INVESTIGATING GEOGRAPHIC ISOLATION IN THE SUTTER BUTTES USING COMPARATIVE NORTHERN PACIFIC RATTLESNAKE (*Crotalus oreganus oreganus*) VENOM PROTEOMICS

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A Thesis/Project

Presented

to the Faculty of

California State University, Chico

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biological Sciences

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by

© Glenn Woodruff

Summer 2015

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DEDICATION

To my family and friends for their love and support.

ACKNOWLEDGEMENTS

I am greatly indebted to CSU, Chico Departments of Biological Sciences and Physical Sciences for their patience and persistence in helping me complete this research. I am grateful to my many field assistants, for their enthusiasm and eagerness in helping with spotting and handling rattlesnakes. My thesis committee, Dr. Tag Engstrom, Dr. Colleen Hatfield, and Dr. Dan Edwards for their time, help, patience and encouragement. Most importantly, I thank my family and friends, especially my parents Sheila and Steve, and my wife Julie, for their support.

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ABSTRACT

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Geographic variation research is an important topic of study, providing insight into trends of organismal origin, dispersal and evolution. By virtue of investigating these trends, this research also exposes areas where conservation efforts are needed to prevent the decline of vulnerable populations. The Sutter Buttes are an isolated patch of mountainous habitat in the middle of the Sacramento Valley. The buttes are separated from the Sierra Nevada by a minimum of nine miles to the east and from the Coast Range by ### miles to the west . The isolation of organisms in the Sutter Buttes has been proposed numerous times, and has also been the subject of research on several other projects looking at the relationship between populations of organisms in the Sutter Buttes and those in neighboring mountain ranges.

This project utilizes liquid chromatography and mass spectrometry to separate and identify proteins in the venom of multiple individuals of northern Pacific rattlesnake (*Crotalus oreganus oreganus*) from two regions: the Sutter Buttes and the nearby Sierra Nevada. I provide a whole venom profile for this subspecies of western rattlesnake, and identify specific proteins found to be significantly different in abundance between populations of *C. o. oreganus* inhabiting the two regions. Significant differences in the relative abundance of three venom proteins provide evidence of restricted connectivity between the two neighboring regions, indicating geographic isolation for certain organisms inhabiting the Sutter Buttes. This has important implications for local adaptation and conservation of unique geographic variants in rattlesnakes.

CHAPTER I

INTRODUCTION

Geographic Variation

The study of geographic variation among populations has been a primary interest of evolutionary biologists since Alfred Russel Wallace and Charles Darwin first proposed the mechanism Natural Selection (Darwin and Wallace, 1858). Both biotic and abiotic selective pressures that drive adaptation differ across varying habitats with differences in vegetation, latitude, precipitation, elevation and human habitation and many others (Ashton and De Queiroz 2001, Clark et al. 2010; Forstner et al. 1997). For venomous snakes, the proteins that comprise the venom are strongly affected by the geographically varying selective pressures (Mackessy 1988, 2003, and 2010, Gibbs and Mackessy 2009, Biardi and Cross 2010). For instance, as a population of rattlesnakes with similar venom composition disperses, variations in selective pressures of different regions can result in differences in the composition of their venom as snakes adapt to local prey populations (Jurado et al. 2007, Mackessy 2010, Gibbs et al. 2011, Glenn et al. 2012). As distinct populations evolve in response to their respective selective pressures, quantifiable variations emerge in traits such as body size (Mayr 1963), behavioral adaptations (Biardi et al. 2006, Biardi and Cross 2010) an on the efficacy of venom on varying prey base (Mackessy 1988, 2010, Mackessy et al. 2003).

