activities of stored samples against activities of samples from animals caught in similar but not exact locations. Thus, it is possible that observed differences are related to variation in venom composition between geographic locations (e.g., Borja et al., 2018; Shashidharamurthy et al., 2002) or to changes in venom composition in these populations over time. In all three studies, proper maintenance of storage conditions appeared to determine the quality of venom samples following storage periods; venom activity and identity are stable, given appropriate handling by researchers.

Beyond analyses of stored venom samples, two groups of researchers have tested experimentally the stability of venom samples under varying preservation and storage conditions. Egen and Russell (1984) tested the stability of the electrophoretic profile of a single sample of venom from a Black Tailed Rattlesnake (*Crotalus molossus*) following a

variety of freezing, desiccation, and lyophilization methods. Qualitatively, the authors saw few differences between handling methods; any minor differences between treatments they ascribed to variation in the assay procedure. They did speculate that there could have been quantitative differences in protein quality or pharmacological differences following handling, but did not test for these changes in sample quality. Munekiyo and Mackessy (1998) also tested for changes in protein and enzymatic quality of a single sample of venom from *C. molossus* following exposure to a variety of temperature and storage conditions and subsequent lyophilization. Following reconstitution, samples were analyzed by comparing electrophoretic profiles, enzyme activities, and toxicity in a cricket model. While some quantitative variation in enzyme activities was detected, electrophoretic, toxicity, and quantitative enzyme activity results were similar, regardless of treatment conditions. In both studies, crude venom appears to be able to withstand a wide variety of handling and storage conditions without compromising the proteomic identities of venom components, their activity, or their toxicity. In combination with investigations of snake venom samples stored long-term, snake venom appears resilient and viable for investigation, given adherence to standard procedures for preserving, storing, and handling proteinaceous samples.

Certainly, there appears to be strong external validity for the use of long-term stored snake venom samples in the present dissertation, but we first sought to validate internally the stability of these samples before making comparisons between historical samples and those collected in recent years. This chapter queries whether samples stored long-term maintain the integrity of their proteomic identity and biochemical activity. Validating the quality of the samples by comparing them to venom samples extracted

from snakes captured in the present day would confound the results of a comparative analysis. If differences are detected between time points, are they caused by true changes in venom characteristics or are they artefacts of changes in long-term sample quality? Alternatively, if no differences are observed between time points, are these results indicative of a stable venom phenotype through time or the result of previously more active samples losing toxic activity in long-term storage so that they appear similar to samples collected more recently? There do not appear to be any published attempts to validate whether the stability of or changes in venom composition of snake populations result from sample storage and handling or result from true biological differences over time. Here, we compared data collected approximately 35 years prior to data collected on the same samples in the present using the same assay procedures to validate that sample quality has not significantly changed in the past four decades.

This study served as our attempt to provide the strongest evidence for sample integrity of long-term stored samples before attempting to make a comparison between historic and recently collected samples. The samples were reconstituted and assayed for two enzyme activities for which assay data, collected around the time of original venom extraction, was available. Assay data collected in the past and in the present were compared to determine whether ~35 years in storage caused degradation in enzyme activity. Original data from ~35 years prior was only available for two enzyme activities, L-amino acid oxidase (LAAO) and phosphodiesterase. While assay protocols are available to assess other enzyme toxins in snake venoms, we did not have original data for these activities in long-term stored samples or access to the appropriate reagents for the assay protocols to make a valid assessment of sample quality following ~35 years of

storage. Considering that several studies mentioned above have indicated no change in venom samples over time, we hypothesized that there would be no difference in activity data following the storage period.

In addition to comparisons between data collected in both the past and present, two qualitative biochemical procedures, reverse phase high performance liquid chromatography and gel electrophoresis under reducing conditions, were used to assess whether degradation was detectable in samples stored ~35 years. These two procedures allowed us to compare changes in biochemical activity with changes in the integrity of the venom proteins themselves. On the one hand, changes in biochemical activity may come from perturbations of enzyme substructure, such as conformational shifts in active site architecture, that leave the protein largely intact and apparently unchanged when analyzed using these two qualitative methods. On the other hand, degradation may completely dismantle the ultrastructure of venom proteins, eliminating or changing their appearance in these qualitative methods. Confirming whether the quality of proteome features, such as the relative number and amount of venom toxins, is stable following long-term storage acts as an alternative to comparing biochemical activity as a means of assessing venom composition change. In the case that the biochemical activity of long-term stored samples was an unreliable source of compositional information, the remaining option was to assess whether proteomic procedures could offer some means of tracking changes within venomous snake populations over time.

Materials and Methods

Sample Preparation and Storage

Dr. Stephen Mackessy (SPM) originally collected a majority of samples discussed in this chapter. Over 400 adult Northern and Southern Pacific Rattlesnakes (*Crotalus oreganus oreganus* and *Crotalus oreganus helleri*) were collected on foot and by nighttime driving in San Luis Obispo, Santa Barbara, Kern, and Ventura Counties, California, between 1978 and 1982. Snake venom was extracted by manual expression and then lyophilized before being stored in glass vials with cork stoppers. Vials were stored at -20 °C in airtight plastic containers with Drierite desiccant. Barring transport between university campuses and emergent freezer malfunctions, these samples have been maintained in a low humidity, low temperature environment to the present. An additional six samples included in the SDS-PAGE analysis were collected from individual adult *C. o. oreganus* captured in Carrizo Plains National Monument. The animals were captured on foot and by nighttime driving during the summer of 2015. Snake venom was extracted by manual expression and lyophilized before storage in screw-capped plastic tubes at -20 °C.

Long-Term Stored Sample Selection

Samples were selected for each of the four analyses (two enzyme activity assays, and two proteomic procedures) based on the availability of original enzyme activity assay and quantity of venom remaining in the stored tube. Out of 498 original samples available, 19 and15 samples had original data and adequate amounts of sample for the L-amino acid oxidase (LAAO) and phosphodiesterase (PDE) enzyme assays, respectively. These 34 samples represented 29 unique individuals, as some individuals had recorded

values for both enzyme assays or were extracted sequentially while in captivity. For the qualitative proteomic analyses, seven samples out of 498 were selected for gel electrophoresis and six were used in liquid chromatography. To provide a preliminary comparison between long-term stored samples and recently extracted samples from snakes caught in the same geographic area, five additional samples collected in recent years were used in the gel electrophoresis procedure.

L-Amino Acid Oxidase Assay

The original L-amino acid oxidase assay and the assay conducted in the present day utilized L-kyurenine (Weissbach et al., 1960; modified by Mackessy, 1988) and activity was expressed as nmol product/min/mg venom protein. The reaction mixture for the assay included 875 μL of 0.1 M EPPS buffer (pH 8.0), 100 μL of 1.041 μg/μL L-kyurenine, and, 50.0 μg of crude snake venom at 2.0 μg/μL. The reaction mixture was incubated at 37 °C for 30.0 minutes before the reaction was stopped using shock cooling in an ice bath and 1.0 mL of a 10% trichloroacetic acid (TCA) solution. The reaction mixture was allowed to return to room temperature before immediate measuring absorbance at 331 nm.

Phosphodiesterase Activity

Both original and present-day phosphodiesterase (PDE) assays were conducted using bis(p-nitrophenyl) phosphate and expressing the activity as ΔA_{400nm}/min/mg venom protein (Mackessy, 1988). The reaction mixture for the assay included 850 μL 0.1 M CHES buffer (pH 9.0), 600 μL 0.001 M bis(p-nitrophenyl) phosphate, and 50.0 μL of crude snake venom at 2.0 μg/μL. The reaction mixture was incubated at 37 °C for 30.0 minutes before being stopped with 1.5 mL 0.05 M NaOH and shock cooling in an ice

bath. The reaction mixture was allowed to return to room temperature before immediate measurement of the absorbance of reaction mixture at 400 nm.

SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

No original gel eltrophoresis data was available for any long-term stored samples. Seven samples of long-term stored and five samples of recently extracted crude venom from snakes captured in Carrizo Plain National Monument were analyzed for the relative abundance of particular venom components using SDS-PAGE under reducing (DTT) conditions on NuPage 12% Bis-Tris gels and MES running buffer as reported previously (Smith and Mackessy, 2016). Twenty μg of crude venom solubilized in double deionized water were electrophoresed for approximately 45 minutes. Mark 12 standards were used as a scale to calculate the approximate mass of each band. Gels were stained with Coomassie Brilliant Blue for a minimum of four hours, destained, and scanned using an HP Scanjet 4570.

Reverse Phase High Performance Liquid Chromatography (HPLC)

No original chromatography data was available for any long-term stored samples. However, to investigate whether storage had affected sample quality, six samples of long-term stored venom were assessed using chromatography. Approximately 1.2 mg of crude venom was dissolved in double deionized water (ddH_2O) and then centrifuged for 10 minutes at 14,000 rpm to remove insoluble debris. The sample was then passed through a 0.45 μm Acrodisc® filter (Gelman). Protein (1 mg) in 200 μL ddH_2O was loaded onto a Phenomenex Jupiter C18 column (250 x 4.6 mm, 5 μm) on a Waters HPLC system using Empower software. The column was equilibrated with 95% of a 0.1%

trifluoroacetic acid (TFA) in water (solvent A) and 5% of 0.1% TFA in 80% acetonitrile (solution B). Elution was performed by a linear gradient to 90% solvent A and 10% solvent B over 10 minutes; to 25% B over 10 minutes; to 32% B over 21 minutes; to 41% B over 1 minute; to 45% B over 10 minutes; to 70% B over 10 minutes; to 90% B over 2 minutes; to 95% B over 3 minutes; to 95% A and 5% B over 2 minutes; and isocratic at 95% A 5% B for 6 minutes. A Waters Fraction Collector II collected fractions at a flow rate of 1.0 mL/min with protein peaks monitored with a Waters 2487 Dual Absorbance Detector at 220 and 280 nm.

Statistical Analysis

For the LAAO and PDE enzyme assays, all samples and a control using ddH_20 were assayed in duplicate or triplicate. The average absorbance of the control replicates was subtracted from each sample replicate before averaging the sample absorbances. A specific activity value was calculated from these averages and used for statistical analysis. Paired, two-tailed Student's t-test (α=0.05) were used to detect differences in LAAO or PDE enzyme activity data collected ~35 years prior and data collected in the present from long-term stored samples.

Results

There was a significant difference from the original measurements to the present day for both PDE ($t(18) = 2.29$; $p = 0.03$; $n = 15$ (Figure 2.1)) and LAAO activities ($t(14) = 2.63$; $p = 0.02$; $n = 19$) (Figure 2.2)). PDE activity decreased from the original activity ($M = 0.24$ ΔAbs_{400nm}/min/mg venom protein; $SD = 0.17$) to ~35 years later ($M = 0.13$ ΔAbs_{400nm}/min/mg venom protein; $SD = 0.096$). LAAO activity also decreased from the

original activity (M = 127.99; SD = 48.96) ~35 years later (M = 98.64 nmol product/min/mg venom protein; SD = 24.29).

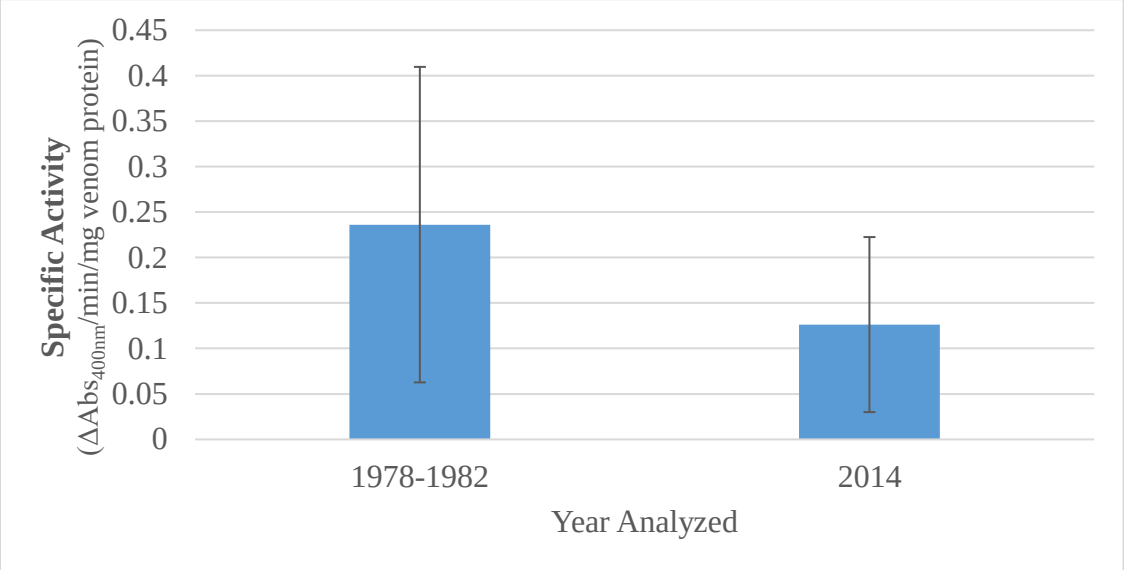

Figure 2.1. Phosphodiesterase activity of crude venom samples following ~35 years in storage. Average specific activity (ΔAbs_{400nm}/min/mg venom protein ± standard deviation (SD)) of 19 samples of *Crotalus oreganus helleri* venom stored lyophilized at -20 °C is shown.

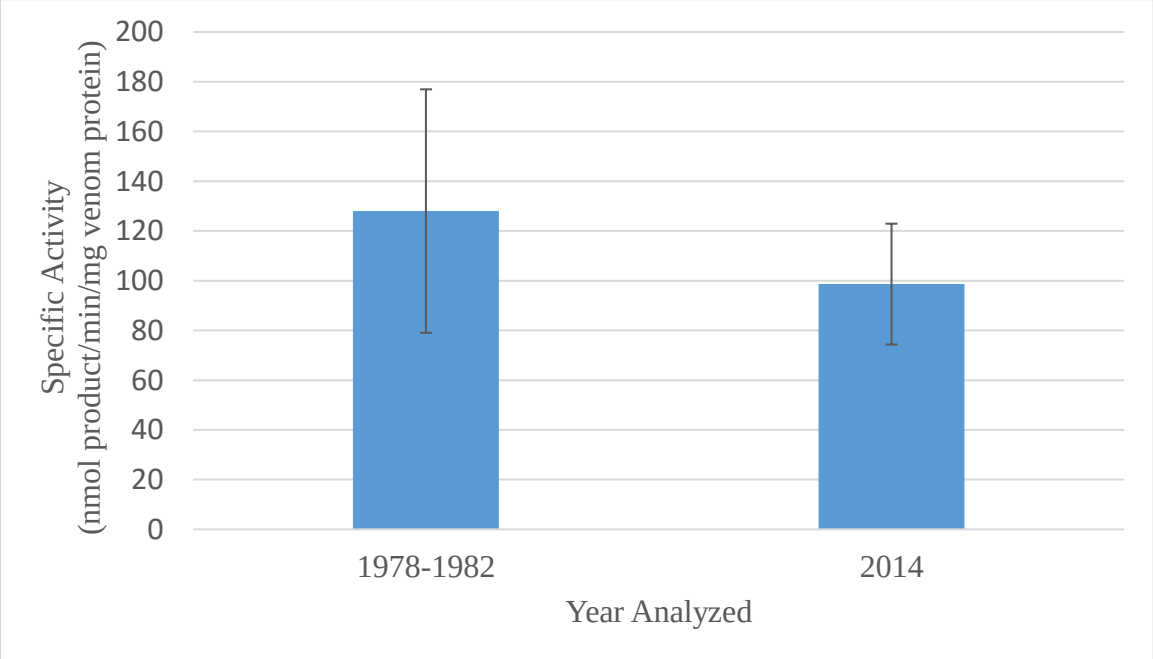

Figure 2.2. L-amino acid oxidase activity of crude venom samples following ~35 years in storage. Average specific activity nmol product/min/mg venom protein ± standard deviation (SD) from 15 samples of *Crotalus oreganus helleri* venom stored lyophilized at -20 °C.

No major shifts in the banding pattern, abundance of bands, or density of bands are observed in the SDS-PAGE of long-term stored and recently extracted samples of *C. o. oreganus* venom from Carrizo Plain National Monument (Figure 2.3). Minor smearing in some of the lanes is apparent but is found in both sample groups. There do not appear to be an overabundance of small peptides as might be expected if proteolytic degradation affected the long-term samples. Variability in the relative abundance of proteins is apparent in some mass ranges, such as those around 31 kDa and 6 kDa, but others, such as bands around 50 kDa, appear consistently abundant across the sample group.

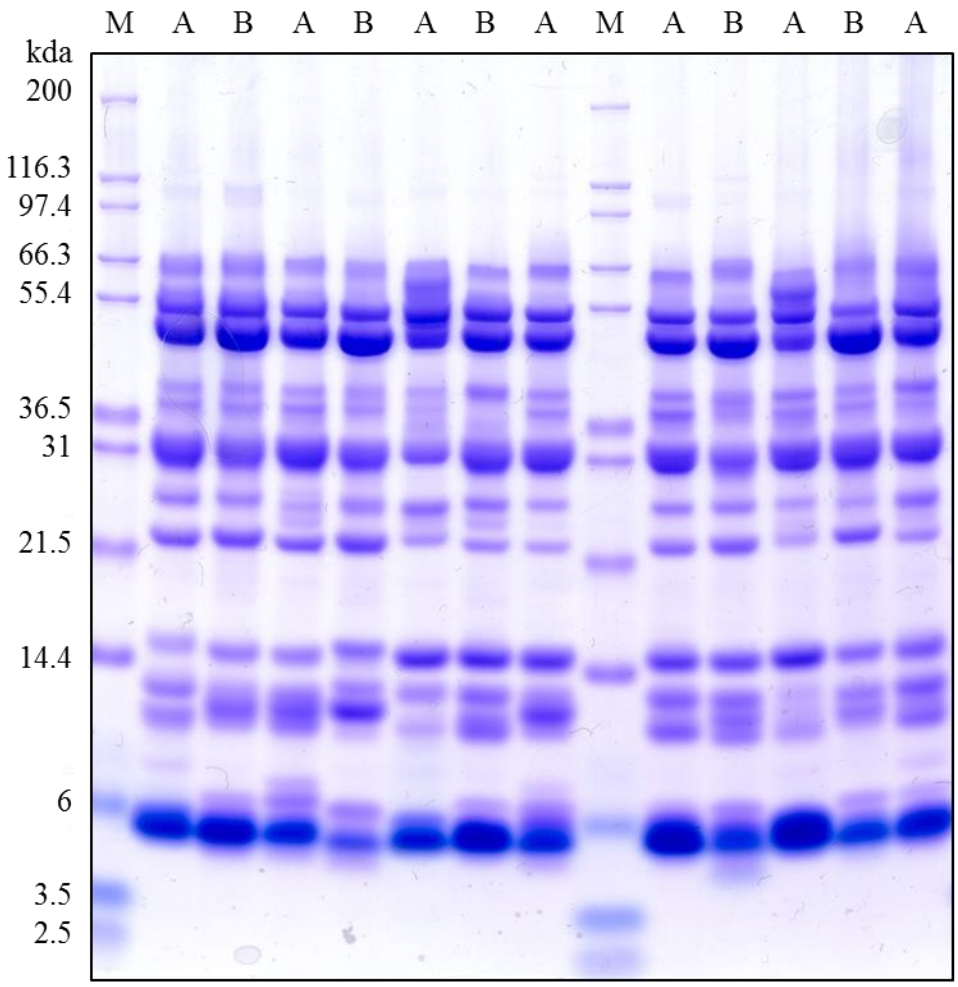

Figure 2.3. Polyacrylamide gel electrophoresis in reducing conditions. SDS - PAGE of 12 samples of *Crotalus oreganus oreganus* venom. Lane key: M, Mark 12™ molecular weight standards; A, samples collected from Carrizo Plains National Monument from 1978-1982 and held in long-term storage; B, samples collected from Carrizo Plains National Monument in the summer of 2015. No major differences in the number of bands or the relative abundance of protein is observed between recently extracted and long-term stored samples.

Reversed phase HPLC for six long-term stored samples showed similar patterns of protein elution (Figures 2.4, 2.5). All six share a similar appearance starting with a thin doublet eluting around 22.0 minutes, a rounded group of peaks around 30 minutes, and a complex group of peaks eluting around 55 minutes. Some exceptionally large peaks late in the run appear in two samples (Figure 2.5 top and bottom); however, the remaining peaks are equivalent in size to the other four samples. Qualitatively, there do

not appear to be markers of degradation, no aberrant peaks appear in any sample and there are no lateral shifts in peaks indicating the loss of a significant portion of the peptide.

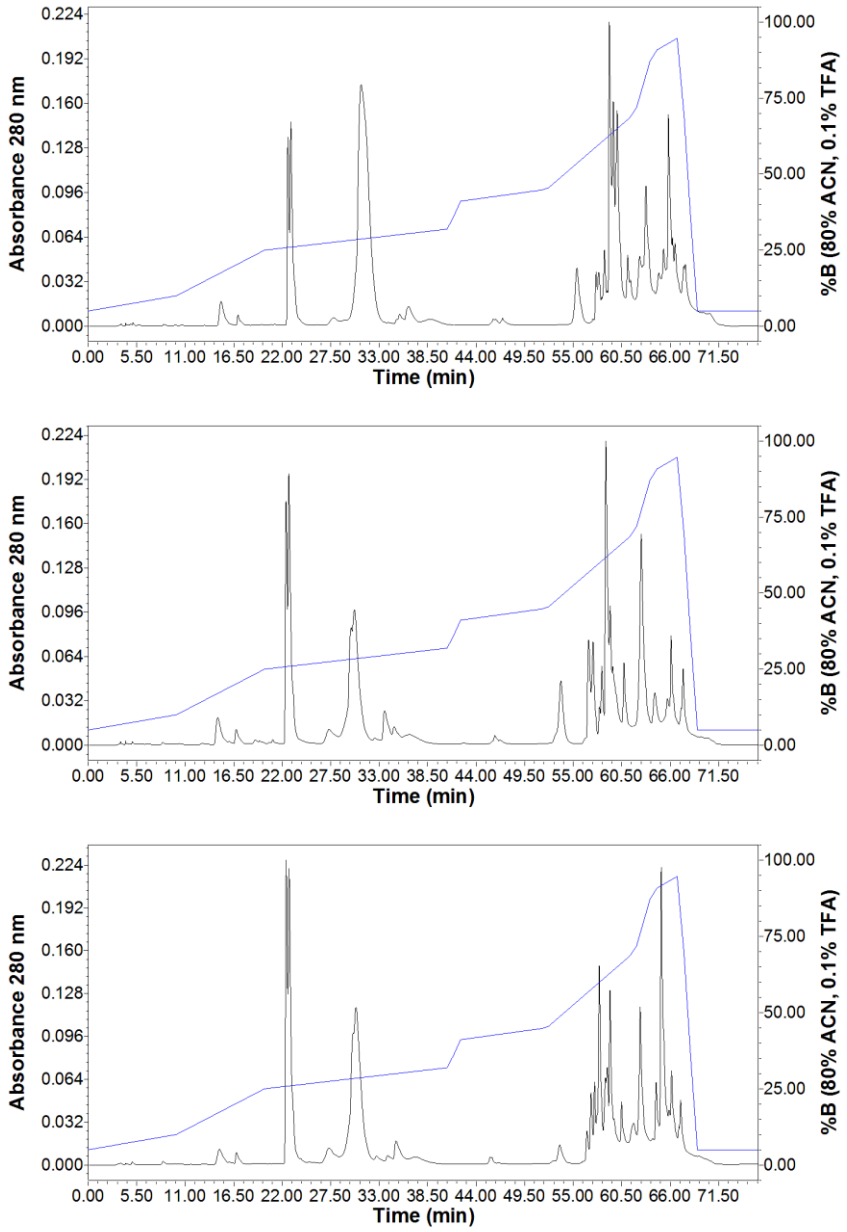

Figure 2.4. Chromatograms of reversed phase HPLC for three of six samples of *Crotalus oreganus oreganus* venom collected between 1978-1982. The number, size and shape of eluted peaks is similar across these three and among the total group of six samples (Figure 2.5).

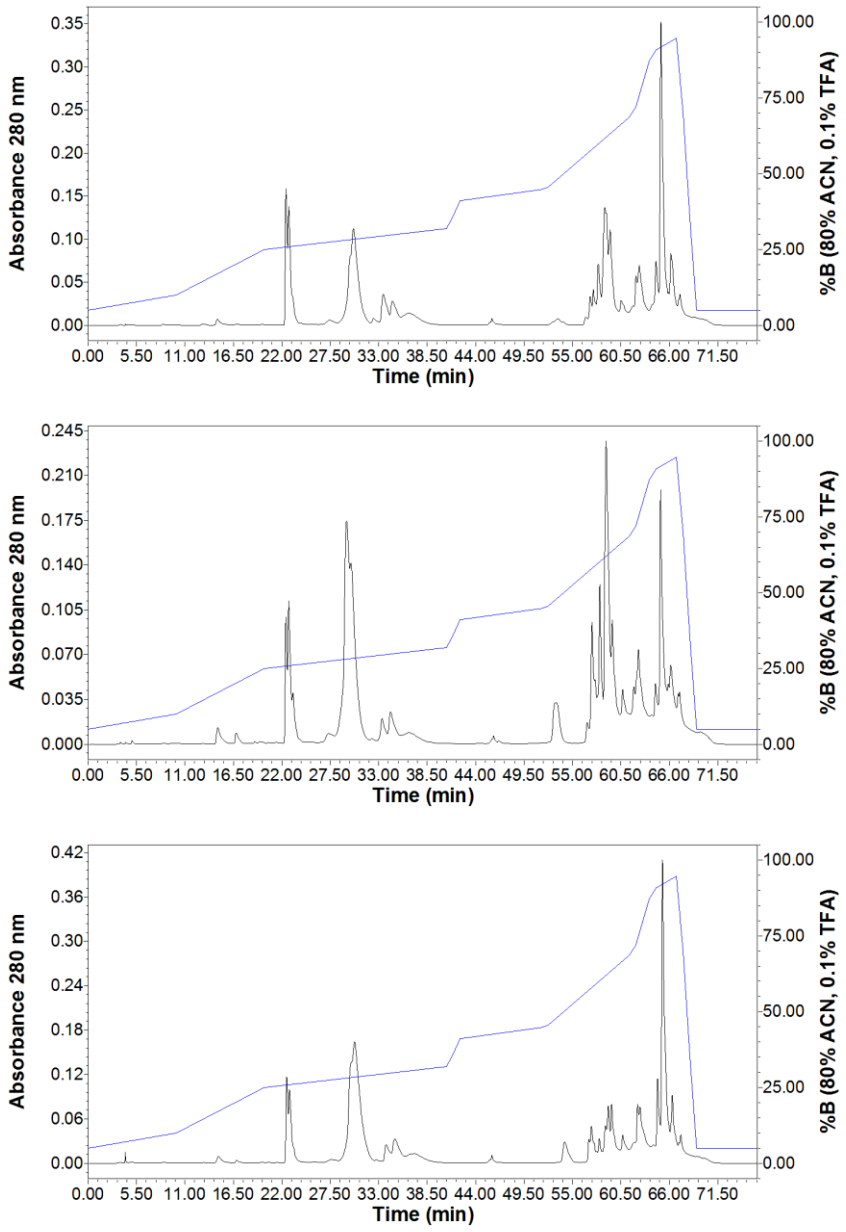

Figure 2.5 Chromatograms of reversed phase HPLC for three of six samples of *Crotalus oreganus oreganus* venom collected between 1978-1982. All three have similar elution patterns and within the total group of six samples (Figure 2.4). Particularly large peaks containing metalloproteases eluted at the end of the run in two samples (A, C); however, there are not obvious signs of degradation that may explain the appearance of these peaks. Apparent abundance of SVMPs was concordant with high SVMP enzyme activity.

Discussion

Biochemical activity assays and qualitative SDS-PAGE and RP-HPLC analyses yielded mixed results when testing for degradation in snake venom samples stored for ~35 years. Phosphodiesterase (PDE) (Figure 2.1) and L-amino acid oxidase (LAAO) (Figure 2.2) activities were significantly lower following ~35 years of storage. We reject our original hypothesis that these venom activities would remain stable following long-term storage. Interestingly, gel electrophoresis of long-term stored samples run side-by-side with samples from snakes captured in near identical locations produced similar banding patterns. The number, sizes, and intensity of protein bands was similar across all samples; long-term stored samples could not be distinguished from samples obtained in the present-day (Figure 2.3). HPLC data also indicated that the structural elements of venom composition remained stable, despite reductions in the activities of two venom enzymes tested.