Mechanisms of Isolation

The geology of northern California, as a whole, is largely dominated by two main mountain systems (Figure 1). The Sierra Nevada was created over four million years ago as the granite that had been formed under the crust began to uplift. In places

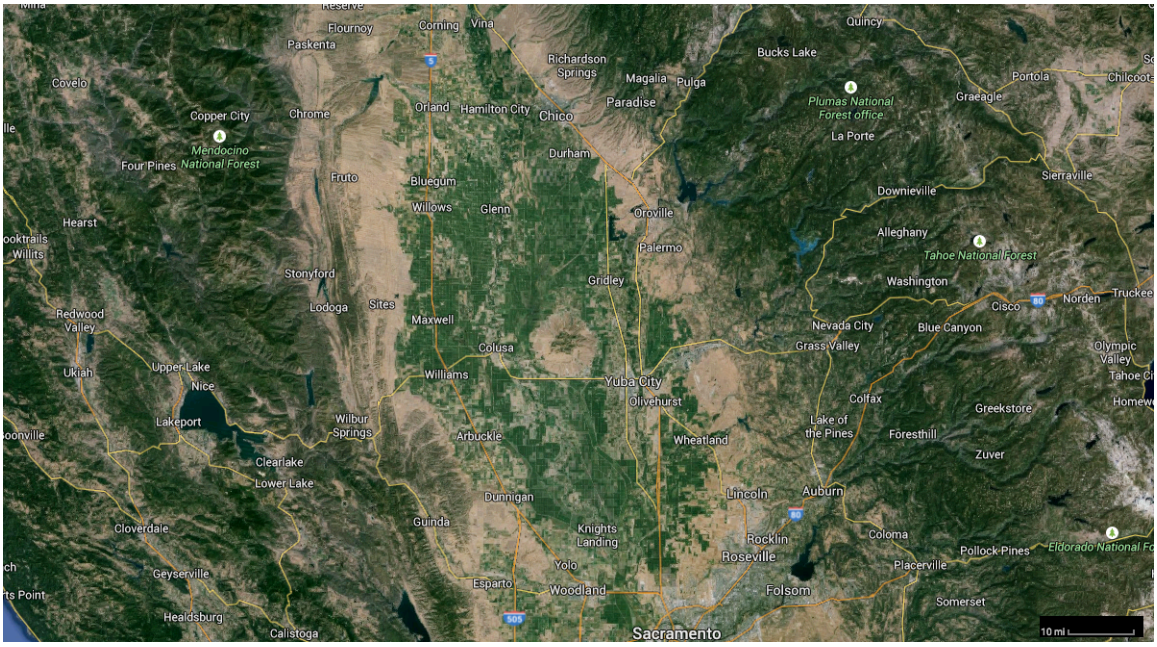


Figure 1: Northern California regional map showing the Sutter Buttes in the center of the Sacramento river valley (Google Maps 2015).

such as Yosemite this granite has been exposed by glacial erosion (Farquar 1925). The Coastal range lies on the San Andres fault line until Mendocino, where the range continues into Oregon and Washington (Oakshott 1972). These two ranges converge in northern California near Redding. Because the two ranges have created a bowl-like feature, all rain that falls on the inside of these mountain systems collects in the valley and brings with it soil, nutrients and seeds from these mountain ranges. Until 1945, the

Sacramento River valley was a vast, largely unpredictable wetland which made permanent settlement by people difficult (Kelley 1953).

The Shasta Dam, completed in 1945, with the storage capacity of Lake Shasta in northern California transformed the central valley (Kelley 1953) by virtually eradicated the annual flooding that made permanent settlement by people in the Central Valley so difficult. Owing to the rich soils formed by millennia of flood deposits from the Sacramento and Feather Rivers, and the high predictability and steady flow of the Sacramento River, agriculture quickly spread after the completion of the Shasta Dam (Kelley 1953). Today, instead of being seasonally surrounded by a sea of water, the Sutter Buttes are surrounded by large expanses of crops and increased urbanization that could severely limit animal movements in this area (Anderson 2004, Clark et al. 2010).