As *C. oreganus ssp.* venom is known to have a large portion of toxins with proteolytic activity (Mackessy, 1988; Mackessy, 2008; Sunagar et al., 2014) one explanation for a loss of activity could be degradative action by other enzyme toxins in the venom or else some process of autolysis. However, the absence of band shifts or the presence of extra low molecular weight bands in gels or chromatograms would indicate levels of proteolytic degradation that are not observable by these methodologies (Figure 2.3). LAAOs are homodimers with masses in the 110-150 kDa range (Aird, 2002; Du and Clemetson, 2002; Costa et al., 2014; Mackessy, 1998; Mackessy, 2010b; Tan and Fung, 2010) and PDEs are also homodimers, with similar native masses of 120-140 kDa (Dhananjaya and D'Souza, 2010; Mackessy, 2010b; Tan, 1998). In this range across the

gel lanes, there are few, faint bands that fit this size criteria (Figure 2.3) so it may be that the prevalence of these toxins is low, so that a small discrepancy in the recorded activity value is exaggerated in the analysis. It would have been ideal to test other, more prevalent venom components; however, original assay data was limited to LAAO and PDE. While we attempted to replicate the protocol exactly, differences in the manufacturing quality of reagents, especially substrates, and the equipment used to measure the results may differ enough to make our comparisons of assays less than identical. However, this explanation is not likely to reconcile the 46.5% average decrease in LAAO activity and 22.9% difference in PDE activity. There appears to be only one study addressing the stability of snake venom PDE; Munekiyo and Mackessy (1998) observed relative stability of PDE activity following a week-long exposure to a number of experimental storage conditions, including 25-fold dilution and heating at 37 °C for 7 days. A number of studies have addressed storage of snake venom LAAOs. In general, snake venom LAAOs are considered to be heat labile (Guo et al., 2012; Samel et al., 2006; Torii et al., 1997), but in several studies LAAOs retained activity after heat treatments or repeated freeze-thaw treatments (Munekiyo and Mackessy, 1998; Sakurai et al., 2003; Samel, 2008). In the case of the venoms tested here, isolation and testing of individual toxins and the lability of their activities would be required to identify a mechanism for loss in activity. In any case, these data indicate that the proteomic identities of the long-term stored samples are stable, whereas these two activities are not. Unfortunately, comparison of other venom toxicities, in particular the hemorrhage-causing and coagulopathic snake venom metalloproteases that are prevalent in this species' venom, were not possible due to changes in commercially available substrates

over the ~35 year period. Replicating any assay procedures used ~35 years prior is possible, but use of altered substrates makes a comparison of enzyme activity data collected ~35 years prior and activity data collected in the present impossible. In other words, no attempt to assess what original enzyme activity data were available because there was no accurate method to measure how that activity had changed or remained the same after ~35 years of storage.

Similar to the SDS-PAGE analysis, reverse phase HPLC produced chromatograms that were qualitatively similar among the older samples tested. All of the major landmarks are shared among the samples and the baseline appears steady through the run with no aberrant, small, early eluting peaks that might indicate the presence of degradation products (Figure 2.4, 2.5). Each of the major landmarks shared among other *C. o. oreganus* venom samples (Sunagar et al., 2014), such as the main doublet appearing around 22 minutes, eluted at approximately the same times; lateral shifts in the positions of these major peaks might have indicated proteolytic processes. Two chromatograms had exceptionally large peaks eluting around 66 minutes (Figure 2.5 top and bottom), but the size of the other peaks in the chromatogram are consistent with the other samples. Saviola et al. (2015) detected degradation peptides from P-1 class SVMPs eluting immediately following the main doublet around 22 minutes; however, a similar effect was not apparent here, lending further support for stable preservation of samples. There do not appear to be overt indications for protein degradation following ~35 years of storage.

However, these data indicate that a certain level of caution is needed when interpreting changes or differences in enzyme toxin activities over time. In the following

chapter, venom samples collected ~35 years apart from the same geographic locations are compared. Assuming that samples collected in the past experience decreases in the activities of at least two enzyme toxins, one may expect that venom samples collected from snakes in the present will appear to have higher activities of these enzymes even if no true change in the quality of the venom has occurred. Degradation of activity following storage may mask the stability of venom composition in a population. Alternatively, if the detected decreases in activity are not generalizable because of differences in assay procedure between the past and present, or because of low statistical power analyses run with small sample sizes, then concerns about the quality of stored venoms may not pose an issue in comparing old and new samples from the same geographic population. The present analysis was only able to address the activities of two families of toxins that already appear to be in relatively low abundance in these venoms (cf. Figure 2.3), and neither is considered a major toxin component influencing envenomation symptoms. Other venom activities are likely be unaffected by the storage conditions, the indication from the literature is that stored venoms samples should retain most toxic activities even after extended periods of storage (Jesupret et al., 2014; Munekiyo and Mackessy, 1998; Russell, 1960).

Questions regarding how snake venom has changed individually or in populations rely on comparing historical, stored samples to those collected more recently. Given proper handling, more recently stored samples will produce proteomic and biochemical data that are indistinguishable from data collected from freshly extracted venom (Munekiyo and Mackessy, 1998). However, there is no evidence to indicate that sample quality is maintained beyond short periods. In the two studies where investigators

compared data collected when the samples were first extracted and following long-term storage, there were several venoms that experienced decreases in toxicity (Schottler, 1951; Russell, 1960). Assuming that identical analyses were conducted on samples when they were first fresh and after being stored, then measured decreases in toxicity, and likely other changes to features of the venom samples, must have resulted from some quality of the storage conditions. The present study highlights the need for quality assurance procedures to be developed to assess sample quality flux over time. A lack of data collected at the time when the samples were newest is likely as researchers are only apt to collect data that is pertinent to current research agendas. The use of historical samples for the creation of new venom related therapeutics, researching questions about snake venom compositional evolution, and researching questions about snake venom diversity in general depend on high quality samples stored long-term. These data suggest that the presumption of stability of all venom components must be reconsidered.

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CHAPTER III

VENOM COMPOSITIONAL STABILITY ACROSS ~35 YEARS IN THREE POPULATIONS OF SOUTHERN PACIFIC RATTLESNAKE (*CROTALUS OREGANUS HELLERI*)

Introduction

Rattlesnake venom is a complex mixture of toxic components, mostly proteins and peptides, which is used by these snakes to incapacitate prey, aid in predigestion of prey, and defend against predators (Arbuckle, 2017; Mackessy, 2010b). The variability of bioactive function within venoms makes them an excellent focus of study for evolutionary biologists interested in questions about the forces that drive predator-prey interactions and potential coevolution of these interactions. The diverse arsenal that venomous snakes deploy to capture prey must be able to disrupt the physiologies of a wide range of possible prey types and sizes, with the caveat that it must do so with the limited volume that can be injected during a strike. Venom must be sufficiently toxic to allow prey capture, and diverse in composition (or evolutionarily labile) to allow for changes in locally available prey taxa. Presumably, a stable prey base (and other ecological factors) should stabilize venom diversity in a local area.

A great deal is known about how venoms vary within and between venomous snakes, including the rattlesnakes. Many snakes experience ontogenetic shifts in venom composition (e.g., Augusto-de-Oliveira et al., 2016; Alape-Girón et al., 2008; Guérico et

al., 2006; Durban et al., 2013; Mackessy, 1988, 1993b; Mackessy et al., 2003, 2006; Margres et al., 2015; Pla et al., 2017; Rokyta et al., 2017; Saldarriaga et al., 2003; Wray et al., 2015; Zelanis et al., 2010; Zelanis et al., 2011). Most often, the shift from neonate to adult venom phenotypes appears to follow switches in prey choice between the two age groups. For example, in the species of focus in this chapter, Mackessy (1988) showed that *C. o. helleri* and *C.o. oreganus* switched from a neonate venom containing high levels of phospholipase A_2 activity and high toxicity to lizard prey to an adult venom containing high levels of proteolytic activity and lower toxicity to lizard prey. The behavioral switch to a diet of rodent prey exclusively was concordant with a change to a venom hypothesized to facilitate capture and digestion. Beyond this initial transition to an adult phenotype, compositional studies have indicated that venom identity and activity remains fairly stable. Two studies have explicitly tested for seasonal fluctuations in venom composition of adult venomous snakes, both finding small or no changes in composition over the study period (Gregory-Dwyer et al., 1986; Gubenšek et al., 1974). Studies in captive venomous snakes similarly found minor or no changes in composition across seasons (Claunch et al., 2017; Freitas-de-Sousa et al., 2015; McCleary et al., 2016; Williams and White, 1992).

Beyond age-related variability in venom composition, snake venom is known to vary within and between venomous snake taxa. Broad phylogenetic trends are seen in venom composition, such as the absence of disintegrins in the venoms of snakes in the family Elapidae (cobras, kraits, coral snakes, and relatives) and the general absence of three-finger toxins in the venoms of snakes in the family Viperidae (vipers, rattlesnakes, and relatives) (Aird et al., 2013; Calvete et al., 2010; Moura-da-Silva et al., 1996; Sanz et

al., 2006). Other trends can be detected within and between genera and species of venomous snakes; for example, rattlesnake species appear to follow one of two trends, having venoms with high concentrations of P-I and/or P-III Metalloproteases and lower toxicity (Type I) versus having a venom with little to no metalloproteases and higher toxicity (Type II) (Mackessy, 2008). However, increasing information about the diversity of venom composition within venomous snake species has uncovered exceptions to apparent trends in venom composition (e.g., Sunagar et al., 2014; Tan et al., 2017), indicating that venom composition alone is not a useful means of discriminating between venomous species (Calvete et al., 2009; Johnson, 1968; Serrano et al., 2005; Soto et al., 1988; Straight et al., 1992).

We understand a great deal about the diversity of snake venom, but information on how venom composition changes in populations through time is not available. A few studies explicitly investigated the quality and viability of venom samples stored long-term (Jesupret et al., 2014; Russell, 1960; Schottler, 1951), but most venom studies analyze samples collected at approximately the same time. This approach provides excellent snapshots into the diversity of venom samples, but it does not allow a long view of how quickly venoms can change over time within and between populations.

The present study describes and compares venom composition in three populations of Northern Pacific Rattlesnakes (*Crotalus oreganus oreganus*). *Crotalus o. oreganus* is a moderately-sized rattlesnake found from San Luis Obispo and Kern Counties in California north into the southern portion of British Columbia, Canada. The subspecies inhabits a wide range of habitat, from coastal chaparral to higher elevation coniferous forest. A large group of samples was collected ~35 years ago and allowed for

a direct comparison of snake venom composition from the same historic and current populations of rattlesnakes. We assessed compositional differences between long-term stored and recently collected samples using three biochemical methods that account for structural and functional characteristics of the venom. To assess the 'structure' of the venom, 1-dimensional SDS polyacrylamide gel electrophoresis was used to assess differences in the presence and absence of toxins. Further, reverse phase high performance liquid chromatography detected differences in the relative abundance of particular toxins. Venom enzyme assays for six toxin families were used to assess changes in the venoms' function. Considering the relatively short difference in time between collection (~35 years), for all three analyses, we hypothesized that there will be no significant difference between groups of samples collected at different time points, regardless of geographic location.

Multivariate analyses allowed detection and description of variation in venom composition within and between the three populations and across the ~35-year period between sample collection dates. Because rattlesnakes depend on the toxicity of their venom to capture prey, we expected that venom composition would remain stable over this evolutionarily short period. To our knowledge, this study represents an unprecedented attempt to assess compositional changes in populations of venomous snakes over time.

Materials and Methods

Permitting and Animal Care and Use Statement

Contemporary venom samples from two populations were collected from rattlesnakes captured and extracted using protocols approved by the University of

Northern Colorado's Institutional Animal Care and Use Committee (1701D-SM-S-20). The remaining samples (seven from the Chimineas Region; 13 from Monatña de Oro) were collected by Matthew Holding and Dr. Emily Taylor at California Polytechnic State University at San Luis Obispo with the approval of their Institutional Animal Care and Use Committee.

Capture Locale Selection

From 1978-1982, Dr. Stephen Mackessy and colleagues at the University of California, Santa Barbara, collected Pacific Rattlesnakes (*Crotalus oreganus* ssp.) across Santa Barbara, Ventura, Kern, and San Luis Obispo counties in southern California. Snake venom was extracted by manual expression and then lyophilized before being stored in glass vials with cork stoppers. Vials were stored at -20 °C in airtight plastic containers with Drierite desiccant. Barring transport between university campuses and emergent freezer malfunctions, these samples have been maintained in a low humidity, low temperature environment to the present. Samples included in this study were chosen from adult snakes (total length ≥ 800 mm) that yielded more than 2 mg of dried crude venom. There was no risk of duplicate sampling as most individuals were sacrificed for the museum collection following extraction; additionally, snake longevity of this duration is exceptionally unlikely. Duplicate samples from a small number of individuals that were kept in captivity and serially extracted were excluded. Rattlesnake collection locations were plotted (Google Earth) and binned by geographical location. From these groupings, three collection locations were selected for ease of access in the present (Figure 3.1). Each sample was collected no more than 20 km from an earlier collected sample, within a likely dispersal distance for these rattlesnakes.

Figure 3.1. Map of three sample locations. Farthest west in green, Montaña de Oro State Park, San Luis Obispo Co. Northeastern area in blue, Carrizo Plain National Monument and surrounding area, San Luis Obispo Co. Southeastern area in red, Chimineas region, Santa Barbara and San Luis Obispo Cos (Digital-Topo-Maps.Com, 2018; Google, 2018).

The first sampling location was the Carrizo Plains National Monument, mostly in the vicinity of the Painted Rock landmark (Figure 3.1, blue). The second sampling location, named here as the Chimineas Region, included Chimineas Ranch (a part of the Carrizo Plain Ecological Reserve) in the headquarters area and along CA Hwy 166 east of Aliso Canyon Road and CA Hwy 33 (Figure 3.1, red). The third collection area was at Montaña de Oro State Park (Figure 3.1, green).

Animal Capture and Extraction

Adult individuals (total length ≥ 800 mm) of Pacific Rattlesnake (*Crotalus oreganus ssp.*) were collected using road surveys and general searches in the habitat. Snakes captured in the present day were collected within the geographical range of samples collected by Dr. Stephen Mackessy; individuals collected outside of this range were sampled within a reasonable dispersal distance (< 20 km from an original capture). There was no risk of duplicate sampling as individuals were either retained in captivity after capture (Carrizo) or actively tracked using radio telemetry devices during a separate study (Holding, 2011; Chimineas and Montaña de Oro). Venom samples were extracted

using standard methods used by the UNC snake venom laboratory, lyophilized in California, and stored at -20 °C in screw top, brown glass dram vials wrapped with Parafilm.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and 1-D Band Analysis

Crude venom from stored and present day samples were analyzed for the relative abundance of particular venom components using SDS-PAGE under reducing (DTT) conditions on NuPage 12% Bis-Tris gels and MES running buffer. Twenty μg of crude venom solubilized in double deionized water were electrophoresed for approximately 45 minutes. Mark 12 standards were used as molecular weight standards. Stored and present day samples were run in alternating lanes across gels to allow for side-by-side comparison and to reduce the effects of any inter-gel bias. Gels were stained with Coomassie Brilliant Blue R-250 for a minimum of four hours, destained, and scanned using an HP Scanjet 4570C.

Uncompressed TIF format photograph files were analyzed using Quantity One® 1-D Analysis Software (Version 4.6.7; Bio-Rad Laboratories, 2011). Bands were scored by adjusting automated band detection to the minimum criteria to identify all bands of the Mark 12 standard ladder. Bad band calls resulting from aberrations in the gel picture were identified by sight and removed manually. An automated feature in the program calculated band size using two lanes of Mark12 ladder to standardize molecular weight. One long-term stored and one recently extracted venom sample were run in triplicate on another gel to calculate mean and standard deviation (SD) of bands. Band size bins were

created by taking the mean band sizes ± 0.96 SD. New bins were created when bands falling outside of these calculated ranges of band size were detected. Individual venom samples were then scored for the presence or absence of these binned band sizes. The resulting presence/absence matrix for band size bins was used to analyze trends in venom compositional change within and between populations across ~35 years of time.

High Performance Liquid Chromatography (HPLC) and Comparison by Integration

Approximately 1.2 mg of crude venom was dissolved in Millipore-filtered water (ddH_2O; 18.2 MΩ) and then centrifuged for 10 minutes at 14,000 rpm to remove insoluble debris. The sample was then passed through a 0.45 μm Acrodisc® filter (Gelman). One mg of protein in 200 μL ddH_2O was loaded onto a Phenomenex Jupiter C_{18} (250 x 4.6 mm, 5 μm) column on a Waters HPLC system using Empower software. The column was equilibrated with 95% of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 5% of 0.1% TFA in 80% acetonitrile (solution B). Elution was performed by a linear gradient to 90% solvent A and 10% solvent B over 10 minutes; to 25% B over 10 minutes; to 32% B over 21 minutes; to 41% B over 1 minutes; to 45% B over 10 minutes; to 70% B over 10 minutes; to 90% B over 2 minutes; to 95% B over 3 minutes; to 5% B over 2 minutes; and at 95% A, 5% B for 6 minutes. A Waters Fraction Collector II collected 1 minute fractions at a flow rate of 1.0 ml/min, with eluting peaks monitored by a Waters 2487 Dual Absorbance Detector at 220 and 280 nm.

A time-based integration procedure was used to calculate the percent area under the curve of each chromatogram. The integration procedure was normalized by starting the integration at the base of a stereotypical doublet that appeared around 20 minutes into

the elution period. Sixty second portions of the chromatogram were serially integrated for a total of 48 portions, or peak areas, for each chromatogram. The matrix of 48 peak areas for each venom sample were used to detect changes in venom composition within and between populations across ~35 years.

The identity of the major protein within each chromatogram peak was determined using SDS-PAGE under reducing (DTT) conditions as described above. A minimum volume of RP-HPLC eluent was run for each minute of time and proteins identified to the level of family based on band size and referencing other published *C. o. oreganus* proteomes (Mackessy, 2010b; Sunagar et al., 2014)

Enzyme Assays

Several enzymatic toxin activities were compared between samples collected in the present and past. Snake venom metalloprotease activity was determined using azocasein as a substrate (AZO; Aird and da Silva, 1991) with the activity expressed as ΔA_{342nm}/min/mg venom protein. Phosphodiesterase (PDE) activity was measured using bis-p-nitrophenylphosphate (Sigma) with specific activity expressed as ΔAbs_{400nm}/min/mg venom protein. Phospholipase A_2 (PLA2) activity was determined using 4-nitro-3-(octanoyloxy) benzoic acid (Sigma) with activity expressed as nmol product/min/mg venom protein (Holzer and Mackessy, 1996). L-amino acid oxidase activity (LAAO) was determined using L-kynurenine as a substrate (Weissbach et al., 1960) and expressing the activity as nmol product/min/mg venom protein. Thrombin-like and kallikrein-like serine protease activity was measured using benzoyl-Phe-Val-Arg-paranitroaniline and benzoyl-Pro-Phe-Arg-paranitroaniline as substrates, respectively,

(KAL and THR, respectively; Mackessy, 1993) and expressing the activity as nmol product/min/mg venom protein.

Statistical Analyses

All three sets of data, 1-D band analysis, RP-HPLC peak areas, and venom enzyme assays, were analyzed using PC-ORD (Version 6.0; McCune and Mefford, 2011). The software is designed for multivariate analysis of ecological data with an emphasis on nonparametric procedures, graphical representation, and randomization tests. Non-Metric Multidimensional Scaling (NMS) was used to analyze variation across the entire data sets of 1-D bands, peak areas, and enzyme activities independently. The procedure iteratively searches for the ranking and placement of individual samples in k dimensions (axes) to determine a solution that has the lowest stress value for the k-dimensional configuration. Once a solution for the number of dimensions with the lowest stress value was found, a graphical representation of the ordination was manually rotated to align input variables along the calculated k dimensions. Lastly, a correlation procedure was used to determine the strength of association between input variables and each axis. PC-ORD calculated correlation coefficients for each input variable in the analysis against each axis of the best solution of the NMS. Pearson's *r* was used to assess the linear relationships between ordination scores and the individual variables used to construct the ordination. Lastly, the age of sample (long-term stored and recently extracted) and geographic capture location (Chimineas Region, Carrizo Plain National Monument, and Montaña de Oro) were overlaid onto the ordination plots to assess visually whether samples clustered along any of the k dimensions of the best NMS solution.

Multiple Response Permutation Procedure (MRPP) was used to determine whether samples statistically differed in gel band presence/absence, RP-HPLC peak area, and enzyme activities between ~35 years of collection. MRPP was conducted piecewise to detect differences between long-term stored samples and recently extracted samples for each data set and independently by each geographic capture location. The null hypothesis that there were no differences between collection times was assessed using an *a priori* $\alpha = 0.05$. If a significant difference between time points was detected, Indicator Species Analysis was used as a post-hoc analysis to detect which input variables were responsible for the difference and the relative importance of these variables for assignment of samples into either time period. Graphical representations of summary data were also constructed to aid in ease of interpretation of statistical tests for differences by date of sample collection.

Results

Comparisons of Enzyme Toxin Activities

Six enzyme assays were conducted for 76 individuals (41 long-term stored; 35 recently collected). NMS recommended a one-dimensional solution based on a randomization test, although this relationship was not stronger than expected by chance ($p = 0.06$). The best solution had a final stress value of 0.69. The final instability was < 0.00001 with 52 iterations in the final solution. Thrombin-like serine protease activity (THR) had the strongest, negative association with this axis ($r = - 0.68$; here and throughout, Pearson's *r*). Kallikrein-like serine protease activity was weakly, positively associated with the axis ($r = 0.38$). Metalloprotease activity detected by azocasein proteolysis (AZO), phosphodiesterase (PDE) activity were positively associated with this

axis, but only weakly with r-values all below 0.07. Phospholipase A_2 and L-amino acid oxidase (LAAO) activity were weakly, negatively associated with r-values above -0.05. There was no clustering of samples by age of sample or capture location, indicating a weak association with this axis (figure not shown). All venom samples appear to vary in similar patterns along this axis, regardless of group membership.

Multiple Response Permutation Procedure (MRPP) indicated that enzyme activities of long-term stored and recently extracted samples did not differ significantly for samples collected at Montaña de Oro ($p = 0.66$). Similarly, enzyme activities for long-term stored and recently extracted samples collected from Carrizo Plain National Monument did not differ significantly ($p = 0.30$). MRPP found a significant difference in enzyme activity between long-term stored and recently extracted samples from the Chimineas region ($p = 0.016$; $A = 0.12$). This effect size ($A = 0.12$) is small; however, values for *A* below 0.1 are often found in community ecology data (McCune and Grace, 2002). An Indicator Species Analysis (ISA) for samples collected in the Chimineas region indicated that AZO activity was higher for recently extracted samples; however, the strength of this activity only had an indicator value of 63.5 ($p = 0.021$) for newer samples (Appendix A). Indicator values range from zero (no indication) to 100 (perfect indication, where perfect indication for a particular variable would point to a particular group without error (McCune & Mefford, 2011). For the purporses of this analysis, only statistically significant indicator values are considered and a stringent cutoff of $IV > 80$ was used to assess the strength of separation between the groups tested. The ISA also indicated that THR activity was higher for stored samples; however, this activity also had a lower indicator value of 61.9 ($p = 0.034$). The strongest difference between recently

extracted and long-term stored samples was PLA_2 activity with a moderate indicator value of 71.1 ($p = 0.036$). Long-term stored samples had higher PLA_2 activity on average, but while this enzyme activity had the highest indicator value of the significantly different acitivities, the separation in values is not strong enough to be a reliable marker for group assignment. AZO, PLA_2, and THR activities differs between collection times, but are not sufficient to discriminate between the two groups of samples collected in the Chimineas region.

Ignoring the age of sample and testing for differences between each location, MRPP indicated a significant difference between the Chimineas and Montaña de Oro populations ($p = 0.039$, $A = 0.03$). Phosphodiesterase (PDE) was significantly higher in samples from Montaña de Oro (ISA, $p = 0.046$) and THR was significantly higher in samples from the Chimineas Region (ISA, $p = 0.018$). However, indicator values (IV) were not large for either population (Montaña de Oro PDE, IV = 43; Chimineas Region THR, IV = 40) precluding these activities from being reliable discriminators between population identities. In other words, there were significant differences between the two populations, but neither predicts group membership with a particular geographic location.

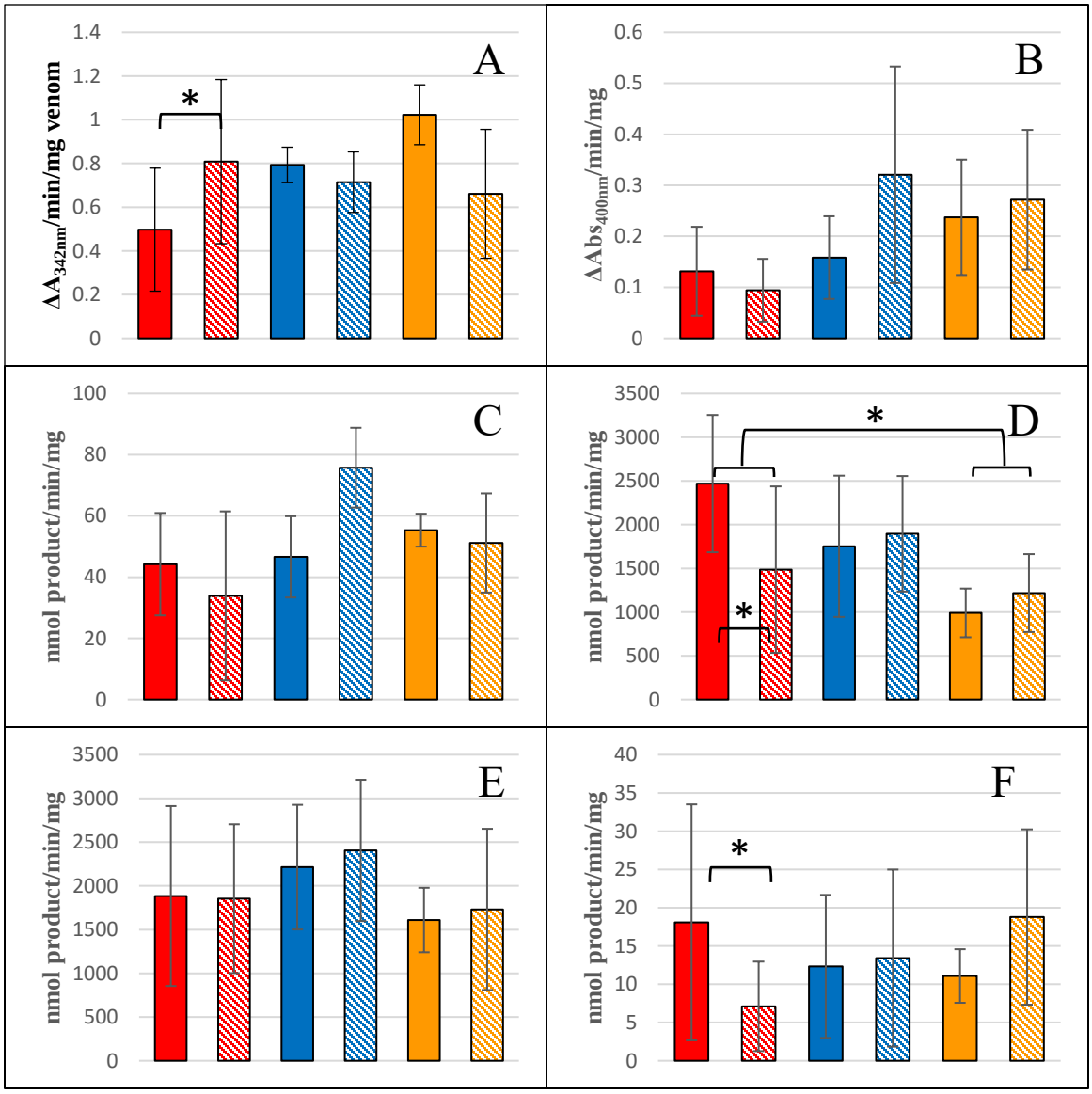

Figure 3.2. Average and standard deviation of six venom enzyme activities for three sample locations collected at two time points. (A) Snake venom metalloprotease activity; (B) Phosphodiesterase activity; (C) L-amino acid oxidase activity; (D) Thrombin-like serine protease activity; (E) Kallikrein-like serine protease; and (F) Phospholipase A_2 activity. Long-term stored samples are represented in solid bars and recently collected in striped bars. Samples are grouped by geographic population with Chimineas Region in red (10 old, 13 new samples), Carrizo Plain in blue (24 old, 15 new samples), and Montaña de Oro in gold (7 old, 7 new samples). Statistically significantly differences in activities (assessed by MRPP) are indicated with an asterisk ($p < 0.05$).

1-Dimensional Band Analysis of Polyacrylamide Gel Electrophoresis under Reducing Conditions

Twenty distinct bands were identified and scored for 73 individuals (41 long-term stored venoms; 32 recently collected) (Appendix B). Using the size of the protein to categorize the bands, one band was classified as L-amino acid oxidase (5%), two bands as unidentified nucleases (e.g. phosphodiesterase) (10%), three bands as P-I and P-III snake venom metalloproteases (SVMPs; 15%), five as serine proteases (25%), one band as cysteine-rich secretory protein (5%), two as phospholipase A_2 (10%), 3 as disintegrins (15%), two as small, non-phospholipase myotoxins (10%), and one as bradykinin potentiating peptide (5%). All samples, regardless of extraction date or capture location, had three conserved bands: serine protease band 30 (SP-30); cysteine-rich secretory protein band 26 (CRiSP-26); and phospholipase A_2 band 15 (PLA2-15). Eleven of 20 bands were present at high frequencies ($f > 0.65$), representing all SVMPs bands and 3 of 5 SP bands. The remaining SP bands, the single LAAO-67 band, and the single bradykinin potentiating peptide band 2 (BPP-2) were found in low frequencies across the data set ($f < 0.35$).

The three dimensional solution recommended by NMS was stronger than expected by chance, based on a randomization test ($p = 0.004$). The best solution had a finals stress value of 14.49. The final instability was <0.00001 with 106 iterations in the final solution. Together, these three axes described 82.0% of band presence/absence variation. The first axis accounted for the greatest amount of the variance, 41.2%. Three bands had strong correlations with this axis: serine protease band 36 (SP-36; $r = -0.61$),

myotoxin band 5 (Myo-5; r = -0.76), and disintegrin band 6 (Dis-6); r = 0.629). Two other bands were weakly associated with this axis: SP-42 (r = 0.40) and PIII snake venom metalloprotease (SVMP-56; r = -0.44). All other bands were not well correlated with this axis, with correlation coefficients r < 0.4. While this axis described the most variance for the data set, there were no obvious trends separating samples by age or location of capture.

Axis 2 explained 21.5% of variance in the data set. Phospholipase A_2 band 13 (PLA-13) and Dis-10 were strongly associated with the positive end of this gradient (r = 0.60 and r = 0.51, respectively). Disintegrin band 11 (Dis-11) was strongly associated with the negative end of this gradient (r = -0.85). All other bands did not strongly associate with this axis, most with -0.5 < r-values < 0.5. Again, samples did not segregate based on age or by location.

Axis 3 explained 19.3% of the variance in gel band presence/absence. Interestingly, two bands with the greatest associations were both nucleases, with nuclease band 58 (Nuc-58) strongly associated with the negative end of the gradient (r = -0.69) and nuclease band 65 strongly associated with the positive end of the gradient (r = 0.74). The final strongly associated band was L-amino acid oxidase band 67 (LAAO-67; r = -0.51). All of the remaining bands were not strongly associated with this axis, with -0.5 > r-values > 0.5. There were no apparent groupings by age or capture location of samples along this axis.

MRPPs indicated no significant difference between long-term stored and recently extracted venom samples for all three capture locations (Chimineas, p = 0.26; CPNM, p = 0.53; MDO, p = 0.22). A subsequent MRPP testing for differences between capture

location, regardless of age of sample, found that there were no significant differences between any capture location ($p = 0.087$). Taken in total, the band analysis did not detect significant differences in the presence or absence of 20 SDS-PAGE bands for any population, for any time point, or for any combination thereof. Visual inspection confirms that the banding patterns are extremely similar across samples, regardless of age group (Figure 3.3; see also Appendix C).

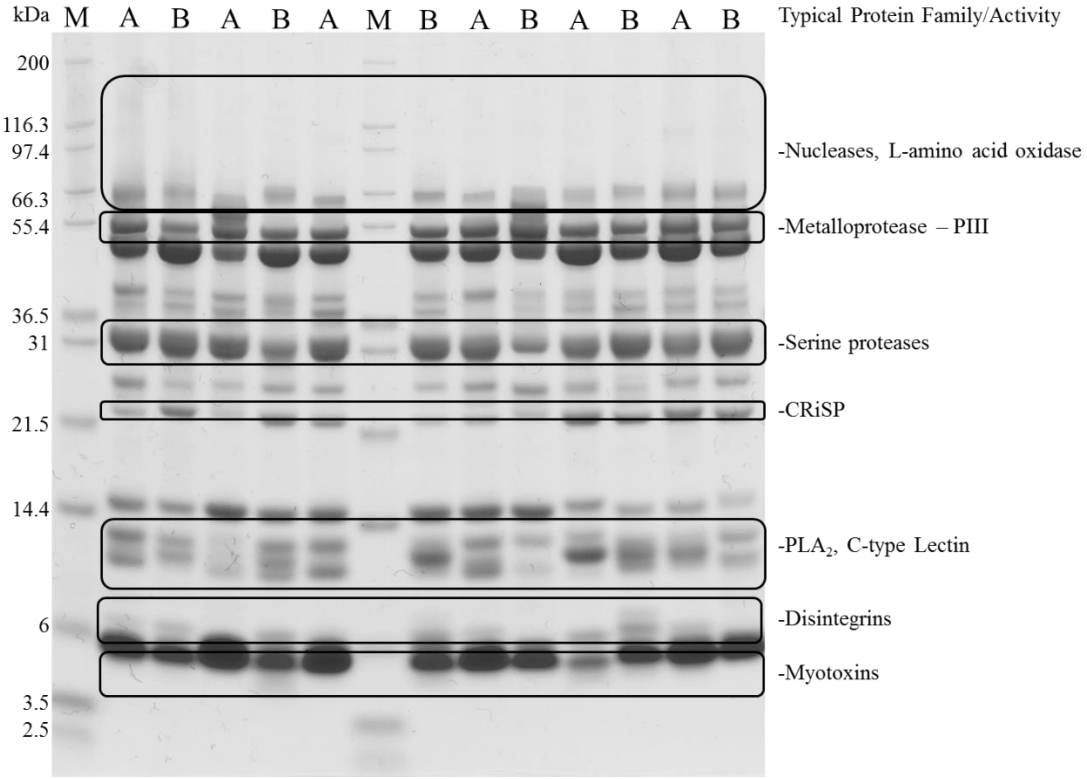

Figure 3.3. Representative SDS polyacrylamide gel electrophoresis photograph of 12 samples from Carrizo Plain National Monument. (M) lanes represent Mark 12 standard; (A) lanes indicate long-term stored samples; and, (B) lanes are more recently collected samples. Reading across the gel, there are a number of bands that vary in their presence/absence; however, they do not vary in statistically significant ways within and between sampling time populations.

Peakwise Integration Comparison

Analysis of 48 1-minute intervals for 77 individuals (42 long-term stored; 35 recently collected) by NMS indicated a three dimensional solution stronger than expected

by chance, based on a randomization test ($p = 0.004$). The best solution had a stress value of 11.6. The final instability was <0.00001 with 109 iterations in the final solution. The three axes explained a cumulative 89.7% of variance in RP-HPLC chromatogram peak area.

The first axis explained the most variance, 45.4%. Three peaks were strongly associated with the negative end of this gradient: Peak 8 (Pk-8), containing myotoxin a-like toxins (MLTs; $r = -0.77$); Pk-42 containing L-amino acid oxidases (LAAOs; $r = -0.81$); and Pk-44, containing metalloproteases (SVMPs; $r = -0.66$). Two peaks had strong association with the positive end of this axis: Pk-43, containing LAAOs ($r = 0.81$), and P-48, containing SVMPs ($r = 0.54$). It is readily apparent that the age of sample is associated with axis 1, as a majority of long-term samples cluster around the positive end of the gradient and recently extracted samples with the negative end (Figure 3.4). There was no apparent effect of capture location with individuals from all populations found scattered across the axis.

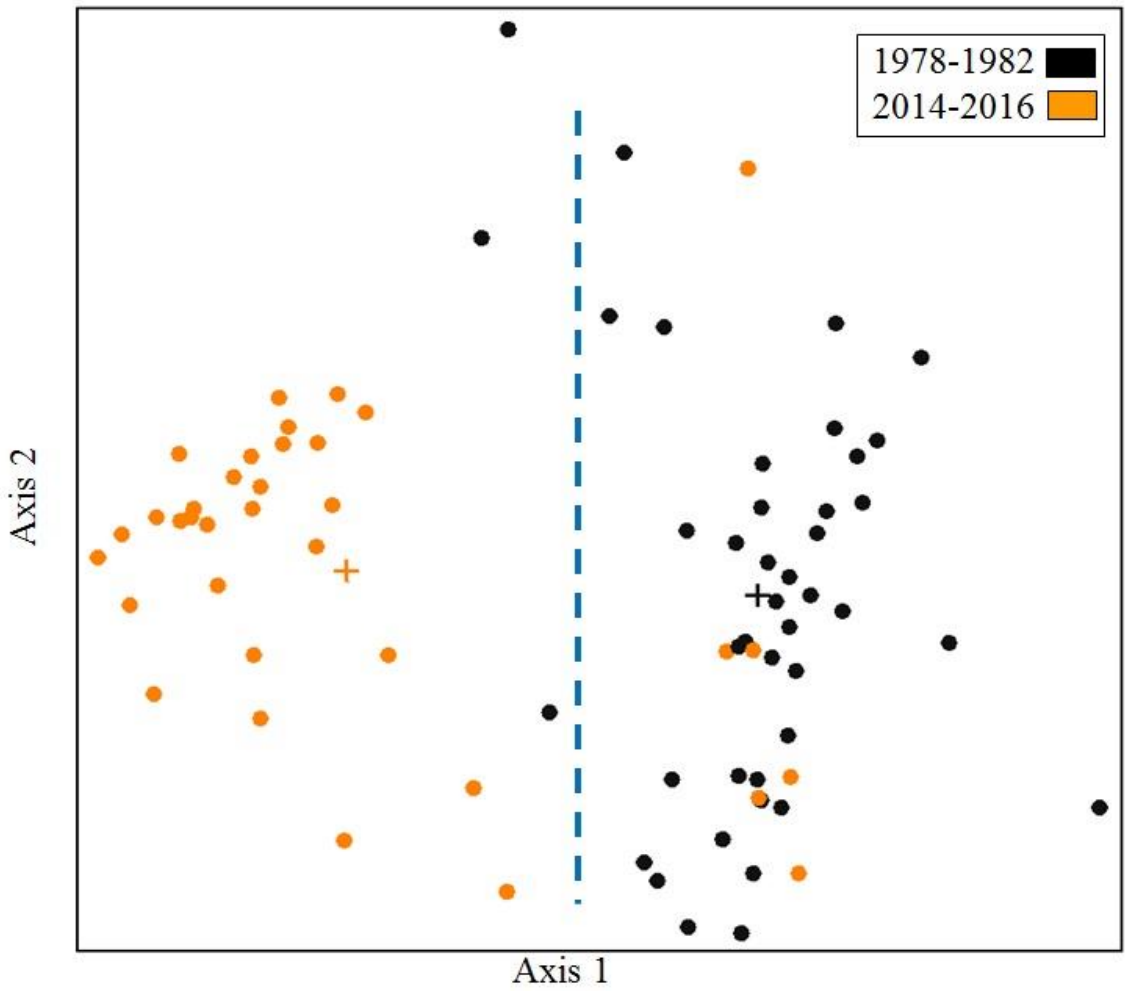

Figure 3.4. NMS ordination of HPLC peak data, axes 1 and 2, rotated 20°. Each point represents an individual sample and all are color coded by their date of collection. Samples appear to separate by their age along this axis, indicated by the blue line.

The second axis explained 27.5% of the variance in peak area. Two peaks were strongly associated with the negative end of the axis, Pk-3, containing bradykinin potentiating peptides (BPPs; r = -0.57) and Pk-45, containing SVMPs (r = -0.75). One peak, Pk-46, containing SVMPs, had a strong association with the positive end of the axis (r = 0.61). Age of sample and capture location did not appear to have an effect on the distribution of samples along this axis.

The final axis explained 16.8% of the variance. One peak, Pk-10 containing MLTs, was moderately associated with the positive end of this axis (r = 0.49). Two

peaks were moderately associated with the negative end of this axis, Pk-11, containing MLT and Pk-40, containing SPs. Individual samples were evenly distributed across this axis, indicating no strong effect by age of sample or capture location.

In general, it appears that variation between samples is dominated by the abundance of a number of SVMPs and MLTs. The greatest segregation of individuals by age in axis 1 is driven primarily by the abundance of SVMPs and an LAAO found in long-term stored samples, and the mix of MLT, SVMP, and LAAO found in recently extracted venom samples. The capture location of the individual snake did not affect the placement of samples in the three-dimensional solution, indicating that individuals captured around the same time are relatively similar to each other, regardless of their locality.

MRPP confirmed that the apparent separation of samples along the first NMS axis represented a statistically significant difference in age for all three capture locations (Table 3.1 CHIM, $p = 0.0059$, $A = 0.075$; CPNM, $p < 0.00000001$, $A = 0.24$; MDO, $p = 0.0076$, $A = 0.07$). The moderate A-value (i.e. effect size), $A = 0.24$, for the CPNM samples indicates that samples from each time point at this location are more homogeneous compared to samples from the other geographic locations. As mentioned above, $A < 0.1$ are not unexpected in ecological data, but a higher A for the CPNM samples supports a stronger within-age relationship that helps discriminate between age group identities. Post-hoc ISA identified a number of peaks that were good predictors of group identity for each age by population combination (Table 3.1). In ISA, each peak is assessed for its relative frequency and abundance to derive an indicator value (IV) statistic that is used to produce a p-value in a Monte Carlo test of significance (Dufrêne

and Legendre, 1997). An IV of 100 for a ‘species,’ here an HPLC peak, is a perfect indicator of group assignment. Peaks with significant p-values and high IVs for each geographic population are summarized in Table 3.1. For each geographic population, there are several peaks that are unique to either long-term stored or recently collected samples, but two peaks, Pk-42 (LAAOs) and Pk-44 (SVMPs), are always significantly larger in more recently collected samples (Table 3.1 highlighted blue). Average percent peak area and standard deviation are provided in the table to indicate which age group is best represented by the selected peaks.

Table 3.1. MRPP and ISA summary table for HPLC percent area under the curve values. MRPP produced both a *p*- and *A*- value to assess the null hypothesis of no significant difference and the effect size of the result, respectively. ISA produced p-value and indicator value (IV) to assess which peaks are significantly different between each age group and how well they represent a group. Gray-shaded boxes - age groups that had a significantly larger amount of a particular peak. Blue-highlighted boxes - peaks that were significantly higher in recently collected samples for all locations.

Chimineas Region						
MRPP	ISA					
p = 0.0059	Peak (Pk)	Contents	Indicator Value (IV)	p-value	1978-1982 Avg. ± SD	2014-2016 Avg. ± SD
A = 0.075	43	LAAOs	70	0.0078	6.79 ± 4.28	1.28 ± 1.97
	42	LAAOs	74	0.0002	0.28 ± 0.67	5.87 ± 3.35
	44	SVMPs	95	0.0024	2.26 ± 4.65	9.58 ± 7.66
Carrizo Plain						
MRPP	ISA					
p < 0.00000001	Peak (Pk)	Contents	Indicator Value (IV)	p-value	1978-1982 Avg. ± SD	2014-2016 Avg. ± SD
A = 0.24	41	LAAOs	80	0.0002	2.43 ± 1.02	0.60 ± 1.09
	43	LAAOs	89	0.0002	6.63 ± 3.04	0.19 ± 0.58
	2	BPPs	99	0.0002	0.086 ± 0.35	5.73 ± 1.60
	8	MLTs	97	0.0002	0.76 ± 0.58	21.52 ± 10.75
	42	LAAOs	84	0.0002	1.51 ± 1.99	8.15 ± 2.18
	44	SVMPs	96	0.0002	0.41 ± 0.74	10.21 ± 8.69
Montaña de Oro						
MRPP	ISA					
p = 0.0076	Peak (Pk)	Contents	Indicator Value (IV)	p-value	1978-1982 Avg. ± SD	2014-2016 Avg. ± SD
A = 0.07	46	SVMPs	71	0.044	7.28 ± 2.95	3.03 ± 4.28
	42	LAAOs	87	0.009	0.73 ± 1.31	4.90 ± 4.33
	44	SVMPs	67	0.045	1.15 ± 2.8	4.79 ± 3.97

Discussion

In general, the hypothesis that venom compositional structure and function would not change over ~35 years was supported. Variability among venom samples was detected by all three methodologies, and each provided a different perspective in the ways in which venoms varied within and between the populations considered here;

however, variability was consistent across the whole population of samples, with few significant separations by age or location of capture. Band analysis of SDS polyacrylamide gel electrophoresis (PAGE) did not detect significant differences between long-term stored and recently collected samples in all three populations. Moreover, ignoring the collection date, all individuals from three capture locations were highly similar. In other words, by gel electrophoretic pattern alone, we could not detect whether a venom came from a particular population or was collected in the past or present. These data indicate that the range of venom phenotypes in these populations is relatively unchanged over time, that is, variability in composition is bounded and measurable and that continuum appears unchanged over time. We did not observe wholesale shifts in the presence or absence of any band (toxin) over time, suggesting that selection favoring phenotypes that are adequately toxic for prey capture has not altered venom phenotype; our inference is that all are sufficient to capture prey in their local environments. A recent study has used 1-D SDS-PAGE band analysis to detect differences in species of snakes (Gibbs and Chiucchi, 2011), but here, all individuals came from the same subspecies and were captured close in proximity, and thus may be too similar for this methodology to detect significant differences. While unable to detect differences in these samples by the presence/absence of particular toxin bands, analysis of HPLC chromatogram peak areas revealed a significant separation of samples by the relative amounts of a small number of toxins.

The peak area analysis provided a finer discrimination of changes in the ‘structure’ of snake venom composition by identifying changes in the amount of toxins, not just their presence/absence in the venom. These data help to explain the lack of differences in the

band analysis data, as a number of the HPLC peaks that were significantly different between age groups were in extremely low abundance (< 1.0% of total venom protein; Table 3.1; Appendix D) in one group and in higher abundance in the other. In SDS-PAGE, the high sensitivity of the band detection procedure would lead to low and high abundance proteins being called 'present' in venoms even if the low abundance proteins were nearly nonexistent. Accounting for their relative abundance in the venom, a number of peaks were significantly different between long-term stored and recently collected samples in all three populations. Interestingly, there were two peaks that increased significantly in more recently sampled individuals in all three populations, Pk-42 containing L-amino acid oxidases (LAAOs) and Pk-44 containing P-III snake venom metalloproteases (Table 3.1).

L-amino acid oxidases are known to induce a number of pathologies including apoptosis, cellular damage, edema, and coagulopathy (Tan and Fung, 2010). However, their relatively low toxicity and low abundance in many snake venoms preclude them as ecologically important toxins (Tan and Saifuddin, 1989). The increase in abundance of Pk-42 may not have functional significance for these predators, as other components may be more critical to determining whether a prey item is incapacitated. However, it may also be that the synergistic effect of a particular LAAO may produce an ecologically more fit venom composition.

P-III snake venom metalloproteases (PIII-SVMPs) are toxins with a wide range of activities affecting tissue integrity and blood chemistry (Gutiérrez et al., 2016a; Gutiérrez et al., 2016b; Moura-da-Silva et al., 1996; Portes-Junior et al., 2014) that are critical in prey capture. In *Crotalus o. oreganus* venom, the ontogenetic increase in this class of

toxins in adults creates a venom composition that is well suited to dismantling mammalian prey physiology (Mackessy, 1988). Here, the increase in PIII-SVMPs may indicate a significant shift in prey base, or a modification of general venom composition that either allows for increased capture success of a particular prey species or improves this venoms capacity to kill a range of prey. Conversely, it may also represent a selectively neutral drift in venom composition. Variability in SVMPs is well-studied in other species of venomous snakes, where the range of specificity of these toxins (i.e. which substrates they are better suited for) may facilitate the ability of populations of snakes to capitalize on particular prey species over others (Amazonas et al. 2018; Sanz et al. 2006).

Beyond Pk-42 (LAAOs) and Pk-44 (PIII-SVMPs) that varied similarly across all populations, other peaks varied uniquely within each capture location. Most notable among these is the dramatic abundance Pk-8, containing myotoxin a-like toxins (MLTs; i.e., small myotoxins and their relatives) in recently extracted Carrizo Plain snakes. As MLTs are non-enzymatic, it was not possible to detect whether this disparity in abundance signals a functional change in this venom. However, studies characterizing the pathological effects of MLTs indicate that all isoforms in this group of toxins are likely to be potent myotoxins (Ponce-Soto et al., 2007; Ponce-Soto et al., 2010; Rádis-Baptista and Kerkis, 2011). Isoforms in this family are known to have a wide range of activities, such as being antibacterial, cytotoxic to cancer cells, or causing hemolysis (Batista da Cunha et al., 2018; Oguiura et al., 2011; Sánchez et al., 2018. However, it is unlikely that these activities are as advantageous to venomous snakes as the rapid onset tetanic contractions caused by these toxins (Bourillet, 1970; Cheymol et al., 1971), which

are likely the most expedient and efficacious mechanism for prey incapacitation. Functional information about the toxins in significantly different peaks is required to determine whether these shifts in relative abundance signal changes in venom toxicity.

Enzyme assay data provided a third means of assessing compositional change, and a means of assessing whether significant differences in HPLC peak analyses are ecologically relevant to prey capture. Previous assays examining venom component stability (Chapter 2) indicated that long-term storage likely only affects the highly labile components L-amino acid oxidase and phosphodiesterase. Other venom components are more stable, as indicated by a lack of significant evidence of breakdown products or proteolytic degradation following SDS-PAGE and RP-HPLC. Interestingly, neither L-amino oxidase or phosphodiesterase activity activity were significantly decreased in any group of long-term stored samples. Thus, it appears that degradation of samples does not explain any differences detected implying that differences in other activities must result from actual biological events. There were no significant differences by time in Carrizo Plain and Chimineas Region samples (Figure 3.2). While MRPP did return a significant difference between long-term stored and recently collected samples from Montaña de Oro, indicator values from ISA were low, indicating that these activities were not strong or reliable predictors of group identity. The distinction here is that these statistically different activities *alone* do not explain the statistical differences between age groups. The multivariate solutions of MRPP and ISA indicate which activities are responsible for separating samples by group identity and determines their importance in this solution by calculating an indicator value. Here, indicator values show that having an abundance of one activity does not reliably predict the time at which a sample was collected. In

addition to low support for the importance of statistically different enzyme activities, this population had the smallest representation in the data set, so it is possible that a significant difference appears because of the influence of a small number of individuals. In total, by the assays conducted here, animals from this locality have similarly toxic venoms.

In total, the functional assays completed in this study are not concordant with any significant differences in HPLC peak areas for enzyme toxins. The general increase in Pk-42 (LAAOs) and Pk-44 (SVMPs) in recently collected samples was not accompanied by an increase in the proteolytic capacity of these venoms. The apparent conclusion from these data is that venom has changed little and in predictable ways for all three populations over ~35 years. In all three approaches, NMS helped to define trends in how venoms varied, but except for the significant difference in a number of relative peak areas, population-level venom composition has remained unchanged. An alternative conclusion is that slight changes in the identity and abundance of toxins over time are ecologically important, but not obvious by our methods in this study. Again, studies have shown that the diversity of isoforms of toxin families confers a range of pathological capacities (e.g. Bernardoni et al., 2014; Gutiérrez et al., 2016a; Gutiérrez et al., 2016b; Moura-da-Silva et al., 1996; Portes-Junior et al., 2014), but more functional assays would be required, including *in vivo* toxicity testing, especially for non-enzymatic toxins like MLTs. Conversely, accepting that the assay procedures used here are good indicators of generalized enzyme function for six toxin families, these data provide strong support of our hypothesis that venom would not change over a short evolutionary time period.

The conclusion that venom composition does not vary dramatically within and between populations of rattlesnakes studied here fits expectations about the likelihood of divergence in this trophic adaptation. If there is a relatively low rate of the appearance of new toxins within a population and there are no barriers to gene flow between local populations (e.g., Clark et al., 2008), then it is reasonable to expect that venom composition may remain relatively stable, especially over a small number of generations. *Crotalus o. helleri* are expected to live up to 20 years in the wild, with females reaching sexual maturity on average at 4 years of age and bearing new litters every two years thereafter (Ernst and Ernst, 2012). Over a period of ~35 years, there may have been a maximum turnover of 10 generations, but considering the longevity of these snakes there may be constant backcrossing between older and younger generations that maintains venom compositional homogeneity. Other studies have documented low levels of inbreeding depression (Anderson, 2010; Gibbs and Chiucchi, 2012), given that there are no barriers to dispersal (Wang et al., 2015). Panmixia within populations is expected to maintain the bounds of venom compositional variability, at least over short time scales, given that there are no dramatic shifts in other selective forces that are expected to affect venom composition (e.g., diet or anthropogenic-caused isolation).

To our knowledge, this study represents an unprecedented approach to analyzing snake venom variation using multivariate analyses to probe for differences within and between populations of snakes. Techniques used in this study provide good data without the cost and time of full proteomic analyses (e.g., Gibbs and Chiucchi, 2011). The specific identification of venom proteins through a venomics approach (Calvete, 2017; Calvete et al., 2007) would be a useful next step toward understanding the complexity of

venom compositional variation over time. In cases where no major differences in venom composition are detectable, finer discrimination may be needed to determine the relative importance of seemingly minor shifts in compositional traits. Further, *in vitro* enzyme activity assays would be bolstered by expanding the investigation to a range of substrates as a means of understanding the functional consequences of shifts within and between enzyme toxins (e.g., Currier et al., 2010; Fox and Serrano, 2008; García et al., 2004; Torres and Kuchel, 2004). The levels of little to no change in enzyme function and relative stability in proteomic measures found in this study indicated that no major differences in the pathological activity of these venoms is probable, but confirmation of sustained toxicity towards locally available prey would be necessary to conclude fully that venom has remained the same. Given that the Chimineas Region and Carrizo Plain populations are geographically adjacent inland grasslands, we might conclude that they share a highly similar prey base, whereas snakes from Montaña de Oro, consisting of very different coastal strand habitat approximately 80 kilometers west from the grassland populations, may have different prey available, in turn potentially influencing venom composition.

Alternatively, there may be differences in the relative abundance of certain prey in each geographic area. Under the assumption that local adaptation of snake venom composition is modulated by the selective pressure of diet (Cipriani et al., 2017; Daltry et al., 1996; Phuong et al., 2016; Sanz et al., 2006), differences in diet should lead to changes in local venom composition, but we found little evidence of this in our study. Further, there was no evidence for major differences between geographic populations at either time point. Rattlesnakes in the present have not developed a different chemical

arsenal that would signal a specialization on a unique population of prey items in the local area. Current venom composition apparently works well as a generalist arsenal, or prey sensitive to these venoms are consistently abundant in all three locations over time. Tracking changes in prey populations in these areas would be difficult, relying on the existence of and access to previous and contemporary natural history data on possible prey, but this data would help to explain why venom appears stable across space and through time. Tracking venom and prey through time, from the present into the future, could also elucidate the dynamics of predator-prey evolution in venomous snakes and their prey in this system.

In summary, snake venom composition did not appear to change dramatically over the course of ~35 years in three populations of *C. o. helleri*. Variability in the expression of a limited number of toxins and toxin families was detected, but the identity of these toxins and families, combined with small differences in toxin activity data, suggest that there may not be a functional consequence towards venom toxicity. Returning to the initial assertion that snake venom must be sufficiently toxic to allow rattlesnakes to capitalize on local prey, no change in venom composition over ~35 years indicates that *C. o. oreganus* from the locations sampled here are still capable of capturing local prey, allowing these populations to persist into the present. This body of evidence adds to a continuing line of research in snake venom evolution investigating the features and drivers of venom compositional changes. Venom, as the sole trophic weapon available to venomous snakes, is logically inseparable from the effects of diet (Barlow et al., 2009; Daltry et al., 1996). However, the specific mechanism of coevolution between venomous snake predators and their prey is not well understood; the

earliest investigations into a mechanism focused on an arms race dynamic (i.e. Van Valen, 1973, Barlow et al., 2009; Daltry et al., 1996; Voss, 2013) and more recent work provided support for a phenotype matching dynamic (Holding et al., 2016). Theoretical coevolutionary studies highlight the importance of understanding the dynamics of predator and prey species through time (Cortez, 2015; Cortez and Weitz, 2014; van Velzen and Gaedke, 2017, 2018) such that future studies testing theoretical predictions in natural systems will benefit from the population-level approaches employed in the present study. Combined with recent advances in –omic technologies that allow researchers to assess the scaling influences of genotype to phenotype to ecological effects, a means of assessing venom compositional flux through time provides access to a wealth of biological data that is not readily available for most systems because historical samples have degraded over time. This study was a novel exploration of changes in population-level venom characteristics over an extended period of time and hopefully a first step towards a broader understanding of the dynamics of snake venom evolution over short and long evolutionary periods.

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CHAPTER IV

LOCALITY-SPECIFIC RESISTANCE IN A POPULATION OF DEER MOUSE (*PEROMYSCUS MANICULATUS*) AGAINST THE VENOM OF THE DESERT MASSASAUGA (*SISTRURUS TERGEMINUS EDWARDSII*) IN SOUTHERN COLORADO

Introduction

Venomous snakes employ their venoms primarily to incapacitate prey (Mackessy, 2010b), and in the rattlesnakes (genera *Crotalus* and *Sistrurus*), venom represents the near singular means by which these snake predators can capture prey (Ernst and Ernst, 2012; Klauber, 1972; Shine and Schwaner, 1985). Rattlesnakes rely on a strike-and-release behavioral pattern, allowing for their venoms to incapacitate prey without continuous contact with the prey (Chiszar et al., 1982), unless prey are small enough to be captured without incurring serious injury to the snake during ingestion. In some species and age groups, a strike-and-hold pattern can be observed (Kardong, 1986), but this is often accompanied by a fast-acting venom or behavioral preference for small-bodied prey. Rattlesnakes lack the muscular structure and force to constrict prey like pythons and boas, and the skull architecture that accommodates the venom apparatus is liable to be damaged by struggling prey that the snake is unable to restrain. Without other means of prey capture, having a venom that is well matched to disrupt the physiologies of local prey is critical to the persistence of rattlesnakes. Noting the importance of this matching between snake toxicity and prey susceptibility, researchers

have proposed a coevolutionary relationship between venomous snake predators and their prey (Barlow et al., 2006; Daltry et al., 1996). In coevolutionary terms, these species appear to compete in a chemical arms race (Dawkins, and Krebs, 1979), in which the appearance of novel toxicities in snake predators maintains the appearance of novel mutations conferring resistance in a closely associated prey species. The relationship between interacting predator and prey phenotypes is expected to last into perpetuity unless a novel selective equilibrium drives one of the partners into extinction or the pair loses contact (Van Valen, 1973).

The present study represents the description of a suspected venomous snake predator and resistant rodent prey pair that may be in a coevolutionary chemical arms race. The rattlesnakes studied were a population of Desert Massasauga (*Sistrurus tergeminus edwardsii*; formerly *S. catenatus edwardsii*) found in Lincoln County, Colorado, one of eleven counties where this subspecies exists in the southeast quadrant of Colorado (Hammerson, 1999). The entire group of Colorado populations generally occur quite distantly from other known localities of the subspecies that occur in the southern portions of Arizona, New Mexico, and Texas and into northern Mexico (Hobert, 1997; Goldberg and Holycross 1999). Individuals of this subspecies are known to prey primarily upon reptilian, mostly lizard, prey (~60% of gut contents), with most of the remainder of their diet composed of mammalian prey (~30% of gut contents) (Holycross and Mackessy 2002). Previous work with the population in Lincoln Co., Colorado indicated that *S. t. edwardsii* make a stereotypical egress from overwintering hibernacula to a foraging ground dominated by sand sage brush microhabitat (Wastell and Mackessy, 2011). Mammalian prey in this microhabitat are limited to a handful of rodent species

that are small enough to be consumed by these small snake predators; small mammal trapping along with gut content analysis indicated that the most likely prey candidate out of these rodent species was the Plains Pocket Mouse (*Perognathus flavescens*) (Holycross and Mackessy 2002; Wastell and Mackessy, 2011). Preliminary toxicity testing of *S. t. edwardsii* venom against *P. flavescens* and another candidate rodent prey species, the Deer Mouse (*Peromyscus maniculatus*), revealed that *P. flavescens* may be very susceptible to the venom while *P. maniculatus* appeared exceptionally resistant (personal communication, S. P. Mackessy). For the purposes of this discussion, resistance is the ability of prey to survive a direct challenge with rattlesnake venom at a dose (μg dry venom/ g body weight of prey) greater than what a rattlesnake would deliver in an average strike and release episode.