In addition to the alteration of the natural flow regime of the Sacramento River basin, and conversion of native habitat to agriculture, increased road construction and vehicle traffic associated with agriculture and urbanization have devastating effects on organisms, particularly snakes (Clark et al. 2010). Roads have been documented to limit migrations and gene flow for organisms due to road mortality (Clark et al. 2010). Surrounding the Sutter Buttes are two-lane highways to the east and a four-lane freeway to the west. Although many animals cross roads and travel at night when car traffic is limited, these structures surrounding the Sutter Buttes could limit migrations and thus narrow the genetic diversity within populations of the Sutter Buttes. Roads are relatively recent additions to the Sacramento River Valley and might not have been in place long enough to have caused genetic isolation of long-lived organisms (Clark et al. 2010), but may serve to keep existing genetic isolation in place by limiting gene flow. However,

even recent anthropogenic additions such as roads can affect the genetic diversity in measurable ways within a few generations (Mitrovich et al. 2013, Clark et al. 2010). Clark et al. (2010) showed that roads had a significant impact on the ability of Timber Rattlesnakes, *Crotalus horridus*, to disperse due to the instances of mortality and natural behavior of snakes as they encounter roads. Even minor roads greatly affected mating frequency between hibernacula (Clark et al. 2010), thus genetically isolating populations. They proposed that roads would have similar effects on other species with similar ecological traits.

The factors mentioned above have left few relatively small and undisturbed areas that remain good habitat within the Sacramento River valley. As a result, some species that used to exist more widely throughout northern California can now only be found in small isolated patches of appropriate habitat. Two examples of montane reptiles that have been studied and found to be isolated in this region include the sagebrush lizards, *Scelopourus graciosus*, (Rodgers 1953) and San Joaquin coachwhip snake, *Coluber flagellum ruddocki* (Stebbins 2003). In the Sierra Nevada and Coast Range mountains *S. graciosus* is typically found at higher elevations (often 5000 feet) than their relative, the western fence lizard, *S. occidentalis* (Stebbins 2003). In the Sutter Buttes however, *S. graciosus* inhabits the tallest peaks ranging from 1100 to 2200 feet (Rogers 1953, Stebbins 2003). This population of *S. graciosus* tolerates the hottest conditions and the lowest elevation of the species' distribution (Rodgers 1953). According to Rogers (1953), the last glacial advance allowed populations of the cold-tolerant *S. graciosus* in the Sierra Nevadas, Coast Range and Sutter Buttes to have relatively continuous contact. However, as the glaciers receded, *S. graciosus* populations in the Sierra Nevada and Coast Range

retreated to higher altitudes. The populations in the Sutter Buttes, with only limited altitude to climb, would have needed to adapt to the warmer, lower elevation climates and competition with their larger relative, *S. occidentalis*.

The San Joaquin coachwhip snake, *Coluber flagellum ruddocki* occurs from southern half of the San Joaquin valley and the eastern slopes of the south Coast Range as well as in two isolated populations north of the bay area: one population in Arbuckle, California and the other in the Sutter Buttes (Stebbins 2003, Anderson 2004). These examples suggest that it may be common for populations of animals to become isolated in the Sutter Buttes. Isolation of reptile populations in the Sutter Buttes may be attributed to a variety of factors in the landscape present between suitable habitats in Sutter Buttes and the Sierra or Coastal range.

The Sutter Buttes

The Sutter Butte mountain range lies in Sutter County, California, and provide habitat for hundreds of species, (Anderson 2004). This mountain range is a small isolated volcanic formation that is well suited for research on geographic variation (Figure 2).