The first goal of this study was to assess whether these two species of mice were resistant or susceptible. Toxicity of venom from Lincoln County *S. c. edwardsii* towards these two mouse species captured in Lincoln County, Colorado, was compared to the toxicity of this venom against mice captured in Weld County, Colorado, where *S. c. edwardsii* are not present (Hammerson, 1999). Biardi and Coss (2011) showed that resistance in ground squirrels (genus *Otospermophilus*) was highest against the venom of rattlesnake captured in the same area as the squirrels and that resistance winnowed as squirrels were challenged with venom from increasingly distant rattlesnake populations. Similarly, we compared the toxicity of venom from Lincoln County *S. t. edwardsii* towards mice captured in the same area to the toxicity of the same venom against mice captured in Weld County, Colorado, where *S. t. edwardsii* are not present (Hammerson,

1999). Ultimately, *P. flavescens* was not found in Lincoln Co. so toxicity testing was only conducted on *P. maniculatus*. We hypothesized that:

H1: *P. maniculatus* from Lincoln Co. will be resistant to *S. t. edwardsii* venom, while *P. maniculatus* from Weld Co. will not be resistant.

Prediction: We predicted that Lincoln Co. mice would be able to survive when challenged with significantly higher amounts of *S. t. edwardsii* venom compared to Weld Co. mice.

The second goal of this study was to confirm whether resistance or susceptibility was specific to *S. t. edwardsii*, or if mice reacted similarly to the venom of a second rattlesnake predator species, the Prairie Rattlesnake (*Crotalus viridis viridis*). This larger rattlesnake is found across the state of Colorado (Hammerson, 1999; Stebbins, 2003) and is large enough to prey on either *P. flavescens* or *P. maniculatus*. Under a chemical arms race hypothesis (Dawkins and Krebs, 1979) it is possible to imagine that resistance may develop against a second predator, if the chemical defense(s) developed in the prey can the withstand the chemical weapons of both predators. Predator weapons may be similar enough that defenses developed in relation to one may provide protection against the other; that is, the mechanism of resistance may be able to defend against a broad range of chemical weapons. For these predators and prey, the latter situation would be necessary to account for venoms that are toxicologically divergent. *Sistrurus t. edwardsii* venom is dominated by degradative and hemorrhagic classes of toxins (Sanz et al., 2006), whereas *C. v. viridis* venom is expected to contain an abundance of myotoxin a, which primarily causes paralysis to incapacitate prey (Saviola et al., 2015). Based on described resistance mechanisms, a defense against both venoms might require a veritable arsenal of mechanisms to account for different toxicities. On the one hand, in systems where prey are resistant to venoms with a prevalence of degradative toxins, such as snake venom

metalloproteinases, endogenous inhibitor molecules appear to be the norm (Biardi et al., 2011; Garcia and Perez, 1984). On the other hand, resistance to toxins that bind to neuromuscular junctions or interfere with nerve conduction arises from structural changes to receptors or ion channels (Barchan, et al. 1992; Neumann et al., 1989; Ovadia and Kochva, 1977). Therefore, we hypothesized:

H_2: If resistance was present in Lincoln Co. *P. maniculatus*, it will be specific to *S. t. edwardsii*.

Prediction: We predicted that Lincoln Co. *P. maniculatus* would survive when challenged with venom from *S. t. edwardsii*, but succumb to smaller amounts of venom from *C. v. viridis*, regardless of whether *C. v. viridis* venom was collected in Lincoln or Weld Cos.

Two rattlesnake predator and resistant rodent prey systems have been described, and in both an endogenous inhibitor molecule confer resistance to prey taxa. Woodrats (genus *Neotoma*) have been shown to tolerate massive doses of Western Diamondback Rattlesnake (*Crotalus atrox*) venom and the venom of another North American pitviper (genus *Agkistrodon*). The mechanism of this resistance was a single, non-enzymatic molecule that specifically bound hemorrhagic toxins in the venoms; however, further descriptive work was not conducted to describe this molecule (de Wit, 1982; Garcia and Perez, 1984; Perez et al., 1978; Perez et al., 1979). A separate case of hemorrhagic toxin neutralizing resistance is described for a number of species of squirrels in the genus *Otospermophilus* (formerly *Spermophilus*) that are preyed upon by a number of rattlesnake predators (genus *Crotalus*) (Biardi and Coss, 2011; Biardi et al., 2011). Based on initial indications of elevated resistance to *S. t. edwardsii* venom in Lincoln Co. *P. maniculatus*, we attempted to describe the mechanism of resistance.

H_3: Resistance towards a venom dominated by degradative toxins would most likely come from an endogenous blood serum protein similar to other rattlesnake predator and rodent prey systems.

Prediction: Isolated resistance molecule(s) will be found in the blood serum, be identifiable as inhibitors of enzyme activity, and capable of significantly limiting the activity of key toxins in *S. t. edwardsii* venom from Lincoln Co. rattlesnakes.

Biochemical and proteomic procedures were used to attempt to isolate and describe a candidate resistance molecule. These data provided a working framework for further elucidation of the evolutionary processes underlying the dynamics of resistance and toxicity in a novel system of venomous snake predator and resistant mammalian prey.

Materials and Methods

Permitting and Animal Care and Use Statement

All animals were captured and used in experimental procedures using protocols approved by the University of Northern Colorado's Institutional Animal Care and Use Committee (protocol 0902E). Permission to capture *S. t. edwardsii* was obtained under IACUC Protocol 1302D-SM-S-16 and Colorado Parks and Wildlife permit 14HP974A1.

Animal and Snake Venom Collection

Individuals from several species of rodents and two rattlesnake species, the Desert Massasauga (*Sistrurus tergeminus edwardsii*) and the Prairie Rattlesnake (*Crotalus viridis viridis*) were captured on a private ranch in Lincoln County from the fall of 2013 through the spring of 2015. *Sistrurus t. edwardsii* and *C. v. viridis* were captured primarily during the months of April, May, and October, during their seasonal egress and ingress, respectively. Individuals were captured through road surveys, searches around known hibernacula, and general searches in the habitat. Snakes were transported to the University of Northern Colorado for venom extraction and processing before release to

their exact capture location within two weeks of capture. All venom samples used for this location were taken from adult snakes (total length ≥325 mm *S. t. edwardsii*; total length ≥800 mm for *C. v. viridis*). Samples were collected and stored individually following lyophilization.

Rodents were captured using small Sherman live traps baited with raw sunflower seeds and/or dried oats. Traps were placed in groups of 50-100 at least 10 m apart and in a rectangular grid pattern. Trapping locations consisted of four main microhabitats: 1) undisturbed sand hills populated by grasses (*Bouteloua* sp., *Andropogon* sp.) and sand sagebrush (*Artimesia filifolia*); 2) sand hills grazed by cattle; 3) along the fence line of a main, graded road running between property allotments of the ranch; and 4) along and around several buildings associated with the ranch operations headquarters. Traps were opened at dusk and recovered within the first two hours of daylight the following day.

Rodents from Weld County, Colorado were compared against rodents captured in Lincoln County to determine if venom resistance was localized only in Lincoln Co. rodents and if resistance was specific towards *S. t. edwardsii*. During the spring and summer of 2014, Deer Mice (*Peromyscus maniculatus*) and Prairie Rattlesnakes (*Crotalus viridis viridis*) were captured at two respective locations in Weld County, Colorado. *Peromyscus maniculatus* and *Mus musculus* were captured on a private residential property approximately 20 km east of Lucerne, Colorado. Individual mice were captured in small Sherman live capture traps baited with raw sunflower seeds and/or dried oats. Traps were placed approximately 10 meters apart along buildings and fence lines on the property. Traps were opened at dusk and recovered within the first two hours of daylight the next day. *Crotalus v. viridis* venom was extracted using standard lab

procedures from individuals caught at a local den site during spring egress and fall ingress. All samples used came from adult snakes (total length ≥ 800 mm). Samples were collected, lyophilized and stored individually.

Lethal Toxicity (LD_{50}) and Evaluation of Resistance

The toxicity of the two rattlesnake venoms was evaluated in two species of rodent, *P.maniculatus* and *M. musculus*. All venom doses were in 100 μL sterile water, injected intraperitoneally. Lethality was expressed as micrograms venom per gram body mass (μg/g) resulting in mortality after 24 hr (Reed and Muench, 1938). A full LD_{50} was determined only in *P. maniculatus* from Lincoln County challenged with *S. t. edwardsii* venom. The remaining toxicity studies included a maximum of only six individuals each in order to adhere to the three R's of animal research (European Commission, 2016). Because the LD_{50} of Lincoln Co. *S. t. edwardsii* venom in local *P. maniculatus* was so high, the remaining trials against other mice using other venoms were reduced, as no mice survived at doses fivefold less than the LD_{50} of resistant *P. maniculatus*.

To test resistance of *P. maniculatus* towards *S. t. edwardsii*, the LD_{50} of *S. t. edwardsii* venom was determined towards *P. maniculatus* captured in Lincoln Co. Doses for Lincoln Co. *S. t. edwardsii* were 10, 12.5,13, 13.4, 13.8, 14.2, 14.3, 14.4, 14.5, 15 and 20 μg/g. This specific LD_{50} in Lincoln Co. *P. maniculatus* was compared against a relative LD_{50} value determined for *P. maniculatus* and *M. musculus* captured in Weld Co. and challenged with *S. t. edwardsii* venom. Groups of three individuals of both species, captured in Weld Co., were challenged with 2.5 and 5 μg/g of *S. t. edwardsii* venom.

To test whether resistance towards *S. t. edwardsii* venom was specific to *P. maniculatus* captured in Lincoln Co., groups of three individuals of *P. maniculatus* and *M.*

musculus, captured in Weld Co., were challenged with 2.5 and 5 μg/g of *S. t. edwardsii* venom.

Finally, to test whether resistance towards *S. t. edwardsii* was specific towards local *S. t. edwardsii* venom or also neutralized the venom of a second sympatric rattlesnake predator, *C v. viridis*, groups of *P. maniculatus* and *M. musculus*, from both Weld and Lincoln Cos., were challenged with *C. v. viridis* venom. *Crotalus v. viridis* occur in both Weld and Lincoln Cos., so separate toxicity trials were conducted using venom from rattlesnakes caught in both counties. Thus, mice from both counties were challenged with venom from rattlesnakes captured in both counties. Six individuals (two groups of three) from both counties of *P. maniculatus* and *M. musculus* were challenged with venom from *C. v. viridis* captured in Lincoln Co. Three *P. maniculatus* from both counties were challenged with *C. v. viridis* venom from Weld Co. *C. v. viridis*. No toxicity trials were conducted challenging *M. musculus* from either location against the venom of Weld Co. *C. v. viridis*, as no *M. musculus* survived any toxicity trial at any dosage for any *S. t. edwardsii* venom.

Mouse Blood Serum Collection

Mouse blood serum from Lincoln County *P. maniculatus* was collected to explore the hypothesis that local resistance was conferred by a serum protein that was capable of neutralizing hemorrhagic and other degradative toxicities. One dozen *P. maniculatus* from Lincoln Co. were exsanguinated for blood serum. Mice were deeply anesthetized by passive inhalation of isoflurane gas and maintained under anesthesia for exsanguination by cardiac puncture. While supine, a 22g needle was inserted at an acute angle to the body, caudal to the xiphoid process of the sternum. Light negative pressure

was applied until flashback appeared in the hub of the syringe; the bolus of blood was extracted gradually to prevent collapse of the ventricle. Once a maximum volume of blood was extracted, the mice were euthanized by cervical dislocation. The blood was expressed into a sterile, non-heparinized 2 ml Eppendorf tube, and allowed to clot at room temperature for approximately 15 minutes. Mouse blood serum was separated by centrifugation at 4 °C for 10 minutes at 1,000 rpm. Individual samples of serum were drawn off and stored at -20°C.

Western Blot to Identify Candidate Resistance Molecule(s) in *P. maniculatus* Blood Serum

A Western blot was performed to identify candidate mouse blood serum protein(s) that may act as a resistance molecule towards *S. t. edwardsii* venom. Whole mouse serum and four 10X serial dilutions were separated by SDS-PAGE under reduced conditions, using commercially produced NuPage 12% Bis-Tris mini gels (Life Technologies, Inc. U.S.A.). The resulting gel was transferred to a nitrocellulose membrane using an XCELL II Blot Module (Invitrogen, Carlsbad, CA) on ice, using the manufacturer's protocol. The membrane was initially blocked using a 3% BSA solution in PBS for 2.5 hours at room temperature and then incubated for four hours at room temperature with 1 mg/ml of *S. t. edwardsii* venom in a 3 % BSA solution in PBS. After a triplicate wash in PBS, the membrane was blotted overnight at 4 °C with the commercially available antivenom CroFab® (BTG International Inc.) diluted 1:1000. After another triplicate wash in PBS, the membrane was incubated with an alkaline phosphatase-linked anti-ovine antibody (Sigma; 5 µL in 15 mL TBS) for one hour at room temperature. The membrane was then rinsed three times with TBS (50 mM Tris-

HCL with 150 mM NaCl) and developed using alkaline phosphatase substrate (Sigma; 5-bromo-4 chloro-3-indoyl phosphate/nitro blue tetrazolium tablet in water). Development was stopped with a 20 mM EDTA solution in PBS once obvious bands had formed.

Isolation of Suspected Resistance Molecule

A series of chromatography techniques followed by LC-MS/MS mass spectrometry were employed to isolate and identify suspected resistance molecules detected using the Western blot protocol described above. First, size exclusion chromatography was used for bulk separation of mouse blood serum proteins. Approximately 10 mg of serum protein was loaded onto a Phenomenex Yarra 2000 3µ size exclusion column (300 x 7.80 mm) on a Waters HPLC system using Empower software. The sample was separated using an isocratic flow of 25 mM HEPES buffer with 5 mM $CaCl_2$ and 100 mM NaCl at pH 6.8 for 120 minutes at a flow rate of 0.15 ml/min. Fractions were collected using a Waters Fraction Collector II, and protein peaks were monitored at 220 and 280 nm with a Waters 2487 Dual Absorbance Detector.

The serum separated into two large peaks, and gel electrophoresis confirmed that the first peak contained a majority of an approximately 60 kDa protein that strongly bound Lincoln County *S. t. edwardsii* venom in the Western blot procedure described above. This single peak from the size exclusion run was further isolated via reverse-phase HPLC using a Phenomenex Jupiter C_{18} (250 x 4.6 mm, 5 µm) column on a Waters HPLC system using Empower software. The column was equilibrated with 95% 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 5% of 0.1% TFA in 100% acetonitrile (solution B). Elution was performed by 95% solvent A and 5% solvent B for 5 minutes; linear gradient to 20% B over 5 minutes; linear gradient to 70% B over 30 minutes; linear

gradient to 100% B over 2 minutes; isocratic at 100% B for 15 minutes; linear gradient to 95% A, 5% B over 2 minutes; and isocratic at 95% A for 6 minutes. A Waters Fraction Collector II collected one minute fractions at a flow rate of 1.0 ml/min, with protein peaks monitored with a Waters 2487 Dual Absorbance Detector. One major peak eluted and was lyophilized and stored at -20 °C until sent out for protein identification by mass spectrometry.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

The candidate resistance molecule peak was analyzed by LC-MS/MS at the Florida State University College of Medicine Translational Science Laboratory (Tallahassee, FL, U.S.A.). Samples were digested using the Calbiochem ProteoExtract All-in-one Trypsin Digestion kit (Merck, Darmstadt, Germany) with LC/MS grade solvents according to the manufacturer's instructions. The LC-MS/MS analyses were performed using an LTQ Orbitrap Velos equipped with a Nanospray Flex ion source and interfaced to an Easy nanoLC II HPLC (Thermo Scientific). Peptide fragments were separated using a vented column configuration consisting of a 0.1 x 20 mm, 3 µm C_{18} trap column and a 0.075 x 100 mm, 3 µm C_{18} analytical column (SC001 and SC200 Easy Column respectively; Thermo Scientific). The elution gradient consisted of 5% buffer B (0.1% formic acid in HPLC grade acetonitrile) and 95% buffer A (0.1% formic acid) at the run start, to 35% B at 60 min, to 98% B from 63 to 78 min with a flow rate of 600 nl/min from 64 to 78 min, and 5% B at 300 nl/min at 79 min. The mass spectrometer was operated in positive mode nanoelectrospray with a spray voltage of +2300 V. A "Top 9" method was used with precursor ion scans in the Orbitrap at 60 K resolving power and fragment ion scans in the linear ion trap. Precursor ion selection using MIPS was enabled

for charge states of 2+, 3+ and 4+. Dynamic exclusion was applied for 60 s at 10 ppm. ITMS scans were performed using collision-induced dissociation (CID) at 35% normalized collision energy. MS/MS peptide spectra produced were interpreted using Mascot (Matrix Science, London, UK; version 1.4.0.288), Sequest (Thermo Fisher Scientific, San Jose, CA, U.S.A; version 1.4.0.288), and X! Tandem (thegpm.org; version CYCLONE 2010.12.01.1), assuming a trypsin digestion. The Mascot5_ Trembl_bony vertebrate database, and the Sequest and X! Tandem Uniprot Serpentes (A8570) databases, were used for homology searches. Sequest and X! Tandem were searched with a fragment ion mass tolerance set to 0.6 Da and a parent ion tolerance of 10 ppm. Mascot was searched with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 10 ppm. Glu/pyro-Glu of the N-terminus, ammonia loss of the N-terminus, Gln/pyro-Glu of the N-terminus, carbamidomethylation of cysteines and carboxymethylation of cysteines were specified as variable posttranslational modifications within X! Tandem. Oxidations of methionine, carbamidomethyl cysteine, and carboxymethyl cysteine were specified as variable post-translational modifications within Mascot and Sequest. Results were viewed and validated within Scaffold (Proteome Software Inc., Portland, OR, U.S.A; version 4.4.6), and protein identities were accepted if they could be established at >99.9% probability and contained at least one identified peptide. Given the sensitivity of the MS/MS instrument, purified protein identities were also restricted to those proteins that consisted of the largest number of fraction spectra, after normalization to protein molecular mass as determined by MALDI-TOF MS.

Enzyme Assays

The ability of *P. maniculatus* and blood serum to disrupt enzymatic toxins in *S. t. edwardsii* venom was tested against the four most abundant enzymatic toxin families in *S. t. edwardsii* venom (Sanz et al., 2006): snake venom metalloprotease (SVMP), phospholipase A_2, L-amino acid oxidase, and serine proteases. Established *in vitro* assay protocols were modified to include the addition of 5 μL of undiluted mouse blood serum to the initial solution of assay buffer and snake venom, incubating this mixture at room temperature (~22 °C) for 30 minutes, and completing the assay protocol as detailed below. Venom-only and substrate-only controls also were run in triplicate. The final volume of the reaction was maintained by adjusting the amount of reaction buffer. To assess the ability of mouse serum to affect enzymatic toxins, percent gain/loss in activity was determined by subtracting the specific activity of venom toxins in the presence of mouse serum from the specific activity of venom toxins alone, divided by the specific activity of venom toxins alone ($(Activity_{venom} - Activity_{venom+serum})/Activity_{venom}) *100\%$). Larger differences between the specific activities indicate a greater capacity to disrupt snake venom enzyme activity. SVMP activity was determined using azocasein as a substrate in the presence of 20 μg of venom (Aird and da Silva, 1991) with the activity expressed as ΔAbs_{342nm}/min/mg venom protein. Phospholipase A_2 activity was determined using Holzer and Mackessy's (1996) protocol using 4-nitro-3-(octanoyloxy) benzoic acid (Sigma) in the presence of 50 μg with activity expressed as nmol product/min/mg venom protein. Thrombin-like and kallikrein-like serine protease activities were measured using benzoyl-Phe-Val-Arg-paranitroaniline and benzoyl-Pro-Phe-Arg-paranitroaniline as substrates, respectively, in the presence of 8 μg of venom (Mackessy, 1993) and

expressing the activity as nmol product/min/mg venom protein. In the SVMP, kallikrein-like serine protese, and thrombin-like serine protease assays, serum from six Lincoln Co. *P. maniculatus* were tested against a sample of venom equally representing ten *S. t. edwardsii* from Lincoln Co. Because the phospholipase A_2 assay required a large sample two samples from individual *P. maniculatus* and one sample equally representing the remaining four *P. maniculatus* were tested against the same combined venom sample of ten *S. t. edwardsii*.

Results

Small Rodent Trapping and Venom Collection

A total of 1917 trap nights were completed in Lincoln County. Five species of rodents were captured: Deer Mouse (*Peromyscus maniculatus*), House Mouse (*Mus musculus*; numbers not recorded), Grasshopper Mouse (*Onychomys leucogaster*), Ord's Kangaroo Rat (*Dipodomys ordii*), and Thirteen-lined Ground Squirrel (*Spermophilus tridecemlineatus*; one individual) (Table 4.1). Accurate counts of *M. musculus* were not recorded; anecdotally, they are abundant, especially adjacent to buildings on the Lincoln County collection site. Only 50 trap nights were necessary in Weld County in order to collect a minimum number of *P. maniculatus* (27) necessary for toxicity testing.

Table 4.1 Species and numbers of small rodents trapped in 1917 trap nights in Lincoln County, CO. Notably, *Perognathus* sp. appear to be locally extirpated, while *Peromyscus maniculatus* are now abundant, differing from Wastell and Mackessy's (2011) rodent capture data.

Species	**# Individuals Captured in 1917 Trap Nights**
Deer Mouse *Peromyscus maniculatus*	105
Pocket Mouse *Perognathus* sp.	0
Grasshopper Mouse *Onychomys leucogaster*	112
Ord's Kangaroo Rat *Dipodomys ordii*	54

Toxicity Determinations

Based on two dose levels (2.5 and 5.0 μg/g), the LD_{50} of *S.c. edwardsii* venom for *M. musculus* in both Weld and Lincoln Counties is below 2.5 μg/g (Table 4.2). The LD_{50} of *S.c. edwardsii* venom for *P. maniculatus* was less than 2.5 μg/g for individuals captured in Weld County, and 13.5 ±0.1 μg/g for individuals captured in Lincoln County (Table 4.2). The LD_{50} of *C. v. viridis* venom for both *M. musculus* and *P. maniculatus* from both Weld and Lincoln Counties was below 2.5 μg/g (Table 4.2).

Table 4.2. Contingency table of LD_{50} values for four populations of rodents against three rattlesnake venoms.

	Lincoln Co. ***S. t. edwardsii***	**Lincoln Co.** ***C. v. viridis***	**Weld Co.** ***C. v. viridis***
Lincoln Co. ***P. maniculatus***	13.5 ± 0.1 μg/g n = 27	<2.5 μg/g n = 6	<2.5 μg/g n = 3
Weld Co. ***P. maniculatus***	<2.5 μg/g n = 6	<2.5 μg/g n = 6	<2.5 μg/g n = 3
Lincoln Co. ***M. musculus***	<2.5 μg/g n = 6	<2.5 μg/g n = 6	Not Tested
Weld Co. ***M. musculus***	<2.5 μg/g n = 6	<2.5 μg/g n = 6	Not Tested

Identification of Molecule Conveying Resistance

Western blot detected a single band in mouse serum (approximately 60 kDa) that bound *S. t. edwardsii* venom (Figure 4.1). This protein was isolated first using size exclusion chromatography followed by a cleanup procedure using reverse phase HPLC. For each chromatography procedure, SDS-PAGE was used to confirm that the 60 kDa band was being isolated. LC MS/MS following trypsin digestion of this isolated protein returned an identity of mouse serum albumin (*M. musculus*; 100% protein identification probability from 53 spectra; NCBI/Uniprot accession number P07724; ALBU_MOUSE).

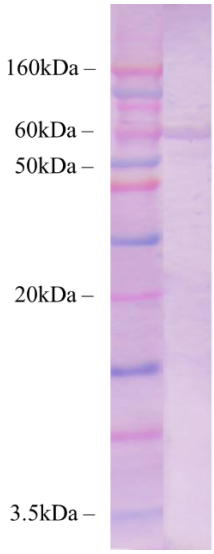

Figure 4.1. Western blot of 1:100 Lincoln Co. *P. maniculatus* serum detected with *S. t. edwardsii* venom. The visible band at ~60 kDa represents the candidate resistance molecule that was in high abundance and readily bound *S. t. edwardsii* venom.

Enzyme Activity Inhibition Assays

A Student's t-test was used to analyze the difference in activities between samples of crude *S. t. edwardsii* venom alone and samples treated with an equal volume of whole blood serum from *P. maniculatus* captured in Lincoln County. There was a statistically significant decrease in metalloprotease activity when mouse serum was added to the reaction mixture ($t(7) = 2.51$; $p = 0.04$) (Figure 4.2A). Activity was inhibited an average of 2.6% from untreated venom (untreated M = 1.04 ΔAbs_{342nm}/min/mg venom protein; SD = 0.016; with serum M = 1.01, SD = 0.015) (Figure 4.2A). Phospholipase A_2 activity was not statistically different in reaction mixtures where mouse serum was included ($t(10) = 1.42$; $p = 0.19$) (Figure 4.2B). The serum had its own endogenous capacity to cleave the assay substrate greatly increasing the total activity in the assay when combined with the venom (untreated venom M = 17.78 nmol product/min/mg; SD = 0.27; with serum M = 45.3 nmol product/min/mg; SD = 32.55). Thrombin-like serine protease activity was not statistically different between the two groups ($t(6) = 0.91$; $p = 0.40$;

untreated venom M = 545.91 nmol/min/mg; SD = 4.29; with serum M = 646.64 nmol/min/mg; SD = 149.32) (Figure 4.3C). Kallikrein-like serine protease activity was significantly higher when mouse serum was added to the reaction mixture ($t(6) = 10.39$; $p < 0.0001$) (untreated M = 1070.18 nmol/min/mg, SD = 38.9; with serum M = 1854.37 nmol/min/mg, SD = 99.76) (Figure 4.2D).

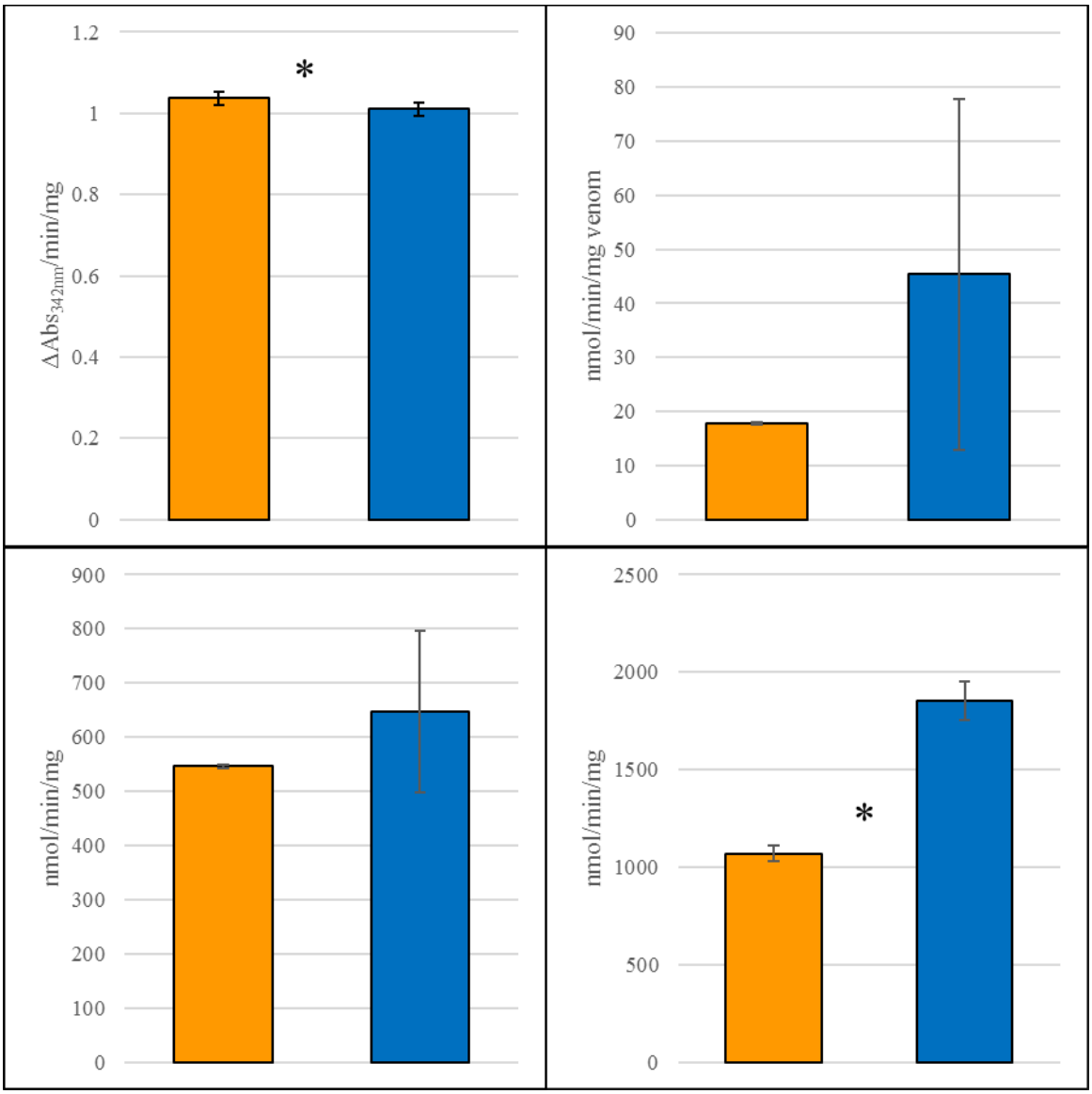

Figure 4.2. Snake venom enzyme activity inhibition assay data. A pooled mixture of venom from 10 individual *S. t. edwardsii* was used in all four assays, the number of individual mice used in assay is listed below. (A) Snake venom metalloprotefase assay (6 serum samples); (B) phosphlipase A_2 assay (3 serum samples); (C) thrombin-like serine protease assay (6 serum samples); and, (D) kallikrein-like serine protease assay (6 serum samples). The averages and standard deviation of untreated *S. t. edwardsii* venom activities are represented in ORANGE, and those of the same venom in the presence of Lincoln Co. *P. maniculatus* serum are represented in BLUE. An asterisk indicates a statistically significant difference ($p < 0.05$).

Discussion

Lethal toxicity assays confirmed partial resistance in a population of *Peromyscus maniculatus* from Lincoln County, Colorado against the venom of a local venomous

snake predator, *Sistrurus tergeminus edwardsii* (Table 4.2). This resistance appears locally restricted, as *P. maniculatus* captured in areas where *S. t. edwardsii* is not found showed no resistance to the venom. The resistant experimental lethal dose, at least 5-fold higher than non-resistant Weld Co. mice, may allow a portion of *P. maniculatus* to avoid capture by *S. t. edwardsii* in Lincoln Co. By venom yield alone, the average *S. t. edwardsii* would be able to kill a *P. maniculatus* with the recorded LD_{50} (13.5 ± 0.1 μg/g) by delivering its full bolus of venom (Glenn and Straight, 1982); however, assuming the snake can only deliver a portion of this (Ernst and Ernst 2012), some mice would survive. Further, in support of our second hypothesis, resistance in Lincoln Co. *P. maniculatus* was specific to *S. t. edwardsii* venom, as these mice showed no resistance against a second sympatric venomous snake predator, *Crotalus viridis viridis* (Table 4.2). Venoms from both species of rattlesnake in Lincoln Co. share certain families of toxins; however, the abundance and identity of individual toxins in these venoms differs (Sanz et al. 2006; Saviola et al., 2015), likely explaining the difference in local *P. maniculatus* ability to withstand their venom.

A majority of toxins in *S. t. edwardsii* venom were identified as snake venom metalloproteases (SVMPs; Sanz et al., 2006), and we tested for the presence of an endogenous resistance molecule against this activity, similar to that found in the well-described ground-squirrel system (Biardi and Coss, 2011; Biardi et al., 2011a; Biardi et al., 2011b). There was a statistically significant reduction in metalloprotease activity when *P. maniculatus* serum was added to the reaction mixture; however, on average this decrease was very small and may not be physiologically relevant (Figure 4.2A). We then tested whether mouse serum neutralized the activity of the next three most abundant and

physiologically relevant enzyme snake venom toxins (Sanz et al., 2006) in *S. t. edwardsii* venom and found they were either unaffected or the endogenous activity of the mouse serum was significantly greater than the venom itself (4.2 B, C, D). The venom enzyme activities selected for the experiment are the most abundant in the venom (Sanz et al., 2006), but it appears that three of four selected may not be ecologically relevant to prey capture as the mouse serum had a greater capacity to cleave assay substrates than did snake venom. Thus resistance, if any, appears specific to SVMPs in *S. t. edwardsii* venom.

A Western blot was used to identify Lincoln Co. *P. maniculatus* serum proteins that bound Lincoln Co. *S. t. edwardsii* venom, and the single band that bound venom proteins was identified as mouse serum albumin. We did not conduct a Western blot against the serum of *P. maniculatus* captured outside of Lincoln Co. and cannot confirm or refute that this protein is involved in a resistance mechanism. Resistance molecules have been described in at least three other systems. California and Mexican Ground Squirrels (Biardi and Coss, 2011, Biardi et al., 2011a; Biardi et al., 2011b; Martinez et al., 1999) and Wood Rats (Garcia and Perez, 1984) have been shown to produce free globular proteins that acted as SVMP inhibitors, and changes in a hemostatic blood protein cause resistance in Didelphid oppossums (Jansa and Voss, 2011). In the *Peromyscus* system, a serum resistance protein does not appear to be present. Another possibility is that there is a serum protein that is able to confer resistance, but is masked by a preponderance of albumin in the serum samples. Following methods similar to Biardi et al. (2011b), we did not use albumin-depleted serum samples, nor did we adjust the amount of venom protein used in the assay reactions to a concentration that was

comparable to concentrations expected in a natural envenomation event. Increased levels of albumin and venom proteins may have masked any protective effects of a free serum protein that provides resistance. On the one hand, the relative 'sticky' nature of albumin may have been able to bind *S. t. edwardsii* toxins in the Western blot, while a true resistance molecule was unable to. On the other hand, a low-abundance resistance molecule may be missed in our assay mixture as the amount of venom used in the assay could exceed the concentration of venom in the bloodstream following envenomation. Thus, the assay as conducted may not be able to assess protection and an assay procedure scaled to biologically relevant concentrations of venom protein would be useful in future assays. It may also be that another mechanism is responsible for resistance in Lincoln Co. *P. maniculatus*.

Assuming that reducing metalloprotease activity in *S. t. edwardsii* venom is sufficient to protect a significant number of *P. maniculatus*, it may be that substrates targeted by SVMPs are resistant to proteolysis and thus unavailable as substrates for enzyme toxins. Hemorrhage, one of the main pathologies associated with SVMPs, is caused by disruption of endothelial cell and basement membrane interactions through cleavage of extracellular proteins on both cell types (Gutiérrez and Rucavado, 2000; Gutiérrez et al., 2016a; Takeda et al., 2012). Escalante et al. (2011) proposed a two-step model for hemorrhage caused by SVMPs in which extracellular membrane proteins surrounding microvasculature are degraded and the subsequent force of normal blood perfusion causes breaks in capillary walls. Possibly, the extracellular matrix of *P. maniculatus* found in Lincoln Co. is resistant to the degradative action of *S. t. edwardsii* venom metalloproteases, preventing hemorrshage as a primary pathology of snakebite

envenomation. The assays conducted in this study used azocasein as a model substrate, but it may not represent the types of structural proteins found in ths population of *P. maniculatus*.

Another possibility is that there is some anatomical or physiological difference in this population that allows them to resist the toxicity of a rattlesnake predator. All doses in the toxicity study were administered by intraperitoneal route; a difference in uptake from the abdominal cavity into general circulation could have impeded the ability of venom to enter the bloodstream. Substances injected by intraperitoneal route are absorbed into the vasculature of the mesentery and pass through the portal system before moving into general circulation (Lukas et al., 1971). Sequestration of the venom in the abdominal cavity may provide enough time for the immune system to target and destroy venom toxins before they are able to cause serious pathology, given there are endogenous antibodies that could recognize venom toxins. Slower uptake into the general circulatory system may allow the body to clear venom at a rate fast enough to maintain a sublethal dose in the bloodstream. Concerning physiological resistance, there may be some differences in degradation of toxins as the venom bolus passes through the hepatic portal system, similar to the differential metabolism of drugs by different parenteral routes (Chaudhary et al., 2010; Fromm et al., 1996; Williams, 1972). Further investigations, including testing the venom-neutralizing capacity of albumin-depleted serum samples, necropsy of resistant and susceptible individuals following administration of a toxic dose, and genetic and proteomic screening for differences in structural proteins, are likely to help uncover the underlying mechanism of resistance in Lincoln Co. *P. maniculatus*.

A final unexplored mechanism of resistance may be the immune state of the populations of mice studied. Recent work in immunology has revealed a gross incompatibility between traditional *in vivo* models and real world immune responses. Researchers conventionally rely on the cleanliness of captive mouse strains to eliminate confounding variation from disease or other environmental factors; however, there is increasing evidence that these clean mice may no longer be good representative model organisms as was once thought. Laboratory mice cohoused with mice purchased in a pet store were more immunologically similar to adult humans than mice maintained in specific pathogen free barrier facilities (Beura et al., 2016). Mice sequentially infected with a number of pathogens showed responses similar to adult humans when vaccinated against yellow fever virus (Reese et al., 2016). Laboratory mice inoculated with the gut microbiome of wild mice were more resistant to infection with influenza virus and mutagen/inflammation-induced colorectal tumorigenesis (Rosshart et al., 2017). However, not all inoculations are supportive of immune function; Leung et al. (2018) demonstrated increased susceptibility to nematodes (*Trichuris muris*) when laboratory mice were 're-wilded' by being housed outdoors. While the mice tested in this study were all wild-caught, differences in immunocompetence related to environmental variation may play a role in the resistance or susceptibility of these populations to the venom of *S. t. edwardsii*.

Regardless of the mechanism of resistance, the toxicity data indicated that we may be witnessing a period of flux in the predator-prey dynamics of this rattlesnake and rodent pair that signals a shift in balance of a chemical arms race. Previous small mammal trapping in the area indicated that *S. t. edwardsii* in Lincoln Co. were

preferentially egressing from hibernacula to microhabitat populated by *Perognathus flavescens* (Wastell and Mackessy, 2011). These mice appeared readily susceptible to the venom of local *S. t. edwardsii* (SPM, personal communication). Trapping data from the present study indicated that *P. flavescens* have been locally extirpated and that *P. maniculatus* have overtaken microhabitat once occupied by *P. flavescens* (Table 4.1). Another rodent species, *Mus musculus*, now also colonizes the area and is readily susceptible to the venom of *S. t. edwardsii*, but it is unknown whether *S. t. edwardsii* capitalize on this new resident species, as it was not recorded in the diet of this rattlesnake (Holycross and Mackessy 2002). The other two abundant small rodents captured in the area, *Onychomys leucogaster* and *Dipodomys ordii*, are too large as adults to be consumed by *S. t. edwardsii*, regardless of their reaction to venom. If *S. t. edwardsii* is attempting to prey upon *O. leucogaster* and *D. ordii*, they must be seeking out young individuals that they are mechanically able to consume. Additionally, the experience of these researchers indicates that juvenile to adult *O. leucogaster* are particularly robust and behaviorally aggressive, suggesting they are not a likely target for *S. t. edwardsii*, a small rattlesnake. In other areas, *Onychomys* species are able to capture large prey items such as scorpions, grasshoppers, and other rodent species (Armstrong et al., 2011; Rowe and Rowe, 2008); a small rattlesnake may therefore be at a disadvantage or become a prey item itself if it attempts to prey on on this species. The recently abundant *M. musculus* may be the most readily available prey source for *S. t. edwardsii*, but capturing them may require some modification of predatory behaviors, as they were not found in the areas that Wastell and Mackessy (2011) defined as their summer forage grounds. In that study, *M. musculus* were most readily captured next to the private

residence of the landowners and adjacent outbuildings. *S. t. edwardsii* were most commonly observed crossing into canonical foraging areas, and none were detected near the aforementioned buildings; however, they are highly cryptic and undetectable in the foraging grounds, so they may also have been present near human habitation.

Overall, there appears to be a detectable shift in mammalian prey diversity and abundance in the foraging areas of *S. t. edwardsii* in this region. Replicating Wastell and Mackessy (2011), trapping in the same locations, during the same times of year, over approximately half the number of trap nights, *P. maniculatus* appeared to have become the predominant rodent species. Following Dawkins and Krebs (1979) predictions about chemical arms races between predators and prey, an overabundance of resistant prey may affect *S. t. edwardsii* by eliminating individuals from the population that do not have a venom phenotype that is able to capture locally available mammalian prey. In response, four main possibilities are expected: first, *S. t. edwardsii* may become locally extirpated if there are no individuals that are able to overcome the chemical defenses of resistant *P. maniculatus*. Second, there may be *S. t. edwardsii* that are able to deliver enough venom capable of incapacitating *P. maniculatus*; thus, persisting individuals would repopulate the area and the average *S. t. edwardsii* venom would be toxic enough to overcome developing resistance in *P. maniculatus*. In this case, the venomous predator would respond with reciprocity, as in Van Valen's (1973) Red Queen Hypothesis. A third possibility is that *S. t. edwardsii* in this area will be able to withstand local shifts in mammalian prey abundance/resistance by shifting to a predominantly non-mammalian diet. Holycross and Mackessy (2002) calculated that mammalian prey made up 40.4% of the diet of *S. t. edwardsii* from Colorado populations. Snakes in Lincoln Co. may persist,

assuming they can capture a sufficient number of non-mammalian prey to make up for a loss of available mammalian prey. Fourth, *S. t. edwardsii* may shift their preference towards other mammalian prey that are abundant and susceptible to their venom, namely *Mus musculus*. Tracking the population dynamics of predators and prey in this local area over time will be required to elucidate which of these outcomes is most likely. What remains clear is that resistance is geographically bound to areas where *S. t. edwardsii* and *P. maniculatus* are sympatric. This local effect indicates that predation by *S. t. edwardsii* has acted as a selective pressure on *P. maniculatus*, driving the development of specific resistance against *S. t. edwardsii* venom.

Conclusion

Suspected resistance in a single mammalian prey species, *Peromyscus maniculatus*, was confirmed through venom toxicity challenges with the venoms of two sympatric rattlesnake predators, *Crotalus viridis viridis* and *Sistrurus tergeminus edwardsii*. Fivefold resistance was found in *P. maniculatus* only where this small rodent is sympatric with *S. t. edwardsii*; *P. maniculatus* captured in areas where *S. t. edwardsii* does not occur had no detectable resistance to the venom, and both rodent populations were equally susceptible to sympatric and non-sympatric *C. v. viridis* venom.

Assaying for a mechanism to explain this elevated resistance in local *P. maniculatus*, we conducted enzyme toxin assays in the presence of resistant mouse serum. There was a statistically significant decrease in the activity of the most abundant enzyme toxin family, metalloproteases. There was no detectable difference in thrombin-like serine protease activity in the presence of resistant mouse serum, and the mouse serum itself had greater levels of kallikrein-like serine protease and phospholipase A_2 activity

than *S. t. edwardsii* alone. Snake venom metalloproteases may be the most likely target of a resistance mechanism in this population of *P. maniculatus*.

Finally, we attempted to isolate a resistance molecule in resistant mouse serum, similar to other resistance molecules found in rodent prey of rattlesnakes. The serum protein that was able to bind *S. t. edwardsii* venom was identified as mouse serum albumin, not a specific metalloprotease inhibitor or anti-hemorrhagic factor as found in other venom-resistant rodent prey. It is possible that a free serum protein is responsible for resistance in this population of *P. maniculatus*, but was undetectable because of a swamping effect of abundant mouse serum albumin. A completely different mechanism from a serum protein, such as resistance of basal lamina matrix to proteolysis or slow uptake into systemic circulation, may provide a means of escaping capture in these mice. Future studies of the interaction between this local predator-prey pair will add to our understanding of the dynamics of venomous snake predators and their prey. As venomous snakes use their venoms primarily for prey capture, prey effects are hypothesized to act as strong selective pressures shaping venom compositional evolution. Identifying and tracking populations of predators and prey where trends in resistance and susceptibility can be observed in real time afford us a vantage point into the long evolutionary history of the development of snake venom as a trophic adaptation.

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CHAPTER V

CONCLUSIONS

The diversity of toxins that compose snake venoms has allowed these predators to capitalize on a wide range of prey species. Found on all continents except Antarctica, venomous snakes employ these chemical weapons to incapacitate food items by disabling any number of physiological processes in a prey's body (Mackessy, 2010b). Immense effort has been taken to document the diversity of these toxins and their functions, defining a set of toxin families that are variably expressed within and between species (and higher taxonomic designations) of snakes, within and between populations of venomous snakes, and across the lifetimes of individual snakes (see Chapter I for a comprehensive review). The rate at which new variants in individual toxins and venom mixtures are described indicates that there is far more that we do not yet know about the breadth of diversity in snake venoms (Calvete, 2013; Tasoulis and Ibister, 2017). This horizon of scientific discovery, in combination with the observation that venom serves as a specialized trophic weapon, has incited new lines of inquiry into the interactions of venomous snakes and their prey, the role of competition in shaping toxicity and resistance, and attempts to match hypothetical predictions in eco-evolutionary theory to the natural system of venomous snake biology (e.g. Daltry et al., 1996; Holding et al., 2016). The major thrust of this dissertation was to add to this growing conversation

surrounding venom compositional diversity and the effects of various ecological factors on its stability or change.

To investigate questions of venom composition change it has become necessary to probe for toxin identities and abundances, as well as functional (pathological) characteristics of historical samples that may have been kept in storage for extended periods. While there is some evidence that venom is a relatively stable biological sample, given proper handling and storage, few attempts have been made to assess the quality of venom samples following long-term storage (Munekiyo and Mackessy, 1998; Russell, 1960; Schottler, 1951). Chapter II provided evidence for stability in the identity and molecular quality of venoms from the Northern Pacific Rattlesnake (*Crotalus oreganus oreganus*) stored for ~35 years. In this data set, however, several toxin enzyme activities appeared to degrade over time; however, it is hard to know whether this conclusion is generalizable, as sample sizes were small. In any case, this chapter lends support to Jesupret et al.'s (2014) conclusion that historical sample are relevant repositories of information given they are properly handled and stored. This and the present study highlight the importance of continued descriptive work on extant populations of venomous snakes and support a call for cataloging and preparing samples and data (e.g. enzyme activity data, proteomic data, etc.) for long term storage and future comparative studies.

Chapter III exemplifies the type of analyses made possible by the availability of long-term stored samples. Having a large number of venom samples with substantial amounts of material allowed for a comparison between individuals from the same geographic populations across a ~35-year period. Enzyme activity assays, 1-dimensional

SDS polyacrylamide gel electrophoresis banding patterns, and reverse phase high performance liquid chromatography (RP-HPLC) peak area analysis were used to assess differences in composition within and between geographic locations, between two time points, and from both a structural (i.e. proteomic/venomic) and functional perspective. Our hypothesis that venom composition would not differ significantly after ~35 years time was supported in most of the analyses. Individual variability was detected in all three analyses, but most samples did not segregate by any variable (except for one instance). For all three localities tested, more recently collected samples had a higher amount of two RP-HPLC peaks containing L-amino acid oxidases and snake venom metalloproteases, respectively. While this difference in peak area was not supported by the enzyme assay data, the data provide a narrowed focus for future investigation to elucidate how venomic (venom proteomic) changes may occur in natural settings. This chapter also provides an excellent example of a holistic approach to assessing venom compositional diversity. So-called 'venomic' approaches have dominated snake venom science in the past decade (Lomonte and Calvete, 2017) and the approach demonstrated in Chapter III of this dissertation represents a complementary analytical process that allows investigators to probe for broad differences in venom composition and activity across large groups of samples. Thus, this method may act as a holistic screening process to identify molecules or activities of particular interest, rather than conducting a full proteomic workup that take longer and require access to mass spectrophotometric equipment. In total, this approach of assessing venom composition promises to expand and enhance our understanding of the dynamics of snake venom in response to ecological pressures.

Chapter IV explored one proposed driver of snake venom diversity, namely diet. Previous work on the Desert Massasauga (*Sistrurus tergeminus edwardsii*) in southeastern Colorado (Wastell and Mackessy, 2011) indicated that a rodent prey species of this rattlesnake might be resistant to the venom of this rattlesnake. Live rodent trapping and toxicity testing revealed that Deer Mice (*Peromyscus maniculatus*) from the study site were resistant to *S. t. edwardsii* venom. Moreover, this appeared to be a local adaptation, as *P. maniculatus* captured in areas without *S. t. edwardsii* were not resistant, and resistance appeared to be specific to *S. t. edwardsii*, as venom from another sympatric rattlesnake, the Prairie Rattlesnake (*Crotalus viridis viridis*), readily caused mortality. It appeared that this system was similar to documented pairs of rattlesnakes and resistant mammalian prey (e.g. Biardi and Coss, 2011; de Wit, 1982; Garcia and Perez, 1984), and we proposed that resistance might be conferred by a free serum protein capable of inhibiting SVMP activity in *S. t. edwardsii*. Enzyme inhibition assays did not support this conclusion and attempts to isolate a resistance molecule only indicated mouse serum albumin, which appeared to bind readily to *S. t. edwardsii* venom proteins.

While the present investigation was unable to uncover an exact mechanism of resistance in this population of *P. maniculatus*, it provides a base of information for continued surveillance of predator-prey dynamics in these two species. Although mouse serum did not inhibit venom activities significantly, toxicity testing indicated that the level of resistance in these mouse would allow a portion of the population to evade prey capture by *S. t. edwardsii*. The present study provides a robust scaffold for future investigations that may attempt to elucidate a resistance mechanism that appears to thwart snake venom toxicity differently than in other published accounts. Results reported here

will support future work tracking population dynamics between this predator-prey pair to determine the coevolutionary mechanisms that are driving the appearance and maintenance (or enhancement) of *P. maniculatus* resistance and the reciprocal effects on *S. t. edwardsii* venom composition.

Understanding population-level venom composition requires access to and means of assessing historical samples, methods that can account for a variety of measurable venom characteristics, and robust preliminary data to initiate new lines of inquiry. This dissertation has provided all three in an attempt to expand our ever growing wealth of knowledge of snake venoms, their diversity, and their roles as trophic weapons driving ecological and subsequently evolutionary processes. Expanding our knowledge in this way makes room for increasingly comprehensive assessments of venom complexity and is also likely to reinforce the notion that snakes and their venoms represent excellent models for investigating ecological and evolutionary processes. This dissertation provides data to support a new trajectory in snake toxinology that takes into account the intricate and variable phenotype of snake venom.

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APPENDIX A

SUMMARY TABLES FOR ENZYME ASSAY DATA

Capture Location	Collection Date	Average Metalloprotease	St. Error	Average Phosphodiesterase	St. Error
		ΔA_{342nm}/min/mg		ΔAbs_{400nm}/min/mg	
Chimineas Region	1978-1982	0.50	0.08	0.13	0.02
	2014-2016	0.81	0.10	0.09	0.02
Carrizo Plain National Monument	1978-1982	0.79	0.02	0.16	0.02
	2014-2016	0.71	0.03	0.32	0.05
Montaña de Oro	1978-1982	0.66	0.11	0.27	0.05
	2014-2016	1.02	0.05	0.24	0.04

Capture Location	Collection Date	Average L-Amino Acid Oxidase	St. Error	Average Thrombin-Like Serine Protease	St. Error
		nmol product/min/mg		nmol product/min/mg	
Chimineas Region	1978-1982	44.22	4.64	2469.25	217.66
	2014-2016	33.88	7.66	1485.22	264.04
Carrizo Plain National Monument	1978-1982	46.63	2.65	1752.33	161.55
	2014-2016	75.75	3.16	1895.57	160.14
Montaña de Oro	1978-1982	51.18	6.12	1217.68	168.30
	2014-2016	55.34	2.03	990.55	105.30

Capture Location	Collection Date	Average Kallikrein-Like Serine Protease	St. Error	Average Phospholipase A_2	St. Error
		nmol product/min/mg		nmol product/min/mg	
Chimineas Region	1978-1982	1883.74	285.29	18.09	4.28
	2014-2016	1853.70	236.00	7.11	1.62
Carrizo Plain National Monument	1978-1982	2214.13	142.53	12.33	1.87
	2014-2016	2405.00	195.91	13.42	2.81
Montaña de Oro	1978-1982	1731.01	348.38	18.79	4.33
	2014-2016	1609.48	139.36	11.08	1.32

APPENDIX B

IDENTITIES OF SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS BANDS

Band Size (kDa)	Protein ID	Band ID
>66.377	L- Amino Acid Oxidase	LAAO-67
62.377-66.377	Nuclease	Nuc-65
56-61	Nuclease	Nuc- 60
55.769-57.769	PIII Metalloprotease	SVMP3-56
47.224-51.224	PIII Metalloprotease	SVMP3-50
42	Serine Protease	SP-42
40	Serine Protease	SP-40
38	Serine Protease	SP-38
36.3-37.9	Serine Protease	SP-37
28.696-32.696	Serine Protease	SP-30
24.04-28.04	Cystein-Rich Secretory Protein	CRiSP-25
20.41-24.41	PI Metalloprotease	SVMP1-22
13.235-17.235	Phospholipase A_2	Pla-15
13	Phospholipase A_2	Pla-13
11	Disintegrin	Dis-11
10	Disintegrin	Dis-10
6.6-6.5	Disintegrin	Dis-6
4.5-5.5	Myotoxin	Myo-5
>3.3	Myotoxin	Myo-3
<3.2	Bradykinin Potentiating Peptide	BPP-2

APPENDIX C

1-D SDS POLYACRYLAMIDE GEL ELECTROPHORESIS BANDING PATTERNS

	SPM146	SPM249	SPM325	SPM347	SPM578	SPM589	SPM658	SPM660	SPM661	MH098	MH102	MH105	MH110	MH115	MH116
Band67	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Band65	0	1	0	0	0	1	0	1	0	0	1	1	0	1	1
Band58	0	1	0	1	1	0	0	0	0	1	0	0	1	0	0
Band56	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
Band48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band42	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band40	1	1	1	1	1	0	1	1	1	0	1	0	1	1	1
Band38	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band36	1	1	1	0	0	0	1	1	0	1	1	0	1	1	0
Band30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band26	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
Band22	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1
Band15	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
Band13	0	0	1	1	1	0	0	1	1	0	1	1	0	1	1
Band11	0	0	0	0	1	1	1	0	0	1	0	1	1	1	1
Band10	0	1	0	0	0	0	0	1	1	1	1	0	1	1	0
Band6	0	1	0	1	1	0	1	1	1	1	0	1	1	0	1
Band5	1	1	1	1	0	1	1	0	0	1	1	1	1	1	0
Band3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

1-d SDS PAGE banding patterns for samples collected in the Chimineas Region. Samples with the prefix, "SPM-", were collected between 1978-1982, and samples with the prefix, "MH-", were collected between 2014-2016 Bins where the band analysis program detected and scored a 'present' band contain a "1" and are highlighted in blue, while absent bands are represented with a "0" and are not highlighted.

	SPM072	SPM073	SPM074	SPM076	SPM077	SPM078	SPM079	SPM082	SPM084	SPM111	SPM340	SPM341	SPM379	SPM380	SPM383
Band67	0	0	0	1	0	0	0	1	1	0	0	0	1	0	1
Band65	1	1	1	0	1	0	0	0	0	1	1	1	0	1	0
Band58	0	0	0	0	1	0	1	0	1	1	0	0	1	0	1
Band56	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Band48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band42	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1
Band40	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
Band38	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
Band36	1	1	1	1	1	1	1	0	1	1	0	0	1	0	0
Band30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Band15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band13	1	0	0	1	1	0	1	1	0	1	1	0	0	1	1
Band11	0	1	1	0	1	1	0	1	1	0	1	1	0	0	1
Band10	1	1	0	1	0	0	1	0	1	1	1	0	1	1	0
Band6	1	0	1	1	1	1	1	1	0	0	1	1	0	1	1
Band5	1	1	0	1	0	1	0	0	1	1	0	1	1	1	0
Band3	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Band2	1	0	0	1	1	1	0	0	1	0	0	0	0	0	1

1-d SDS PAGE banding patterns for samples collected in Carrizo Plain National Monument. All samples in this table were collected from 1978-1982. Bins where the band analysis program detected and scored a 'present' band contain a "1" and are highlighted in blue, while absent bands are represented with a "0" and are not highlighted.

	SPM440	SPM560	SPM579	SPM580	SPM581	SPM582	SPM641	SPM715	SPM750	SPM806
Band67	0	0	0	0	0	0	0	1	0	0
Band65	1	1	1	1	0	1	1	1	1	1
Band58	1	0	0	0	1	0	1	0	0	1
Band56	1	1	1	1	1	1	1	1	1	1
Band48	1	1	1	1	1	1	1	1	1	1
Band42	0	0	0	0	0	0	0	1	0	0
Band40	1	1	0	1	1	1	1	1	1	1
Band38	0	0	0	0	0	0	0	0	0	0
Band36	0	1	0	1	1	1	1	0	1	0
Band30	1	1	1	1	1	1	1	1	1	1
Band26	1	1	1	1	1	1	1	1	1	1
Band22	0	1	1	1	1	1	0	1	0	0
Band15	1	1	1	1	1	1	1	1	1	1
Band13	1	1	1	0	1	0	1	1	1	1
Band11	0	0	0	1	0	0	1	0	0	0
Band10	1	1	1	1	1	1	0	1	1	1
Band6	1	0	1	0	0	1	1	0	0	0
Band5	0	1	0	1	1	1	0	1	1	1
Band3	1	0	0	0	1	0	0	0	0	0
Band2	1	0	1	0	0	0	1	0	1	1

1-d SDS PAGE banding patterns for samples collected in Carrizo Plain National Monument. All samples in this table were collected from 1978-1982. Bins where the band analysis program detected and scored a 'present' band contain a "1" and are highlighted in blue, while absent bands are represented with a "0" and are not highlighted.