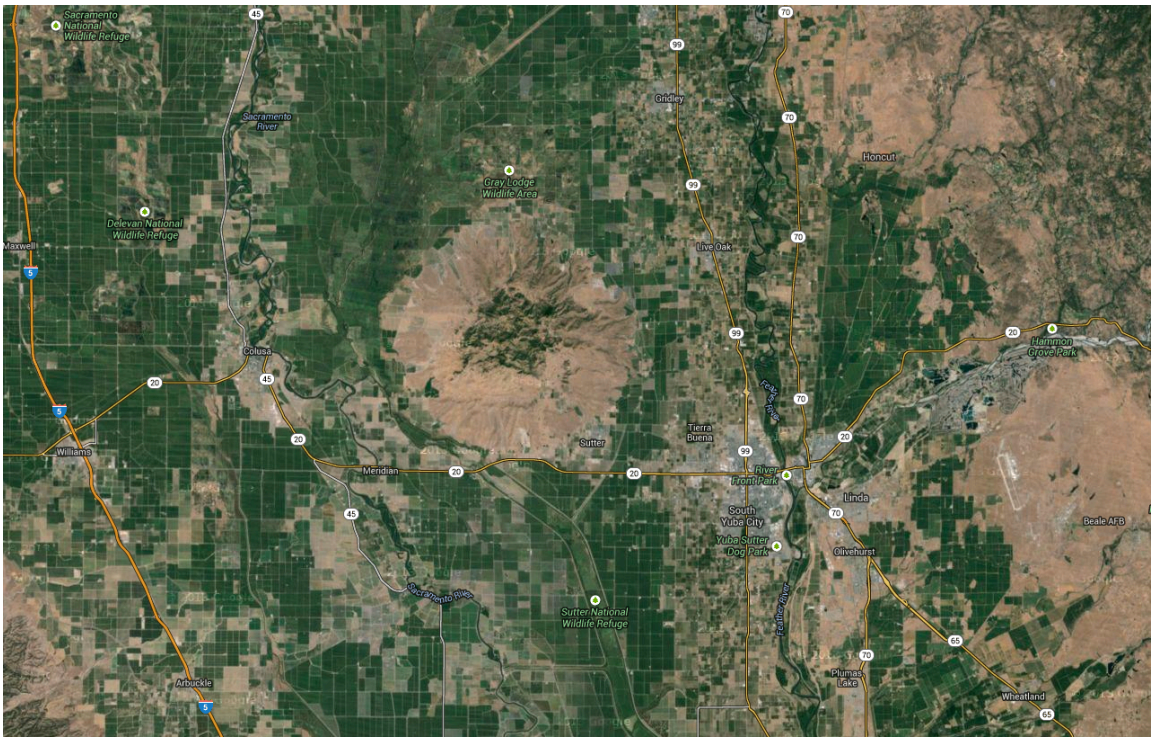


Figure 2: The Sutter Buttes of Sutter County in Northern California (Google Maps 2015)

This mountain range, within the Sacramento River valley, was created by a volcanic upheaval 1.6 million years ago (Williams and Curtis 1977, Anderson 2004). It is comprised of four geological subunits, containing a spectrum of different soils and vegetation composition (Williams and Curtis 1977, Anderson 2004). Today, the Sutter Buttes rise to a maximum height of 2070 feet above the valley floor and 2130 feet above sea level (Anderson 2004). The mountains form a ten-mile diameter circle, surrounded by a relatively flat valley floor (Williams and Curtis 1977, Anderson 2004). Because of the contrasting habitat that surrounds the Sutter Buttes by a minimum of nine miles, they are considered an "inland island" (Figure 1). The nearest habitat similar to the Sutter Buttes exists nine miles to the east, in the Sierra Nevada foothills.

Due to the volcanic origin of the Sutter Buttes, the contrast between this range and the valley floor is more than topography alone. The geological formations within the four major regions of the Sutter Buttes have different underlying structure, which gives way to soils of different composition, varying rates of erosion and also water permeability (Kelley 1998, Anderson 2004). These features set the Sutter Buttes apart from the valley floor which, in contrast, has loamy soils deposited by the numerous riparian systems (Kelley 1998). Prior to the construction of the Shasta Dam, the Sacramento River Valley floor would flood in large storm events (Kelley 1998). This influx of water and nutrients from historic floods would inundate only the perimeter of the Buttes leaving the higher elevation portions out of the water, and free from alluvial soil and nutrient deposition (Williams and Curtis 1977, Kelley 1998, Anderson 2004). Therefore, factors such as elevation changes, differing soil types, vegetation composition and the seasonal flooding of the Sacramento River Valley has served to isolate organisms in the Sutter Buttes (Rogers 1953, Stebbins 2003, Anderson 2004). Isolation that was initiated by geology and maintained by flooding, could persist due to agriculture, urbanization and roads.