	JGM01	JGM02	JGM03	JGM04	JGM06	JGM07	JGM08	JGM09	JGM10	JGM11	JGM12	JGM13	JGM14	JGM15	JGM16	JGM17	JGM18
Band67	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band65	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1
Band58	0	0	0	1	0	1	0	0	0	1	1	1	0	1	0	0	0
Band56	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band48	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
Band42	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1
Band40	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Band38	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
Band36	1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	0
Band30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band22	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Band15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band13	1	1	0	0	1	1	1	0	0	1	1	1	0	0	1	0	1
Band11	0	1	1	1	0	1	0	1	1	1	0	1	0	1	0	1	1
Band10	1	0	1	1	1	0	1	1	0	0	1	1	1	1	1	0	0
Band6	1	1	1	1	1	1	1	0	1	1	1	0	0	1	1	1	1
Band5	0	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0
Band3	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1	1	0
Band2	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	1	0

1-d SDS PAGE banding patterns for samples collected in Carrizo Plain National Monument. All samples in this table were collected from 2014-2016. Bins where the band analysis program detected and scored a 'present' band contain a "1" and are highlighted in blue, while absent bands are represented with a "0" and are not highlighted.

	SPM029	SPM033	SPM034	SPM035	SPM036	SPM037	SPM038	MH109	MH191	MH192	MH193	MH200	MH204	MH209	MH210	MH337
Band67	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0
Band65	1	1	1	1	1	1	0	0	1	1	1	0	0	1	0	1
Band58	0	0	0	1	0	0	1	1	1	0	0	1	0	0	1	0
Band56	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
Band48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band36	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1
Band30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band26	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band22	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1
Band15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Band13	1	0	0	0	1	1	1	1	0	0	0	0	0	0	1	0
Band11	0	1	1	1	0	0	0	1	1	1	0	1	1	1	0	1
Band10	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0
Band6	0	1	1	1	0	0	1	1	1	1	0	0	1	1	0	1
Band5	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0
Band3	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band2	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0

1-d SDS PAGE banding patterns for samples collected in the Montaña de Oro State Park. Samples with the prefix, "SPM-", were collected between 1978-1982, and samples with the prefix, "MH-", were collected between 2014-2016 Bins where the band analysis program detected and scored a 'present' band contain a "1" and are highlighted in blue, while absent bands are represented with a "0" and are not highlighted.

APPENDIX D

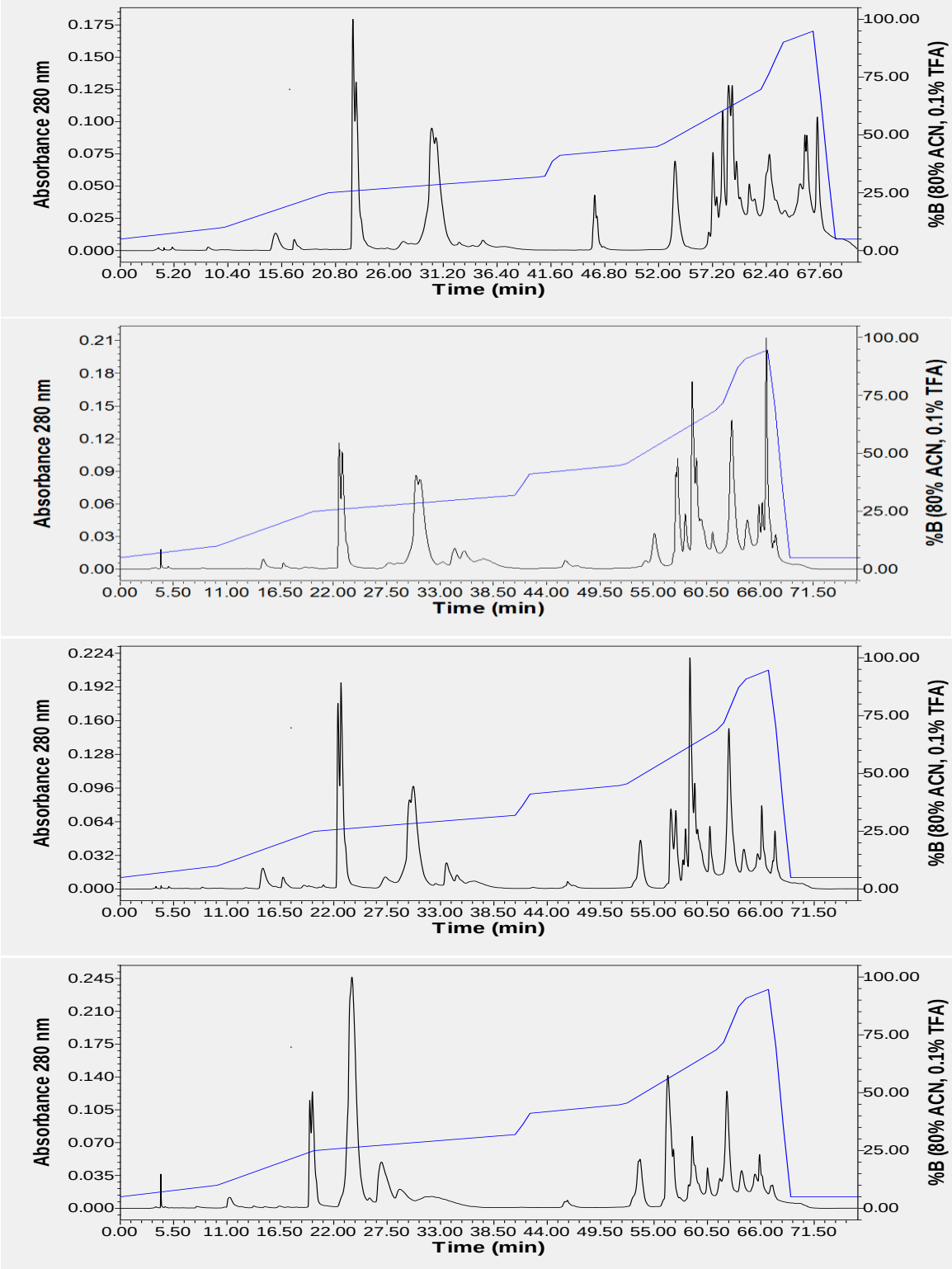
SUMMARY TABLES FOR PERCENT AREA UNDER THE CURVE FROM REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY CHROMATOGRAMS FOR CALIFORNIA COMPARISON SAMPLES

	Chimineas Region				Carrizo Plain National Monument				Montaña de Oro State Park			
	1978-1982		2014-2016		1978-1982		2014-2016		1978-1982		2014-2016	
	Average	St. Error	Average	St. Error	Average	St. Error	Average	St. Error	Average	St. Error	Average	St. Error
Peak 1	0.490	0.418	0.005	0.004	0.117	0.106	0.004	0.003	0.476	0.467	0.013	0.009
Peak 2	1.012	0.863	3.393	0.937	0.086	0.073	5.731	0.463	0.673	0.666	2.313	1.064
Peak 3	12.392	2.605	9.356	1.337	14.706	0.876	8.336	0.645	11.289	1.503	9.173	1.475
Peak 4	3.851	2.080	0.253	0.170	0.551	0.351	0.014	0.010	0.559	0.528	1.120	0.927
Peak 5	0.141	0.126	0.005	0.004	0.042	0.029	0.035	0.022	0.013	0.010	0.073	0.030
Peak 6	0.909	0.700	0.203	0.123	0.269	0.250	0.046	0.040	1.267	1.256	0.007	0.005
Peak 7	0.698	0.451	3.993	3.671	0.256	0.108	2.064	1.464	0.014	0.014	6.238	5.781
Peak 8	0.640	0.154	8.103	2.473	0.762	0.120	21.520	3.102	1.353	0.654	3.607	3.058
Peak 9	0.764	0.482	1.805	1.288	3.817	1.708	0.000	0.000	0.839	0.690	2.742	2.742
Peak 10	4.451	1.781	5.068	3.054	8.291	1.613	0.432	0.092	6.300	3.836	5.808	3.087
Peak 11	7.173	1.974	2.428	0.988	3.718	1.235	1.340	0.431	19.793	5.569	4.048	1.990
Peak 12	0.453	0.295	0.988	0.466	1.273	1.102	2.264	0.885	0.000	0.000	0.483	0.483
Peak 13	0.368	0.206	1.511	0.335	0.534	0.162	0.464	0.239	0.301	0.153	2.310	0.881
Peak 14	2.050	1.165	0.520	0.207	0.963	0.216	1.116	0.555	1.324	0.615	1.862	0.934
Peak 15	1.436	0.290	0.319	0.174	0.662	0.224	0.046	0.040	3.567	1.168	2.112	0.785
Peak 16	0.136	0.088	0.066	0.066	0.543	0.143	0.012	0.014	0.046	0.046	0.000	0.000
Peak 17	1.292	0.583	0.203	0.138	0.500	0.227	0.709	0.844	1.500	0.724	1.505	0.694
Peak 18	0.643	0.403	0.000	0.000	0.000	0.000	0.000	0.000	0.906	0.700	0.000	0.000
Peak 19	0.163	0.142	0.000	0.000	0.027	0.027	0.000	0.000	0.000	0.000	0.005	0.005
Peak 20	0.000	0.000	0.000	0.000	0.146	0.146	0.000	0.000	0.000	0.000	0.000	0.000
Peak 21	0.007	0.006	0.732	0.732	0.307	0.307	0.000	0.000	0.000	0.000	0.000	0.000
Peak 22	0.276	0.236	0.003	0.003	0.002	0.002	0.019	0.019	0.007	0.007	0.003	0.003
Peak 23	0.007	0.006	0.000	0.000	0.014	0.014	0.002	0.002	0.084	0.084	0.000	0.000
Peak 24	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

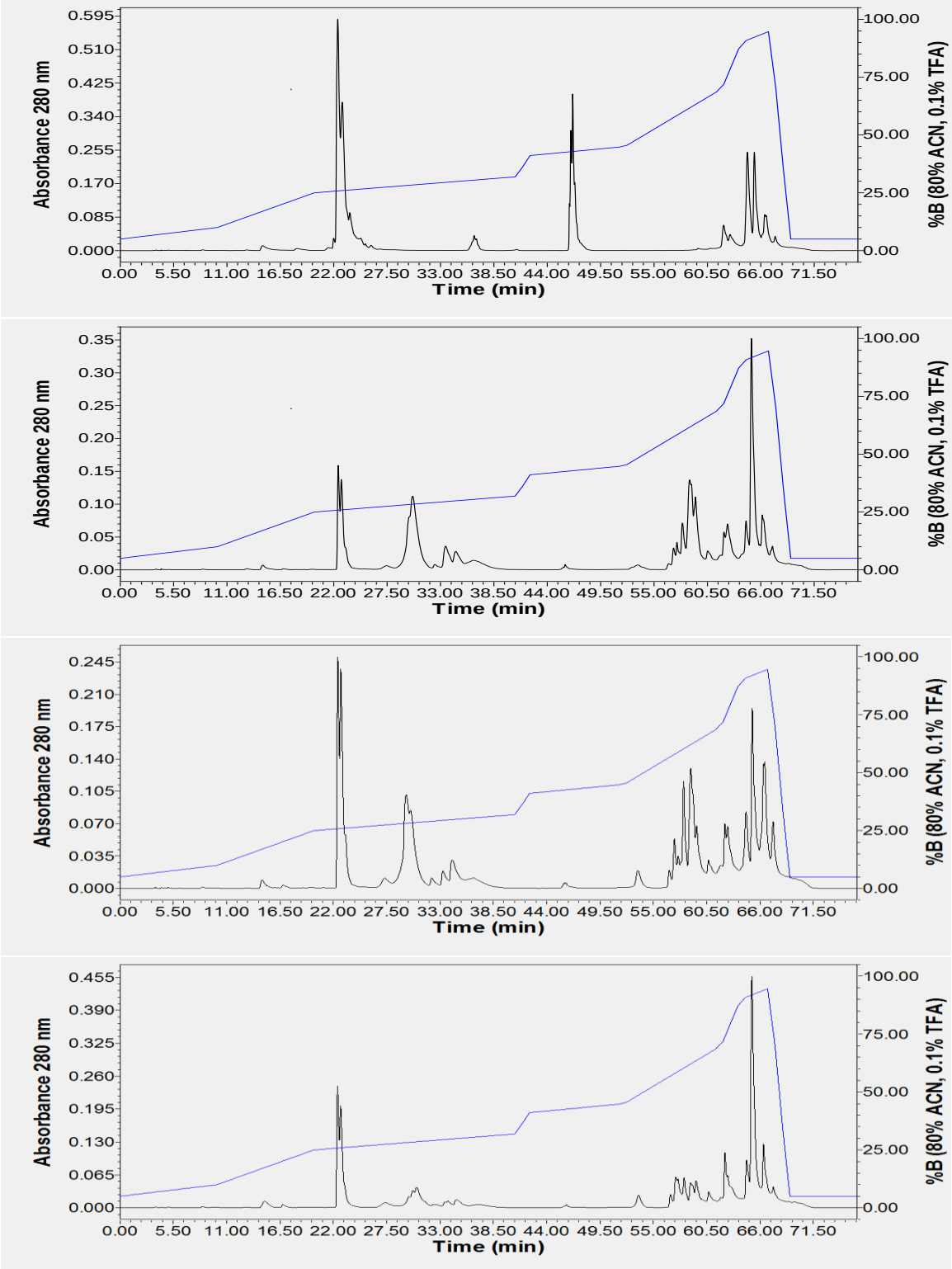
	Chimineas Region				Carrizo Plain National Monument				Montaña de Oro State Park			
	1978-1982		2014-2016		1978-1982		2014-2016		1978-1982		2014-2016	
	Average	St. Error	Average	St. Error	Average	St. Error	Average	St. Error	Average	St. Error	Average	St. Error
Peak 25	0.000	0.000	0.799	0.799	0.000	0.000	0.717	0.831	0.000	0.000	0.512	0.512
Peak 26	0.200	0.088	0.129	0.037	1.050	0.764	0.265	0.056	0.201	0.117	0.288	0.146
Peak 27	1.545	1.199	0.360	0.325	0.087	0.025	0.034	0.015	0.104	0.048	0.098	0.054
Peak 28	0.001	0.001	0.039	0.039	0.155	0.155	0.214	0.178	0.000	0.000	0.000	0.000
Peak 29	0.000	0.000	0.847	0.254	0.000	0.000	1.633	0.280	0.000	0.000	0.725	0.492
Peak 30	0.000	0.000	0.346	0.264	0.000	0.000	0.130	0.107	0.000	0.000	0.280	0.280
Peak 31	0.223	0.199	0.000	0.000	0.000	0.000	0.000	0.000	0.549	0.549	0.000	0.000
Peak 32	0.000	0.000	0.078	0.078	0.825	0.274	0.452	0.538	0.000	0.000	0.008	0.008
Peak 33	0.253	0.227	0.569	0.221	1.775	1.247	1.245	0.432	0.031	0.031	1.023	0.376
Peak 34	1.527	0.451	0.892	0.190	0.665	0.317	0.532	0.179	1.416	0.978	0.342	0.219
Peak 35	0.640	0.327	0.334	0.197	0.333	0.262	0.695	0.231	0.000	0.000	0.000	0.000
Peak 36	0.538	0.425	0.442	0.157	0.611	0.296	0.298	0.154	0.497	0.380	0.067	0.067
Peak 37	1.356	0.698	2.537	0.584	1.840	0.410	1.515	0.408	0.331	0.240	0.857	0.628
Peak 38	4.525	0.726	5.413	0.786	5.207	0.794	6.233	0.935	4.823	2.928	5.003	0.607
Peak 39	6.408	1.508	6.291	0.759	7.183	0.661	5.582	0.755	7.867	2.989	3.340	0.733
Peak 40	6.278	1.495	4.348	1.238	4.410	0.795	3.175	0.539	7.339	2.722	2.485	0.687
Peak 41	2.269	0.426	1.346	0.331	2.431	0.212	0.601	0.314	1.620	0.556	2.092	0.607
Peak 42	0.284	0.173	5.872	0.968	1.510	0.415	8.148	0.629	0.733	0.495	4.902	1.769
Peak 43	6.786	1.105	1.275	0.569	6.630	0.634	0.191	0.168	5.786	1.609	2.920	1.408
Peak 44	2.256	1.200	9.578	2.211	0.406	0.154	10.213	2.508	1.149	1.060	4.793	1.620
Peak 45	3.600	0.736	3.476	1.661	11.741	1.785	2.572	1.315	1.267	0.462	11.617	4.641
Peak 46	10.457	1.848	6.821	1.746	4.709	1.323	5.583	0.730	7.276	1.116	3.033	1.745
Peak 47	5.578	0.885	4.472	0.809	5.493	0.567	2.774	0.597	4.227	1.967	6.582	1.432
Peak 48	2.956	0.457	1.322	0.317	2.527	0.295	0.638	0.308	1.811	0.644	1.948	0.474

APPENDIX E

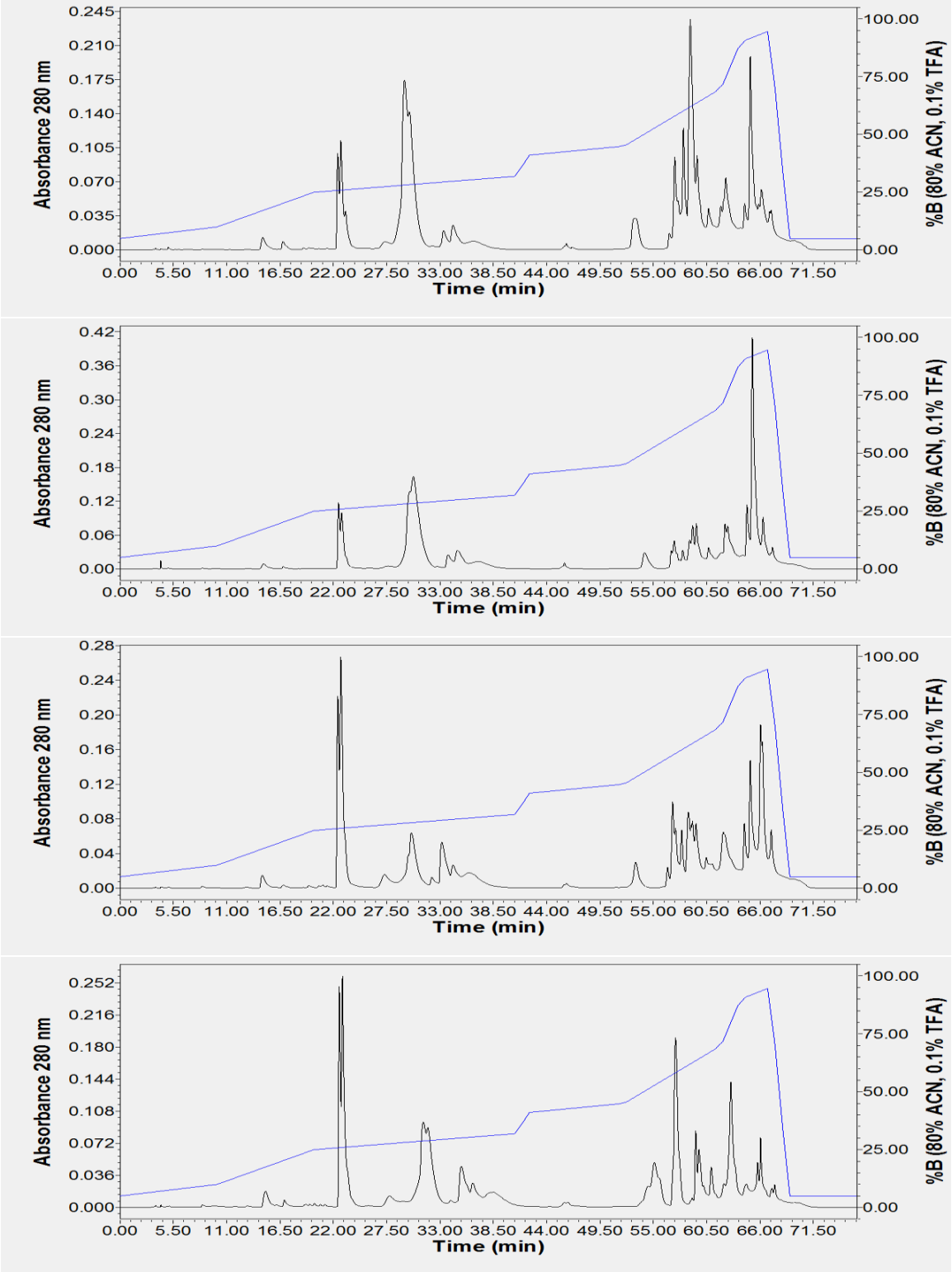
REVERSE PHASE HPLC CHROMATOGRAMS FOR CALIFORNIA COMPARISON SAMPLES



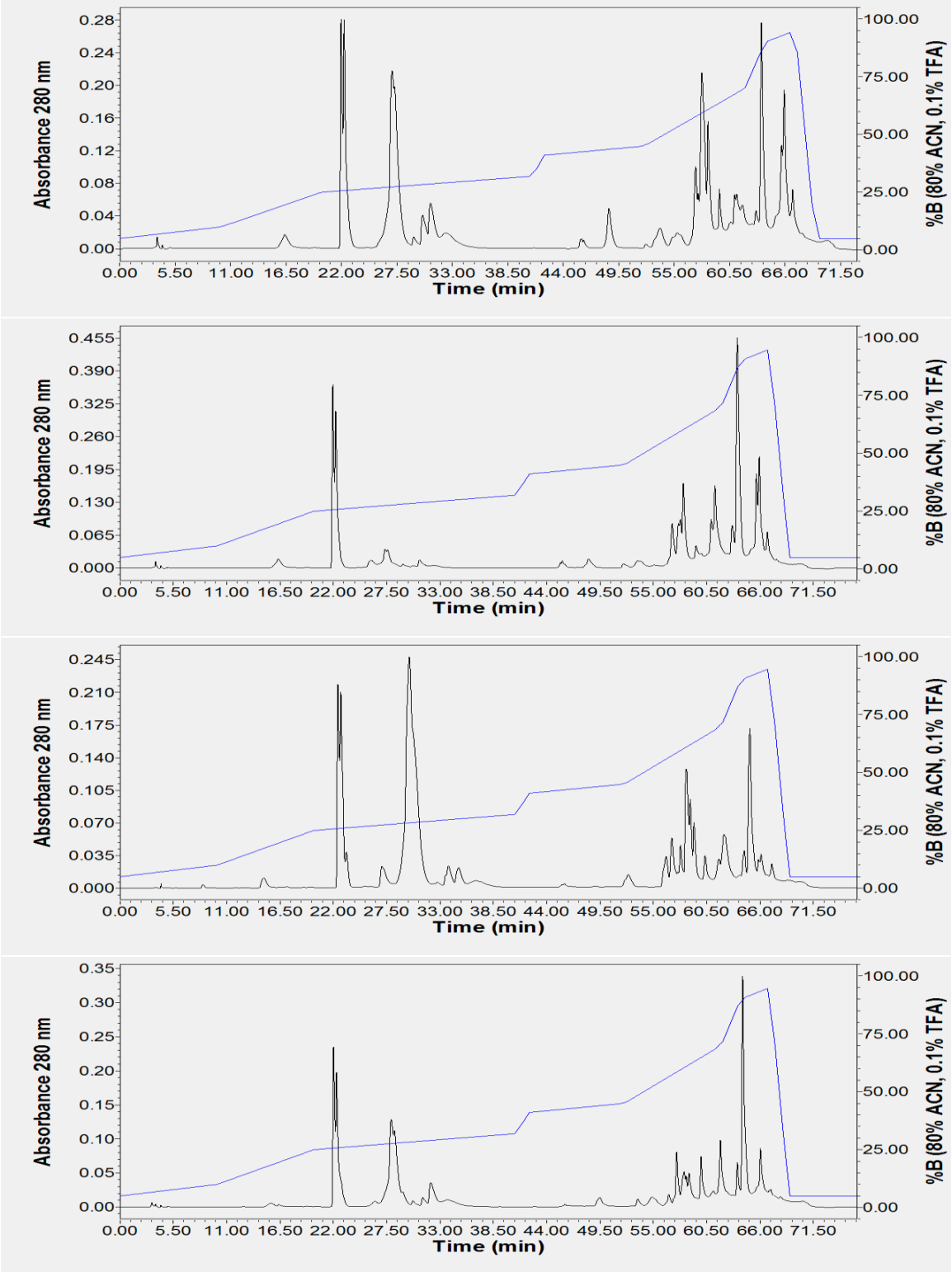
HPLC Chromatograms for four samples collected 1978-1982 in Chimineas Region. Top to bottom: SPM 249; SPM 323; SPM 325; and, SPM 347.



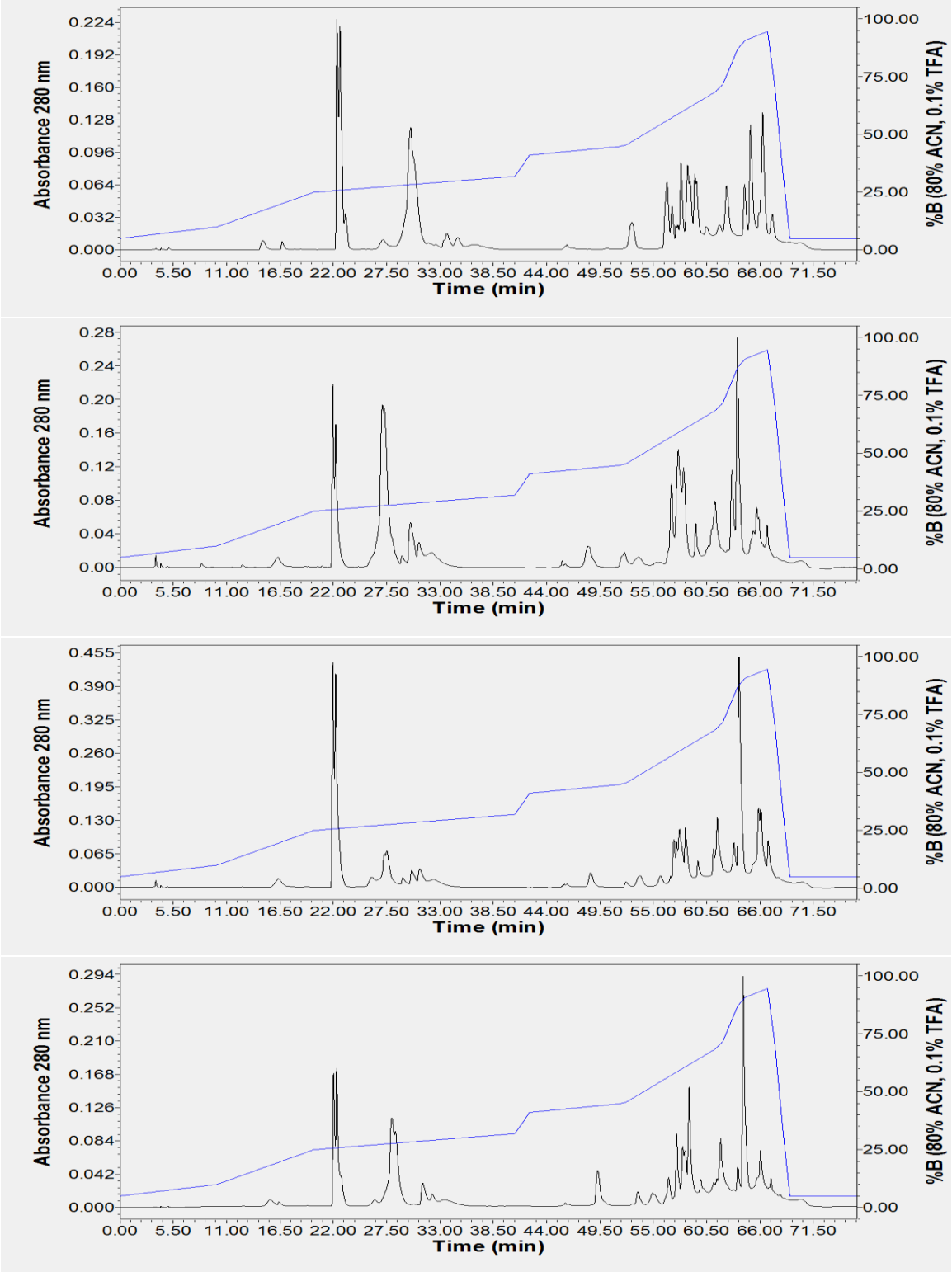
HPLC Chromatograms for four samples collected 1978-1982 in Chimineas Region. Top to bottom: SPM 578; SPM 589; SPM 590; and, SPM 591.



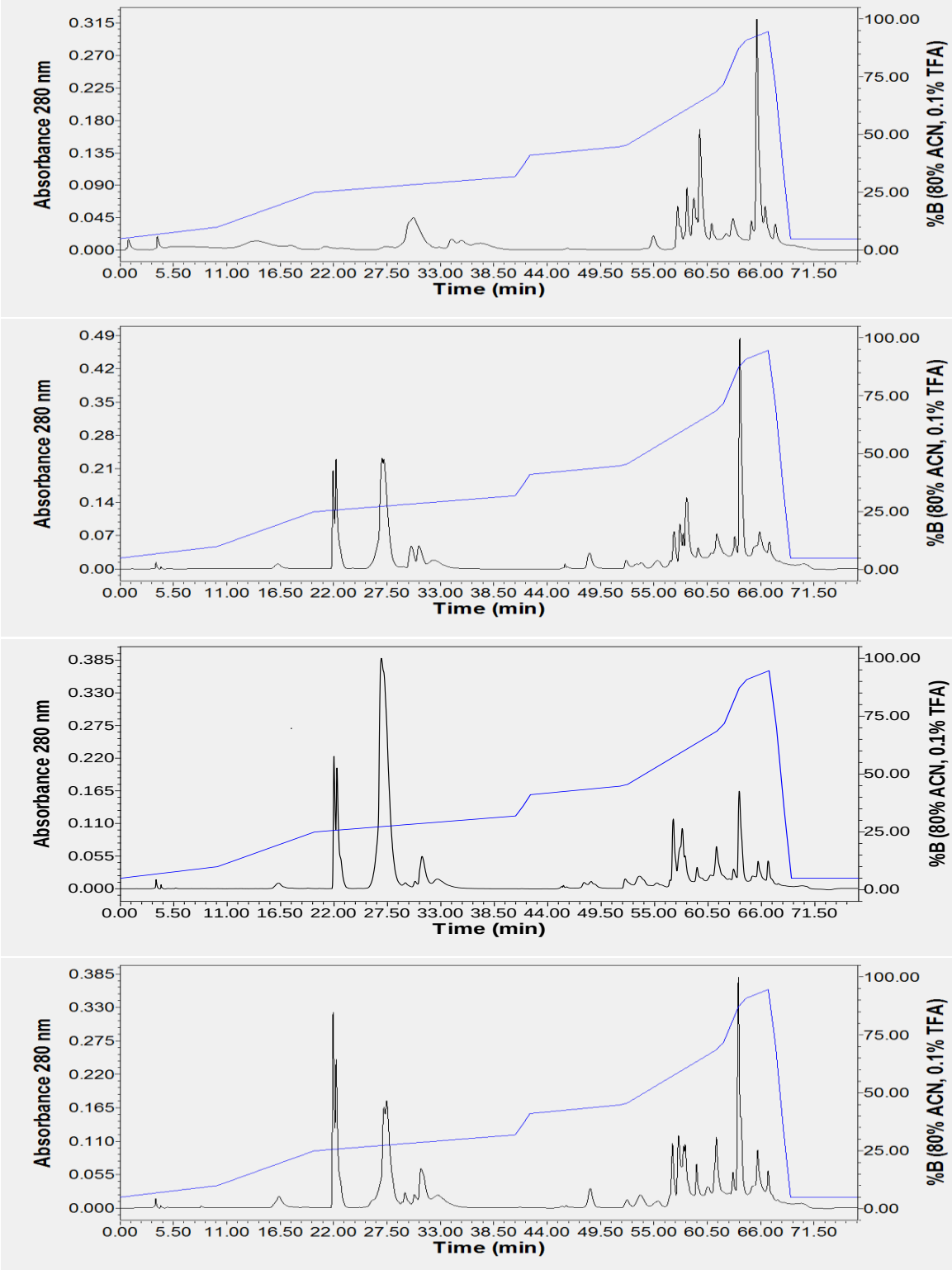
HPLC Chromatograms for four samples collected 1978-1982 in Chimineas Region. Top to bottom: SPM 592; SPM 593; SPM 660; and, SPM 661.



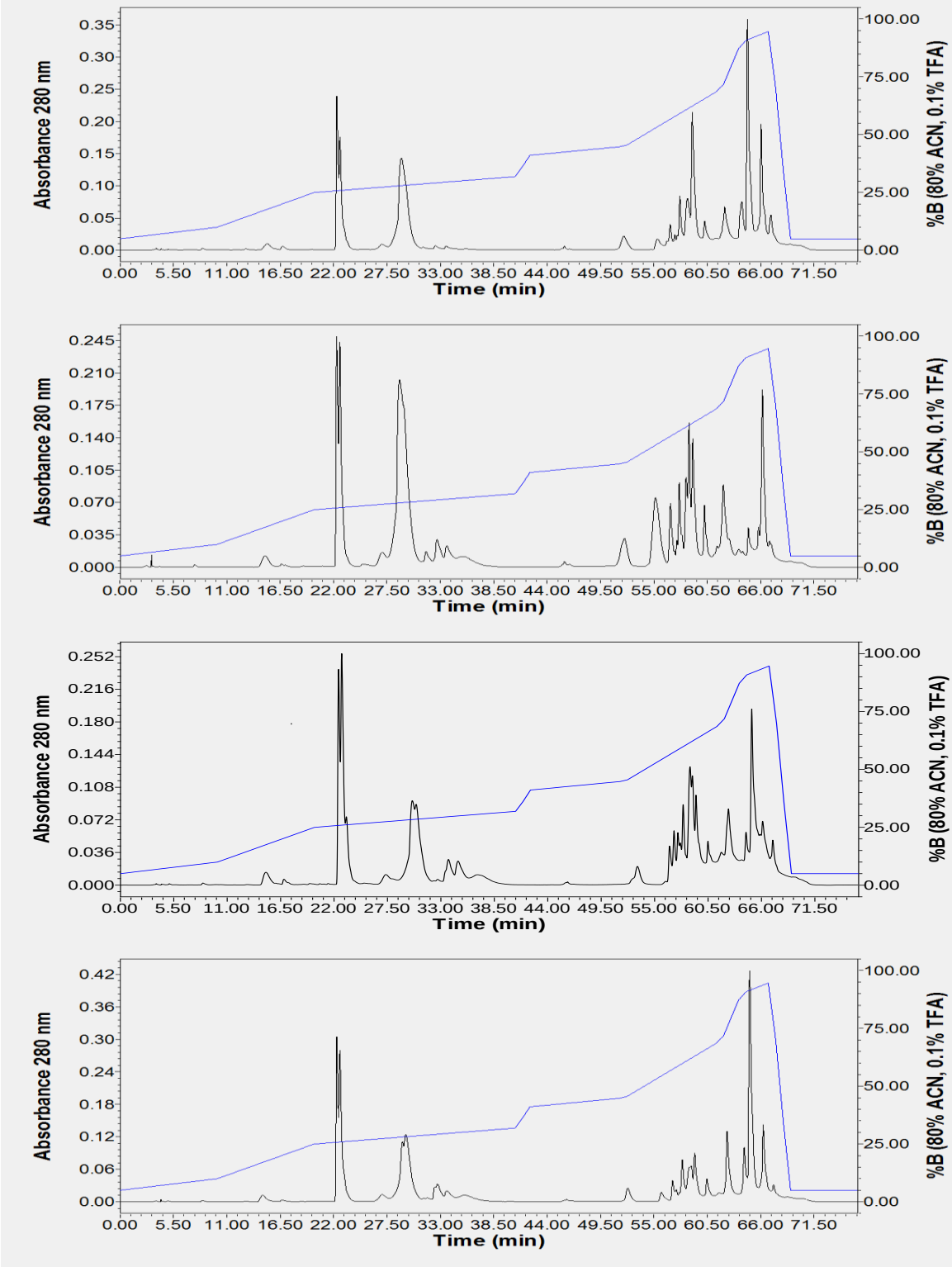
HPLC Chromatograms for four samples collected 2014-2016 in Chimineas Region. Top to bottom: MH 098; MH 100; SPM 102; and, SPM 105.



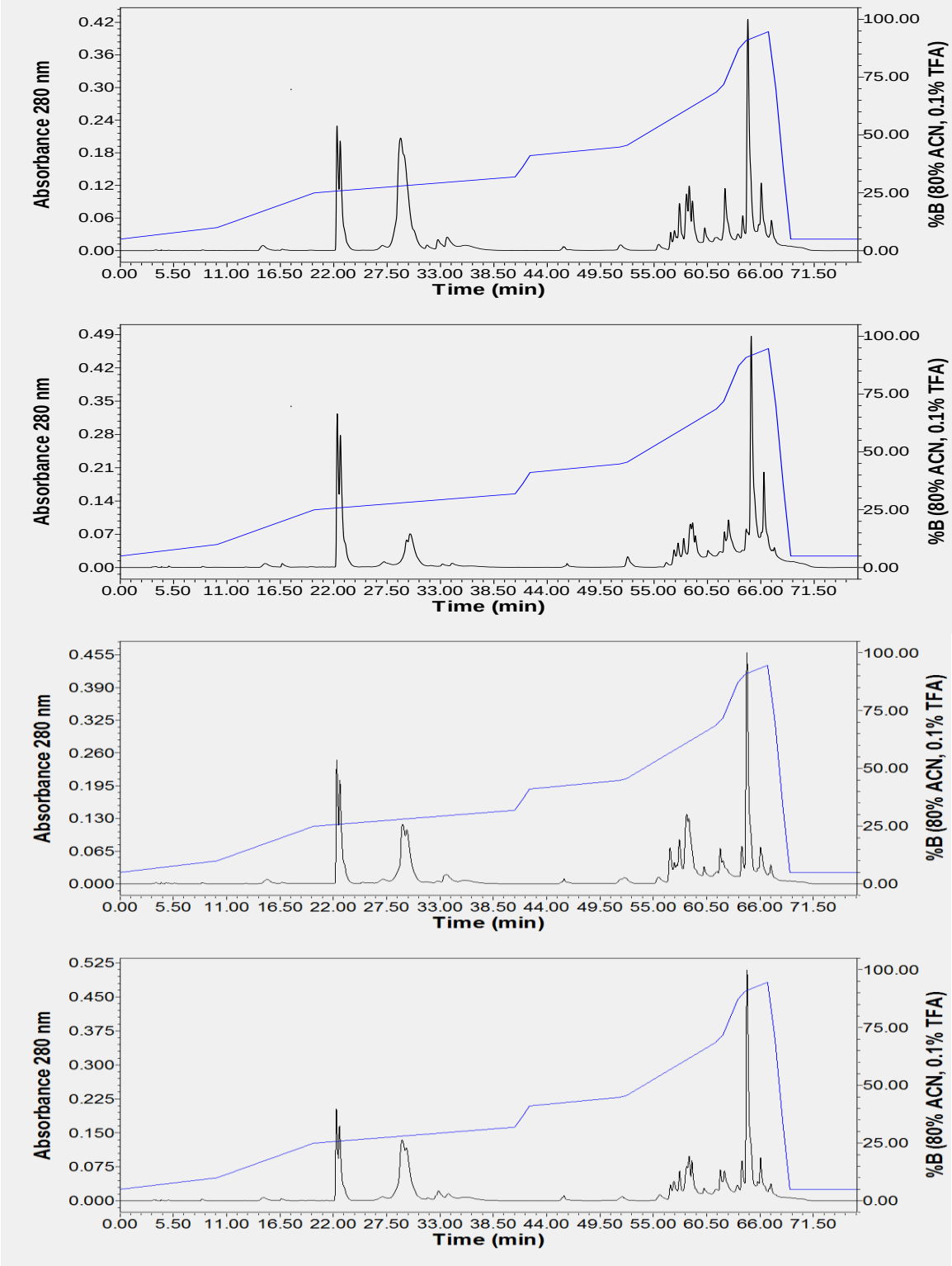
HPLC Chromatograms for four samples collected 2014-2016 in Chimineas Region. Top to bottom: MH 106; MH109; MH 110; and, MH 115.



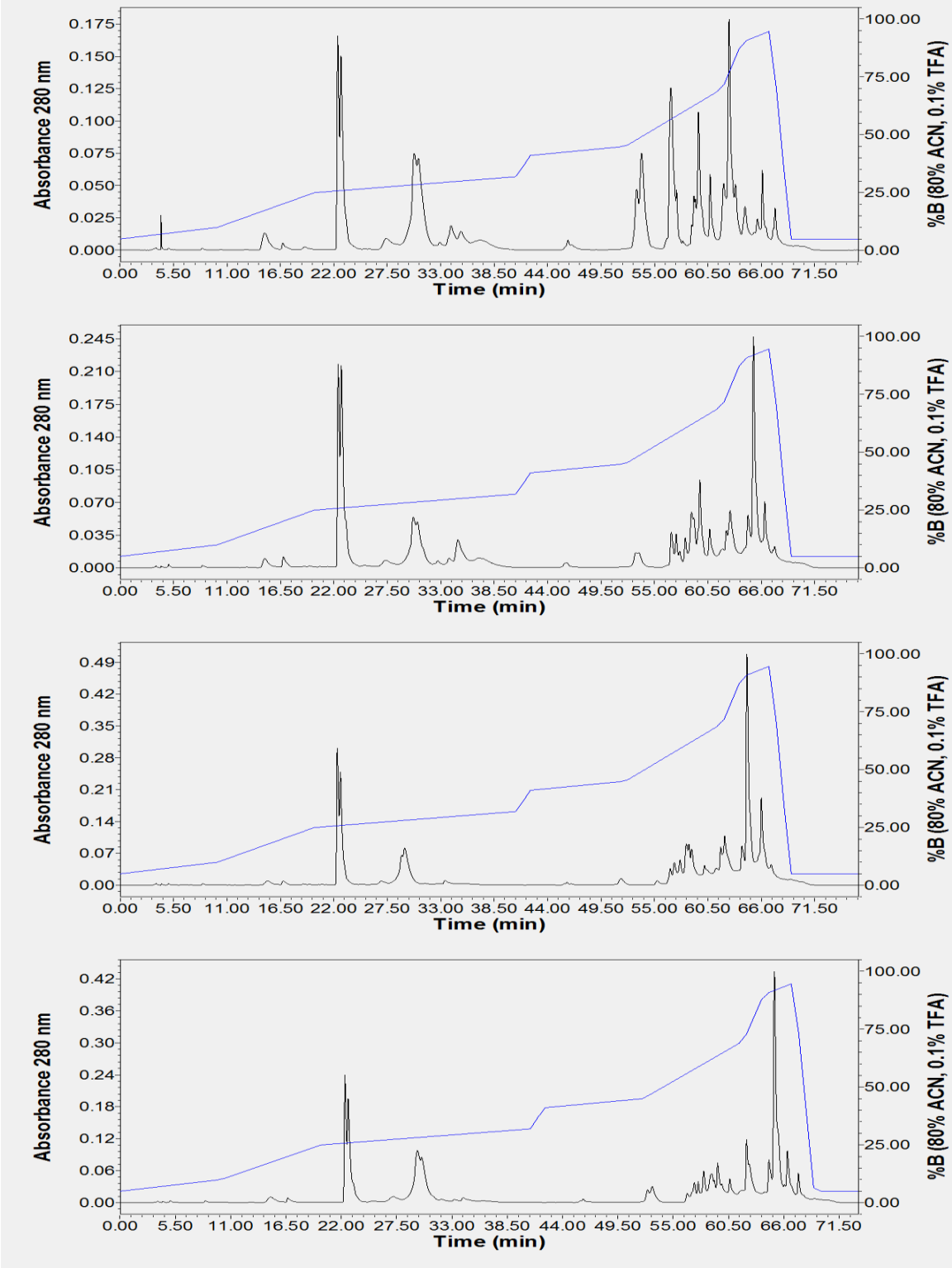
HPLC Chromatograms for four samples collected 2014-2016 in Chimineas Region. Top to bottom: MH 116; MH 117; MH 118; MH 337.



HPLC Chromatograms for four samples collected 1978-1982 at Carrizo Plain National Monument. Top to bottom: SPM 072; SPM 073; SPM 074; and, SPM 076.

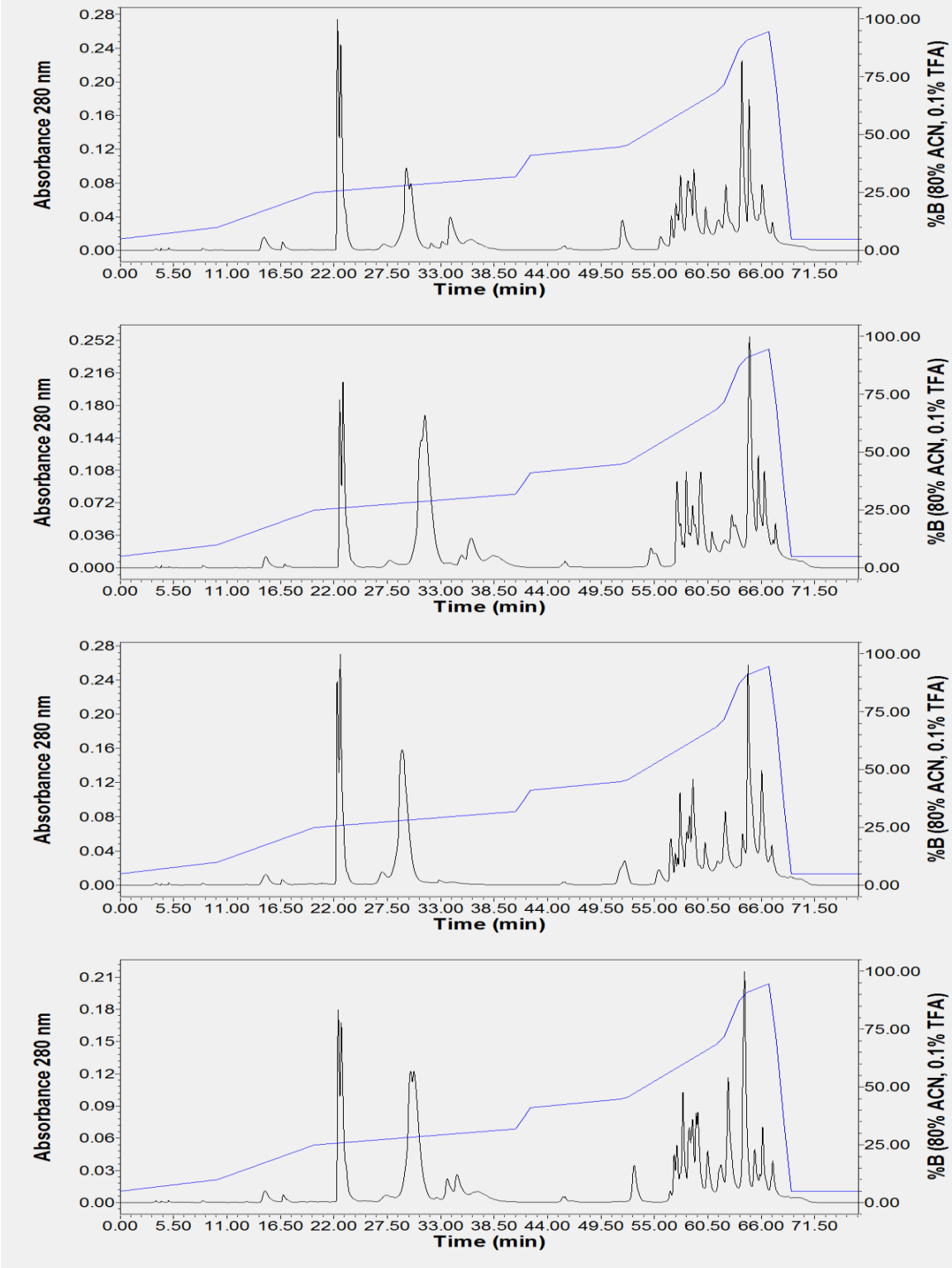


HPLC Chromatograms for four samples collected 1978-1982 at Carrizo Plain National Monument. Top to bottom: SPM 077; SPM 078; SPM 082; and, SPM 084.

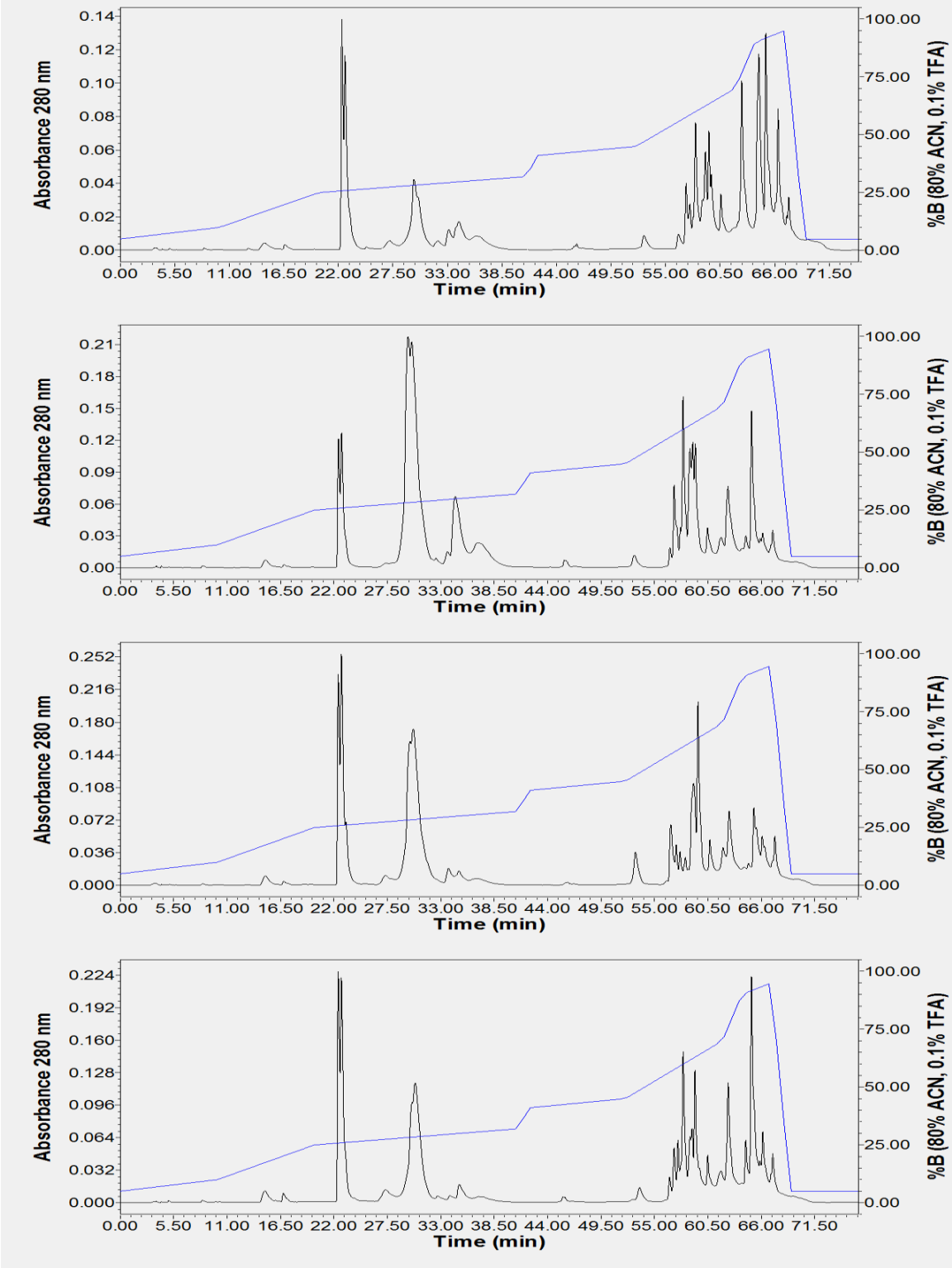


HPLC Chromatograms for four samples collected 1978-1982 at Carrizo Plain National Monument. Top to bottom: SPM 111; SPM 340; SPM 341; and, SPM 379.

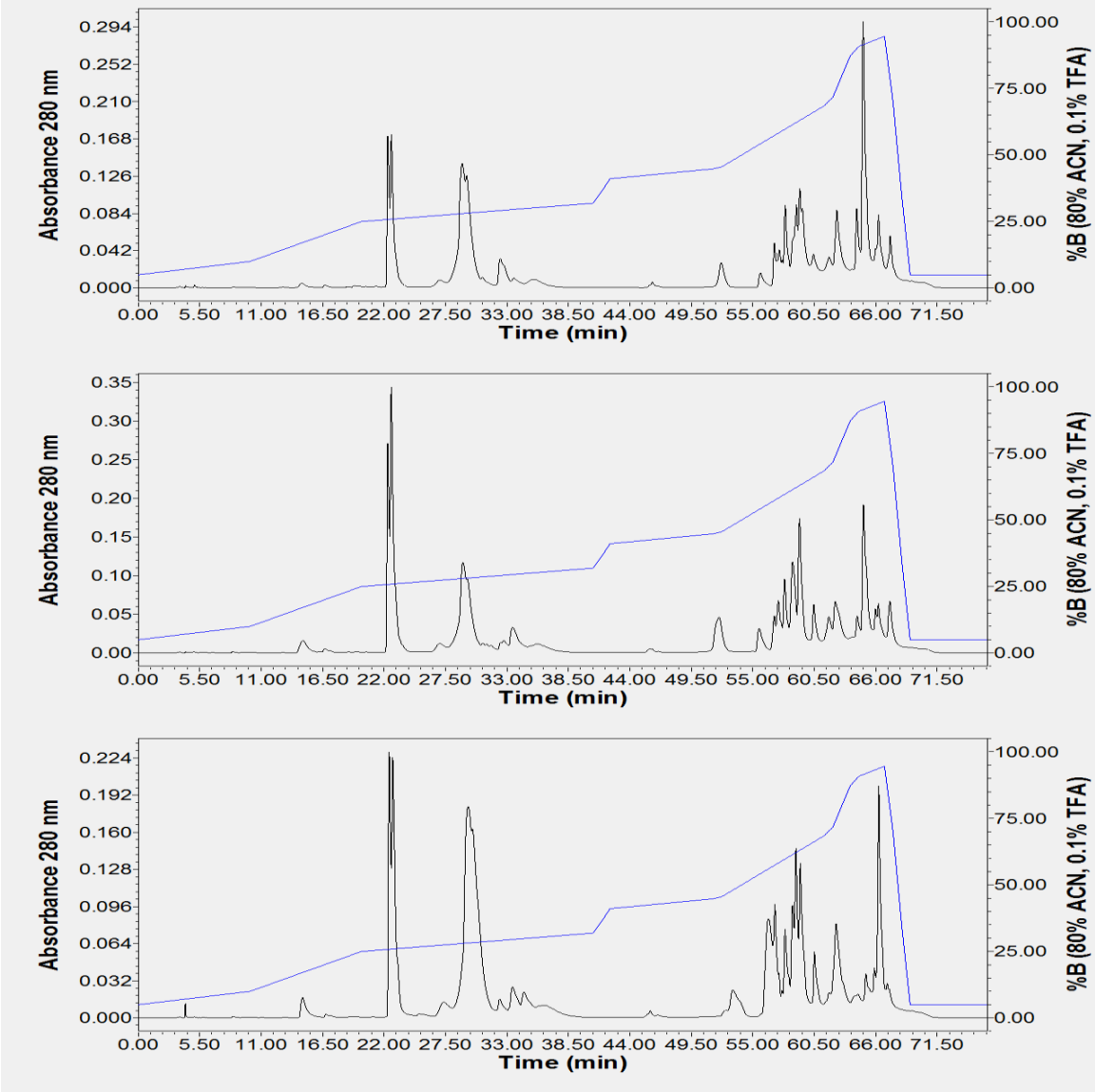
SPM 111



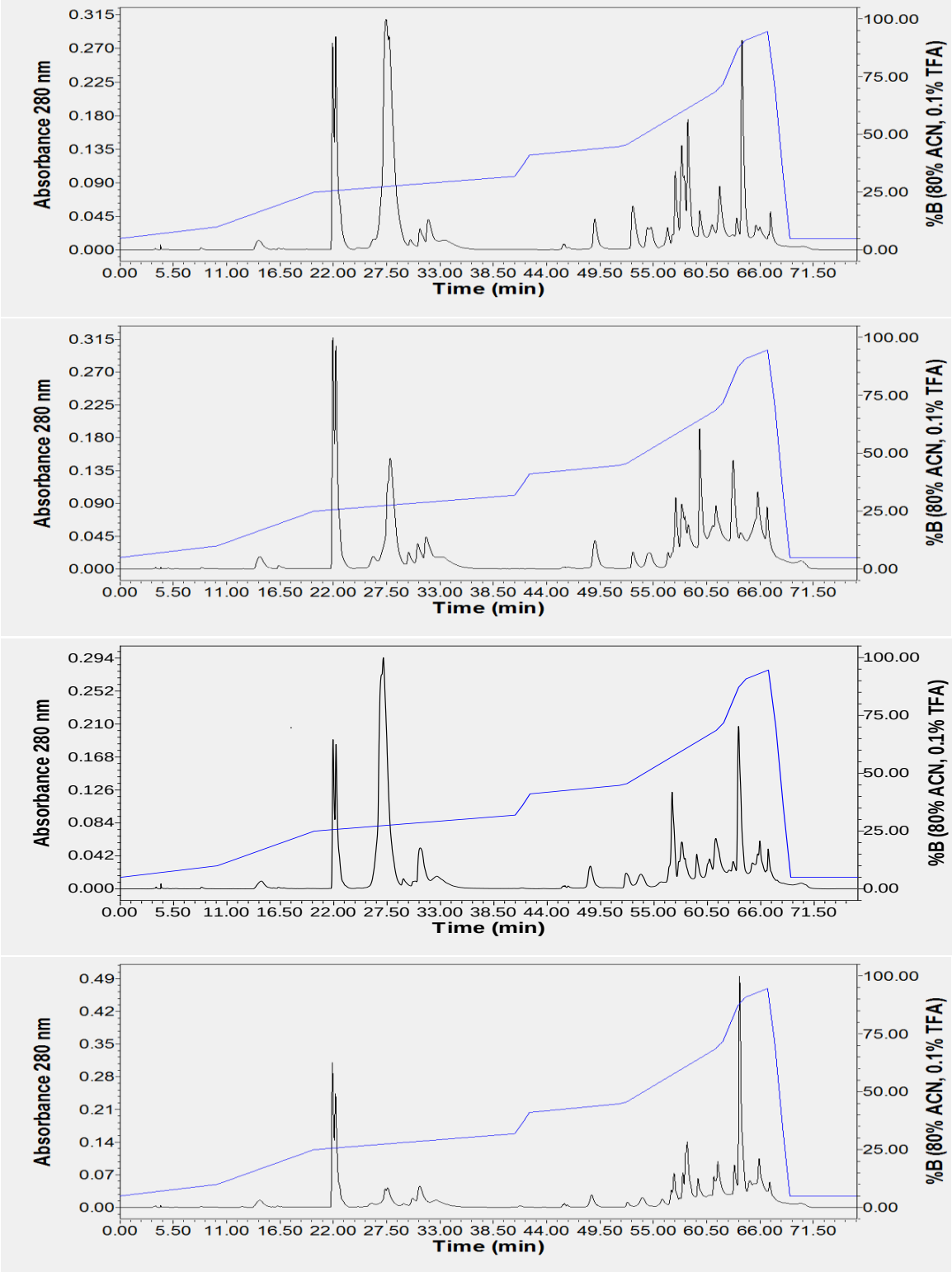
HPLC Chromatograms for four samples collected 1978-1982 at Carrizo Plain National Monument. Top to bottom: SPM 380; SPM 383; SPM 440; and, SPM 560.