Northern Pacific rattlesnakes

The northern Pacific rattlesnake, *Crotalus oreganus oreganus,* is a widely distributed subspecies in the western rattlesnake complex (Stebbins 2003). The western rattlesnake complex is comprised of five subspecies of *C. oreganus* whose combined range spans much of the western United States. The range of *C. o. oreganus* extends

from Santa Barbara County, north throughout California into Oregon, Washington and into British Colombia (Figure 3) (Nafis 2004, Stebbins 2003).

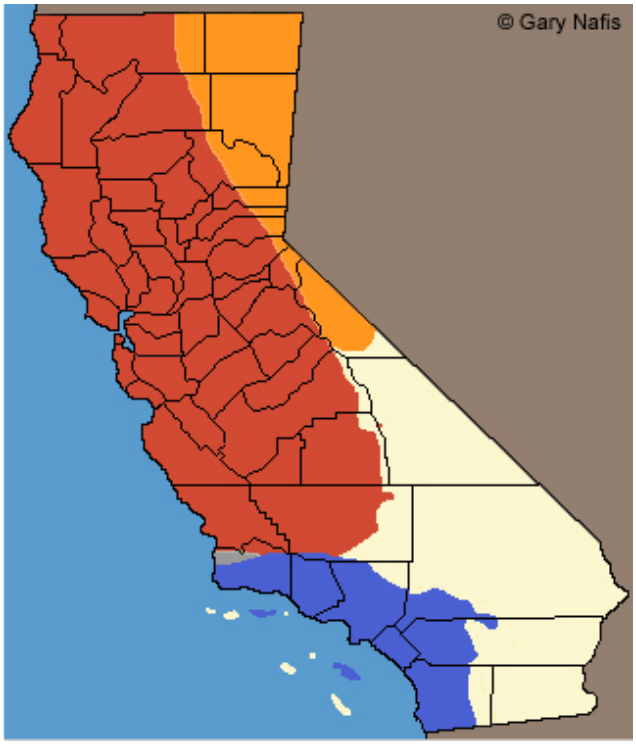


Figure 3: California distribution of *C. o. oreganus* in red. Other colors represent distributions of other *Crotalus oreganus* subspecies (Nafis 2004).

Crotalus oreganus oreganus, is a member of the family Crotalidae, one of five families of venomous of snakes. The evolution of venom in many species of snakes has allowed them to dispatch prey and begin digestion of their prey with enzymes and other proteins, instead of using the ancestral prey capture method of constricting prey still used by most non-venomous snakes (Mackessy 1988, Wilkinson et al. 1991, Biardi and Cross 2010 and Gibbs and Mackessy 2009). Throughout all venomous snakes, a multitude of venom adaptations exist to more effectively subdue the particular prey species with

which the population has evolved (Gibbs and Mackessy 2009, Mackessy 2003, Mackessy 2010). Venom can evolve in response to ecological factors such as prey diversity, type and abundance, as well as habitat and ancestry (Randy et al. 2004, Michael et al. 2008, Lind et al. 2010).

Venom Variation

Geographically isolated rattlesnake populations have been shown to exhibit micro-geographic variation in venom proteins over a relatively short period of time (Jurado et al. 2007). Within populations, the exchange of genetic material by interbreeding allows the population to pass on traits adapted to environmental pressures specific to their region (Highton and Webster 1976, Calvette et al. 2012, Biardi and Cross 2010, Bob et al. 2011). Traits from populations that are genetically isolated will diverge, developing adaptations specific to their region (Calvette et al. 2012, Oyler-McCance and Parker 2010, Mackessy 2010). Relevant traits including venom proteins are constantly fine-tuned and improved, while superfluous traits such as venom proteins that have little or no affect on the prey may be lost or left unchanged (Highton and Webster 1976, Forstner et al. 1997, Mackessy and Williams 2003, Mackessy 2010). Evolution of venoms that require less energy from the snake and kill prey more rapidly is an example of one such adaptation (Chippaux et al. 1991, Forstner et al. 1997, Gibbs and Mackessy 2009, Mackessy 2010).

Doley et al. (2009) found that many snake venom protein superfamilies undergo mutations at a much higher rate than other proteins not associated with venom. The phospholipase A2 family in venoms, for instance, was found to undergo rapid single base nucleotide mutations in its protein coding regions. These mutations change the surface

characteristics of the protein and thus their functionality (Doley et al., 2009, Nakashima et al., 1995). Additionally, substitutions leading to changes in amino acid residues are more common than substitutions that produce no change in amino acid residues of the cDNA protein-coding region (Doley et al., 2009, Nakashima et al., 1995). The increase in non-synonymous substitutions is evidence for positive selection for diversity (Charlesworth and Charlesworth 2010) in venom proteins. This change over time has lead to the great diversity of proteins in snake venoms, and the diverse effects they exhibit on their victim. Doley et al. (2009) called this phenomenon "the accelerated segment switch in exons to alter targeting" (ASSET). Doley et al. (2009) looked at several venom protein superfamilies and found these results in elapid and viperid snake venoms. Their research suggested this relatively high rate of change allows successful snakes to pass on the best venom cocktail for their region to their progeny (Wilkinson et al.1991, Biardi et al. 2006, Mackessy 2010) to assist in the killing and digestion of prey. It also could mean that if snakes become isolated from other populations, differences in venom protein composition can evolve over a relatively short period of time.

Identifying and comparing proteins in venom profiles can show where differences between individuals and populations are starting to emerge. The rapid rate of substitutions and mutations that occur in venom proteins allow for venoms to gain greater affinity towards prey in an evolutionary arms race (Biardi et al. 2006, Doley et al. 2009). Because of this, snake venoms do not affect all potential prey equally. Venom affinity for specific species can demonstrate how snake populations have evolved to more effectively subdue a specific prey guild (Biardi et al. 2006, Gibbs and Mackessy 2009, Calvete et al. 2012). Gibbs and Mackessy (2009) demonstrated that venom from four different

subspecies of *Sistrurus* rattlesnakes exhibited greater lethality toward mammals than other experimental prey groups including frogs and other reptiles. Within all four *Sistrurus* subspecies sampled, relatively little venom was needed to kill mice; whereas this same venom showed less effectiveness towards frogs, and required more volume for a lethal dose indicating that venom in these snakes has evolved primarily for mammals. Within the four subspecies of these snakes, the amount of venom needed to kill the mice varied from subspecies to subspecies. This variation can reflect co-evolution between snakes and prey, where venom in the "more toxic" specimens was likely adapted to subdue prey most similar to the lab mice used. As explained by Gibbs and Mackessy (2009), had the sympatric prey species been used in this experiment, the amount of venom required to kill each type of animal would likely be higher due to co-evolved resistances in the the prey to counter specific snake toxins (Brodie and Brodie 1999, Biardi et al. 2006, and Gibbs and Mackessy 2009). If populations of rattlesnake and their prey species in the Sutter Buttes are genetically isolated from other populations in the Sierra Nevadas and Coast Range, it is possible that rattlesnake and prey are co-evolving (Brodie and Brodie 1999, Biardi et al. 2006). An affinity for a specific prey type can be shown when venom demonstrates greater lethality toward that species (Gibbs and Mackessy 2009, Dos-Santos et al. 2011, and Lomonte et al. 2012).

Similar studies have examined intraspecific variation in the venom of rattlesnakes across geographically isolated regions (Forstner et al. 1997, Wilkinson et al.1991, Jurado et al. 2007). Forstner et al. (1997) utilized gel electrophoresis of the whole venom profile to study intraspecific variation in the mottled rock rattlesnake, *Crotalus lepidus Lepidus,*

but this and other early studies, relied solely on the comparison of unidentified proteins for relative differences in venom profiles (Wilkinson et al. 1991, Forstner et al. 1997). More recently, combined techniques of separating the proteins by gel electrophoresis and liquid chromatography or MALDI-TOF have been used (Calvete et al. 2012, Lomonte et al. 2012). Lomonte et al. (2012) analyzed the toxicological properties of a Central American pitviper, *Bothriechis supraciliaris,* using a combination of gel electrophoresis, reverse phase HPLC (RP-HPLC), MALDI-TOF and a lethal dosage experiment. This variety of information helped give a more complete picture of the venom properties for *B. supraciliaris* and allowed them to better evaluate hypotheses about the differences between venom properties of this species compared with from its sympatric relatives.