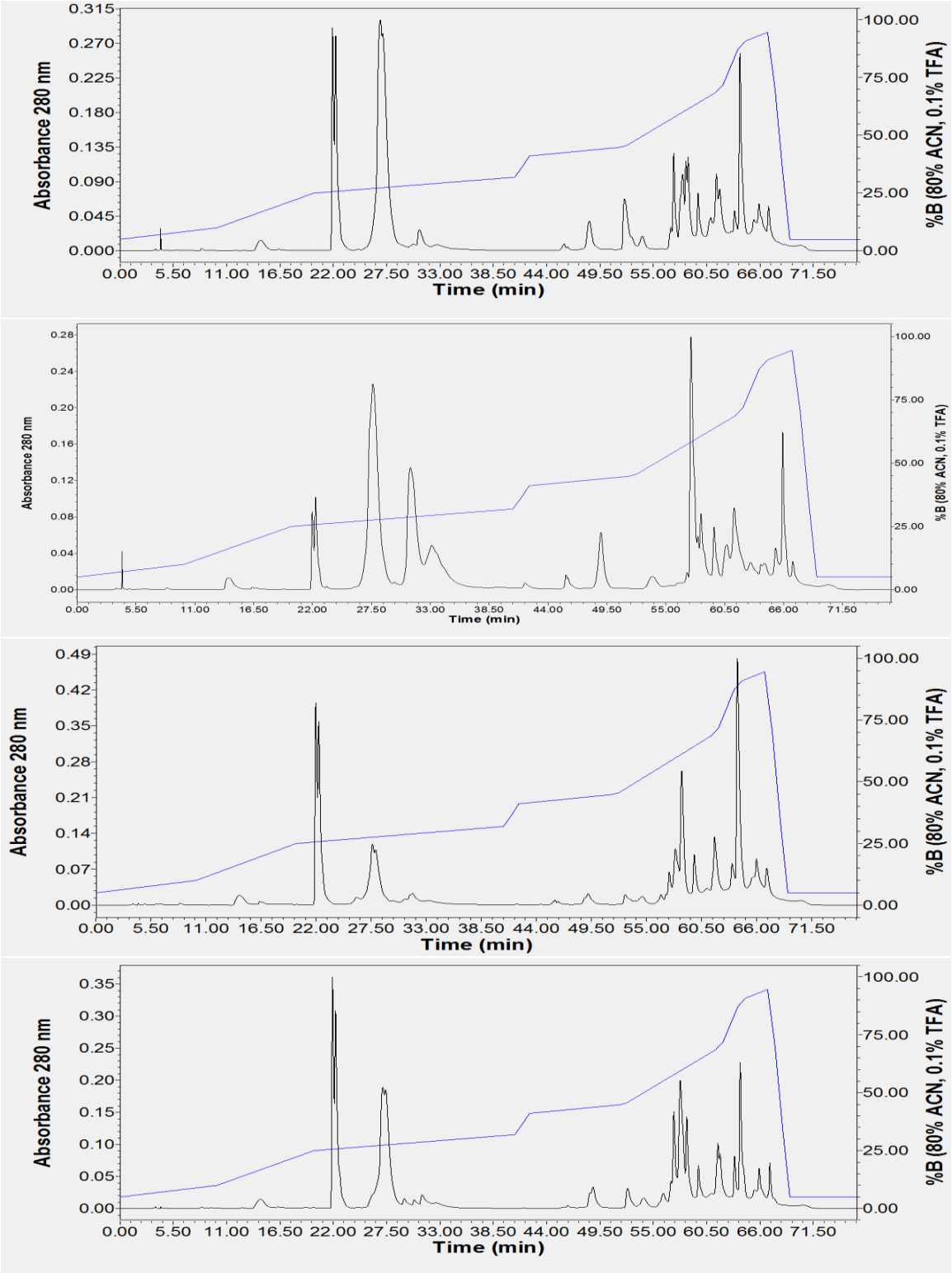
HPLC Chromatograms for four samples collected 1978-1982 at Carrizo Plain National Monument. Top to bottom: SPM 579; SPM 580; SPM 581; and, SPM 582.



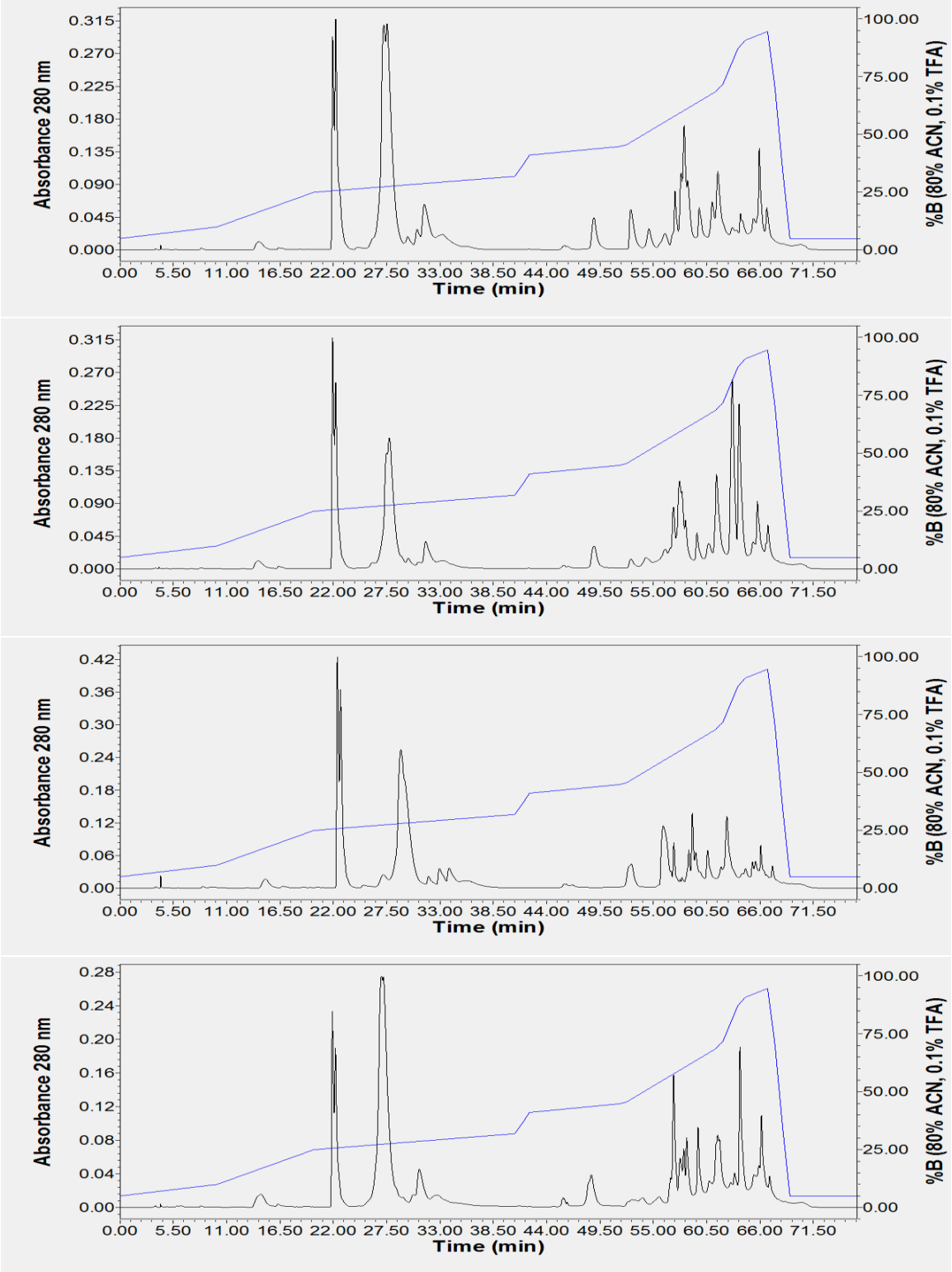
HPLC Chromatograms for three samples collected 1978-1982 at Carrizo Plain National Monument. Top to bottom: SPM 715; SPM 750; and SPM 806.



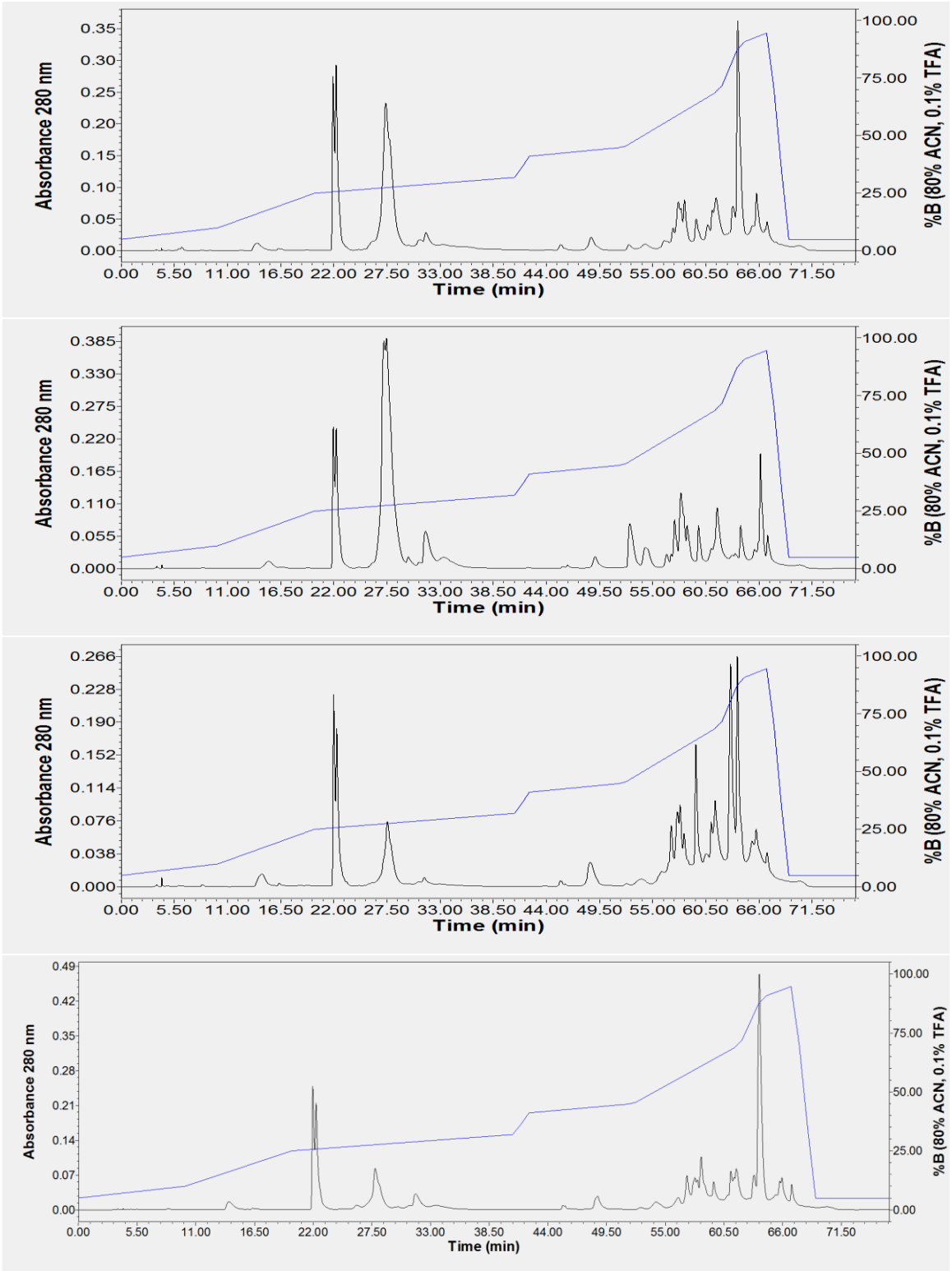
HPLC Chromatograms for four samples collected 2014-2016 at Carrizo Plain National Monument. Top to bottom: JGM 01; JGM 02; JGM 03; and, JGM 04.



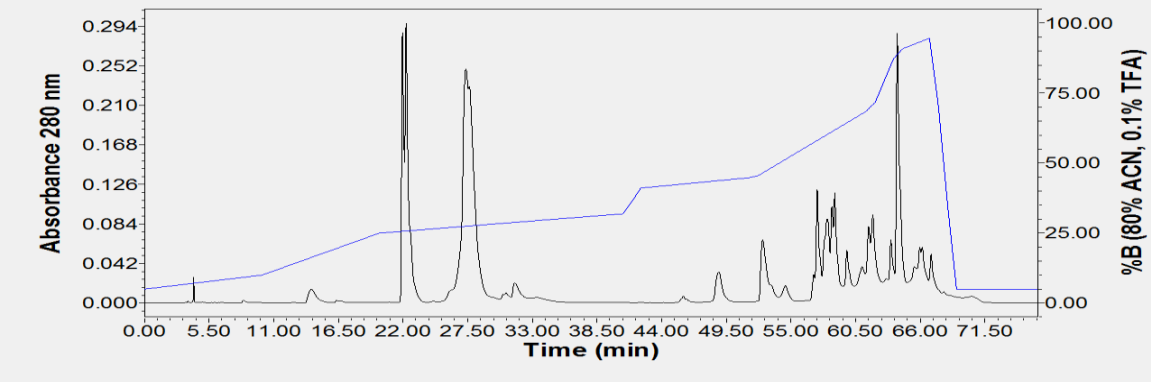
HPLC Chromatograms for four samples collected 2014-2016 at Carrizo Plain National Monument. Top to bottom: JGM 06; JGM 07; JGM 08; and, JGM 09.



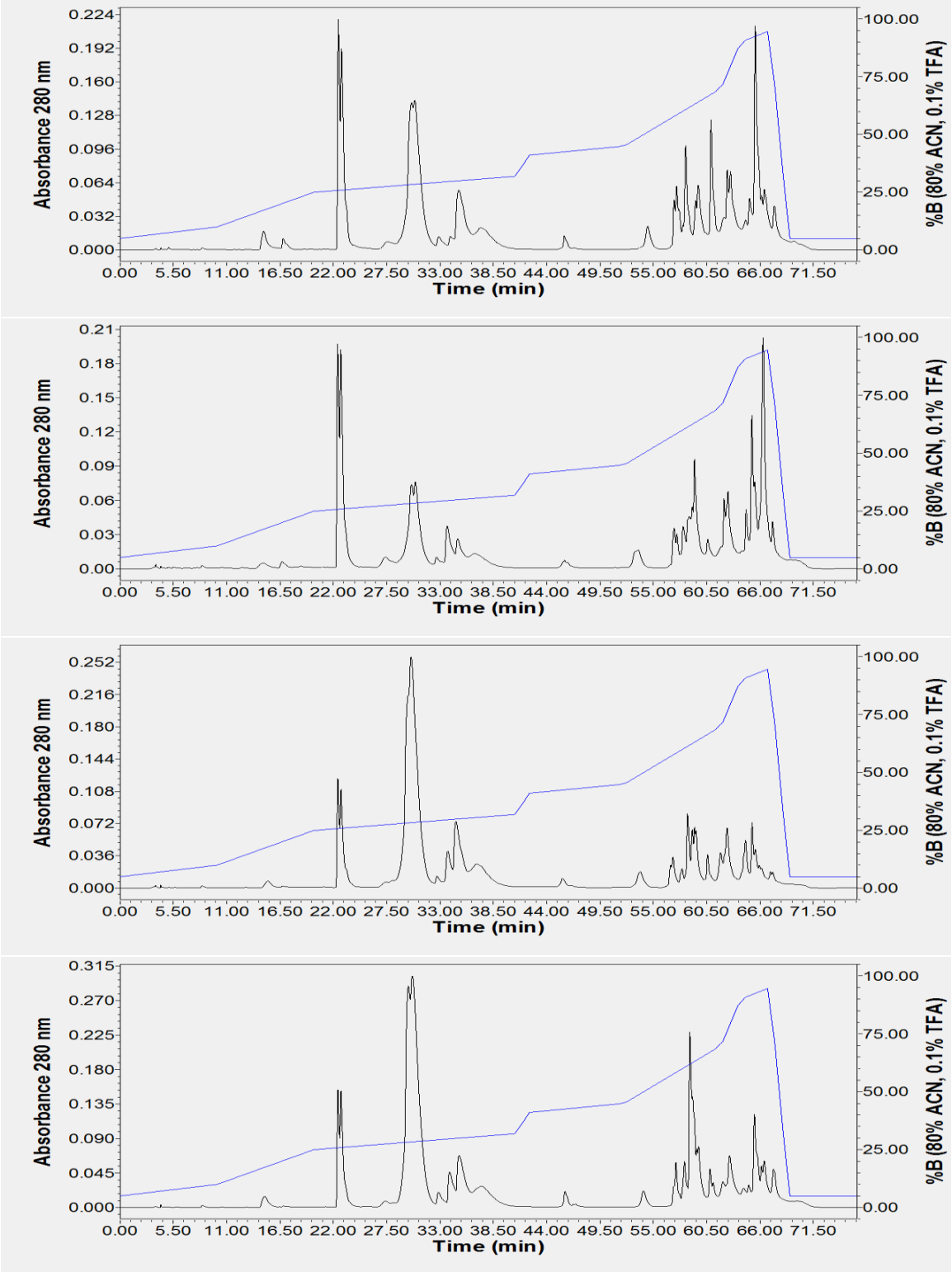
HPLC Chromatograms for four samples collected 2014-2016 at Carrizo Plain National Monument. Top to bottom: JGM 10; JGM 11; JGM 12; and, JGM 13.



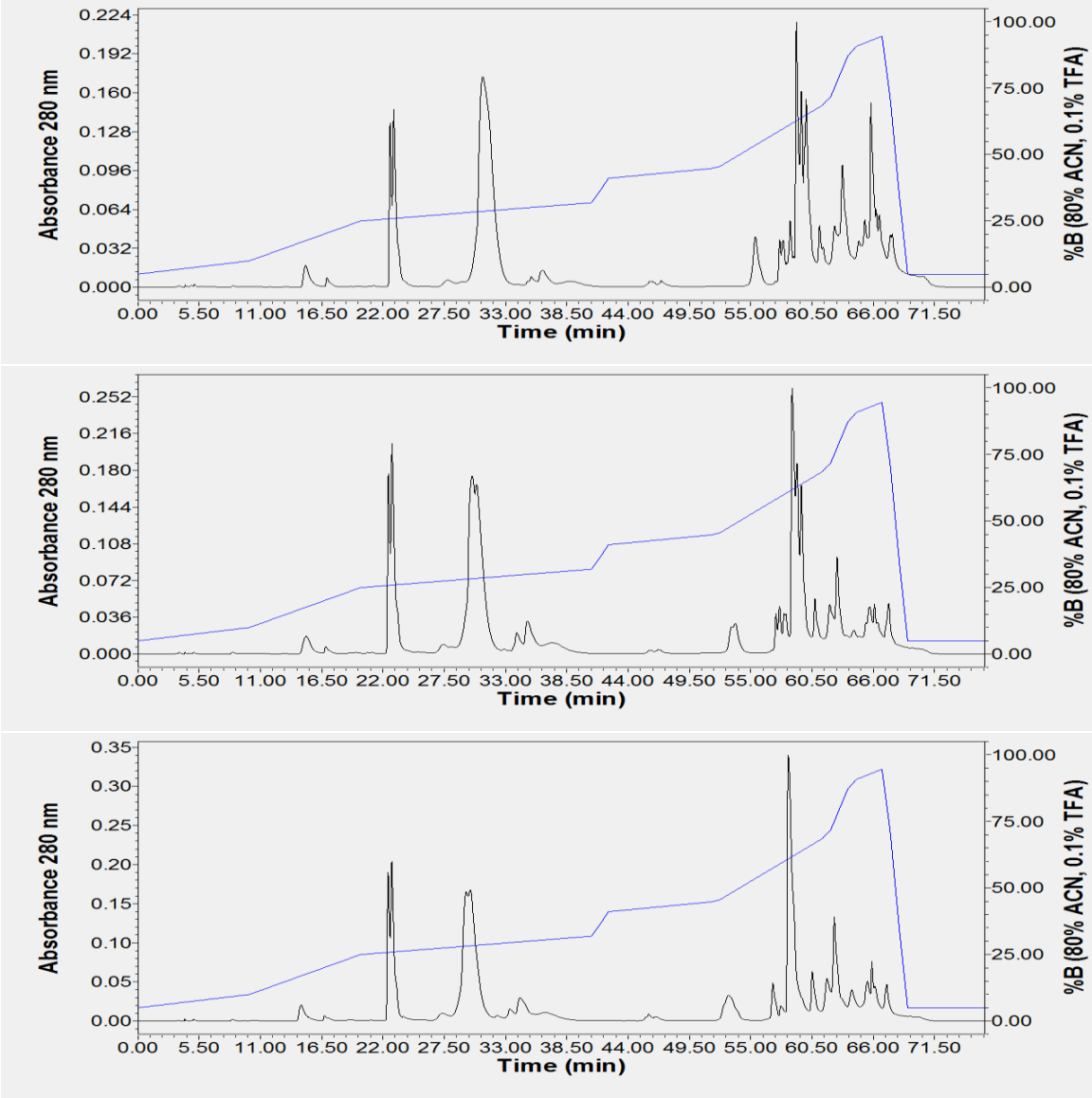
HPLC Chromatograms for four samples collected 2014-2016 at Carrizo Plain National Monument. Top to bottom: JGM 14; JGM 15; JGM 16; and, JGM 17.



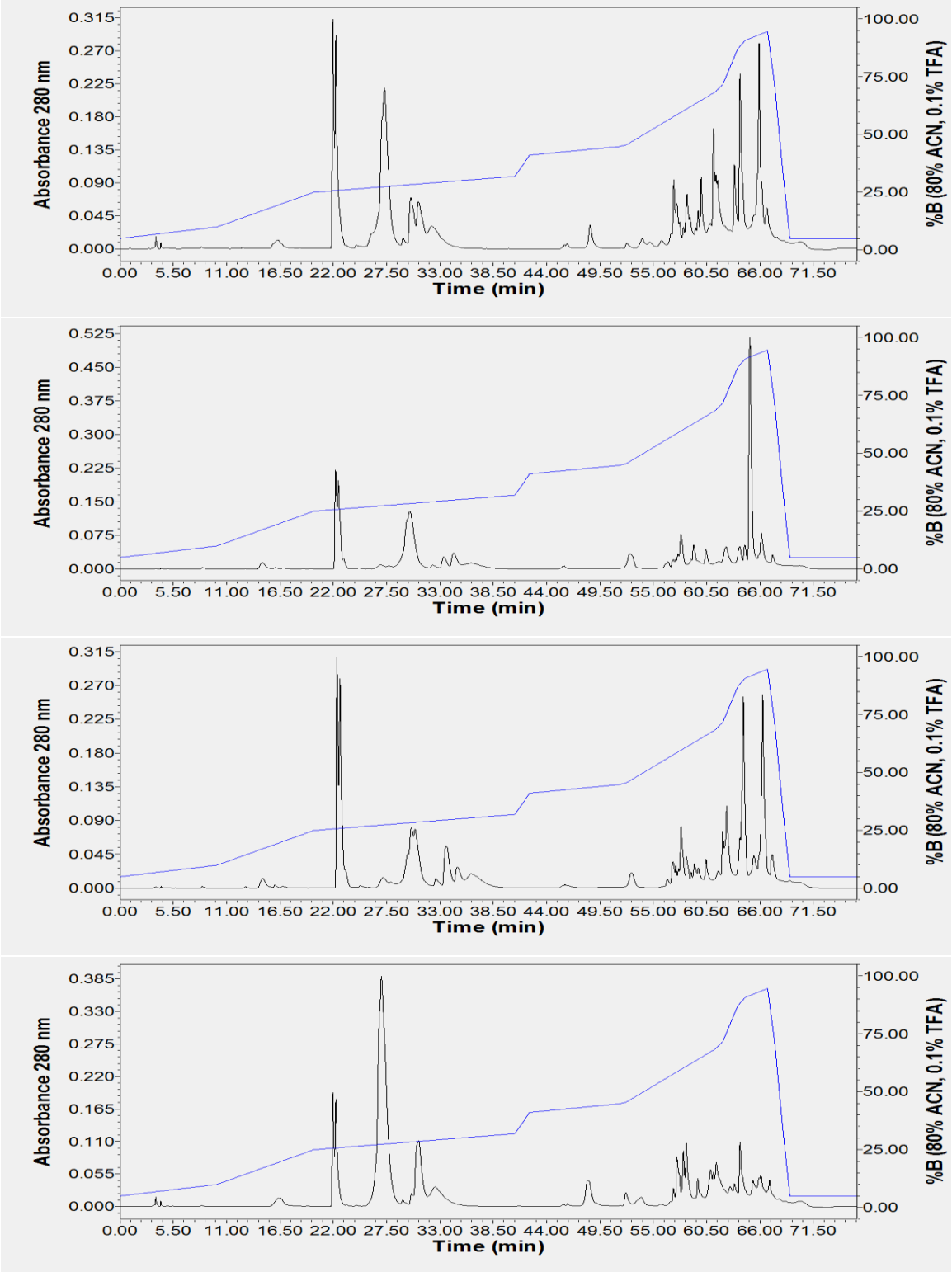
HPLC Chromatograms for one sample collected 2014-2016 at Carrizo Plain National Monument. JGM 018.



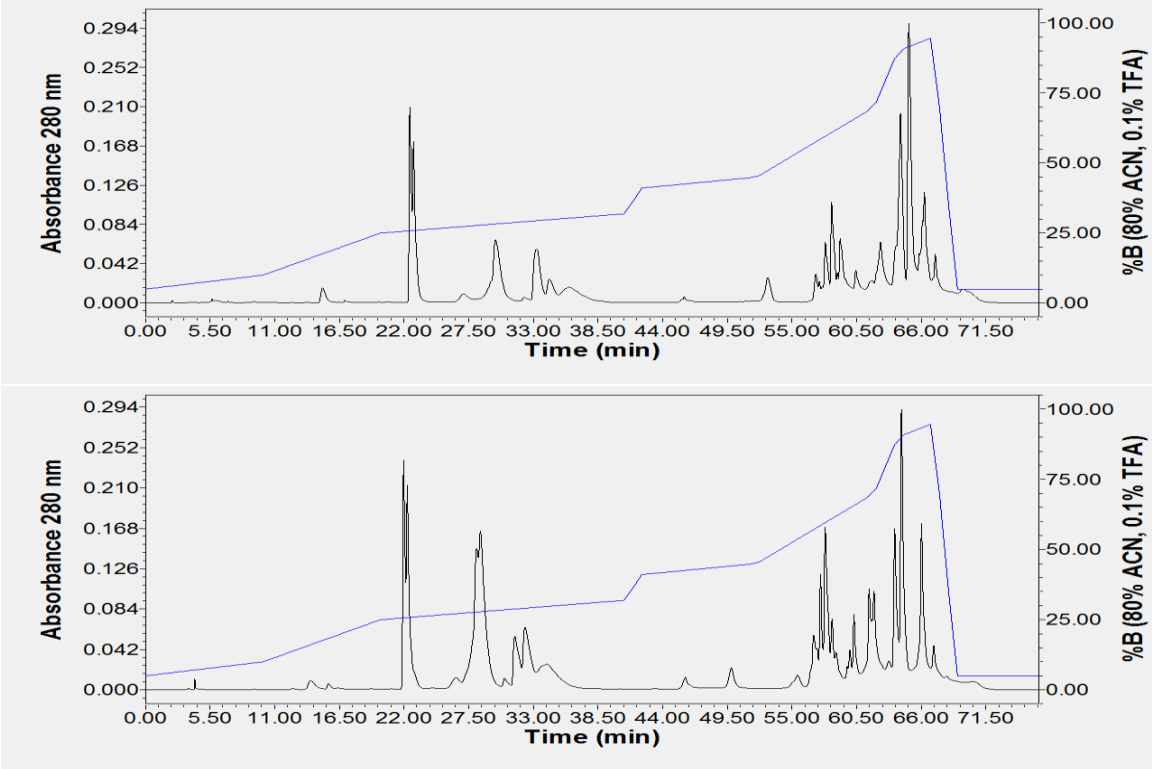
HPLC Chromatograms for four samples collected 1978-1982 at Montaña de Oro State Park. Top to bottom: SPM 029; SPM 033; SPM 034; and, SPM 035.



HPLC Chromatograms for three samples collected 1978-1982 at Montaña de Oro State Park. Top to bottom: SPM 036; SPM 037; and, SPM 038.



HPLC Chromatograms for four samples collected 2014-2016 at Montaña de Oro State Park. Top to bottom: MH 191; MH 192; MH 193; and, MH 200.



HPLC Chromatograms for two samples collected 2014-2016 at Montaña de Oro State Park. Top to bottom: MH 204; and, MH 209.

APPENDIX F

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

UNIVERSITY of NORTHERN COLORADO

IACUC Memorandum

To: Dr. Steve Mackessy

From: Laura Martin, Director of Compliance and Operations

CC: IACUC Files

Date: 2/11/2015

Re: IACUC Protocol **1504D-SM-SMLBirds-18** Approval

The University of Northern Colorado Institutional Animal Care and Use Committee has completed a final review of your protocol "**Toxicity of Venoms and Purified Toxins to Mice, Birds & Lizards**". 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 **1504D-SM-SMLBirds-18**.

The next annual review will need to be approved prior to February 11, 2016.

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: 4/28/2015

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, 2015**.

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**.