Statement of Research Investigation

The goals of this research are (1) to produce a venom profile for *C.o. oreganus* to allow broad comparisons between the venom characteristics of other subspecies within the western rattlesnake complex. And (2) to determine if the Sutter Butte populations of *C. o. oreganus* possess variation in their venom proteins that could be attributed to geographic isolation from other populations outside the Sutter Buttes. *C. o. oreganus* of the Sutter Buttes have not yet been the focal point of a major research project, and whether or not they are isolated from other regions is unknown. If venom profiles of rattlesnakes are different between regions, further research can shed more light on the functional and ecological importance of these venom differences and the mechanism for isolation of the Sutter Buttes populations. High Pressure Liquid Chromatography (HPLC), Sodium Dodecyl Sulfate Polyacrylamide Gel-Electrophoresis (SDS-PAGE) and

Liquid Chromatography Mass Spectrometry (LC-MS) analysis were used to purify, separate and identify all possible proteins in the crude venom of *C. o. oreganus*. This information will be used to compare differences and similarities between populations of *C. o. oreganus* from the Sutter Buttes and from the nearby Sierra Nevadas.

CHAPTER II

Methods

Study Sites

The snakes for this project were caught in two main regions classified as either Sierra Nevada snakes, or Sutter Butte snakes. The Sierra Nevada sampling locations included; The Big Chico Creek Ecological Reserve (BCCER), Humboldt Road, and Saddleback Ranch. The BCCER is a property located in the foothills of the Sierra Nevada and managed as an ecological reserve by the Institute for Sustainable Development, Chico, CA, and owned by California State University, Chico. The BCCER is located at 39°50’41 N, 121°42’06 W. Elevation in the reserve ranges from 700- 2044 feet and contains 3,995 acres of oak woodlands, chaparral, pine forest, and riparian zone including 4.5 miles of Big Chico Creek. Given this diversity of habitat, the BCCER is home to many animals, including a sustainable population of *C. o. oreganus.* The BCCER has many uses and is open to public use on a restricted basis. Due to its close affiliation with the university, the BCCER is the focus of many master’s thesis research projects. It is also open to nature hikes, hunting, learning seminars, and student field trips.

The Humboldt Road site is a section of road paralleling grasslands and a productive rock wall. Only a small section of Humboldt Road was sampled and this section will hereby be referred to as the Humboldt Road site. The section sampled is a public, 3.55 mi (5.68 km), poorly maintained road that parallels highway 32, east of Chico, Ca. The center of sampling on Humboldt Road is located at 39°44’46 N,

121°46’06 W. Elevation ranges from 270 feet to 1015 feet as it heads into the Sierra Nevada foothills. This area is a blue oak woodland savannah containing an understory of mostly non-native grasses and Yellow Star Thistle (*Centaurea solstitialis*). *C. o. oreganus* sampling was restricted to rock walls and nearby blue oak savannah woodland that runs parallel to Humboldt road. The Humboldt Road site has relatively low vehicle traffic, and as a result is often used for illegal refuse disposal. The surrounding lands are also used for grazing of cattle. Humboldt Road lies approximately 1.6 mi (3 km) to the south of Big Chico Creek and .6 mi (.7 km) north of Little Chico Creek.

Saddleback Ranch is a private ranch in the Sierra Nevada foothills located due east of the Sutter Buttes. The ranch is located at 39°16’35 N, 121°27’37 W. Elevation on the ranch ranges from 230 feet to 750 feet. It contains approximately 5000 acres of blue oak woodland with numerous large rocky outcrops. The dominant understory is invasive grasses and Himalayan blackberry. Saddleback Ranch is designated as a ranching property used for grazing, and rice farming. It also has four residences, a landing strip and several manmade lakes and streams for recreation.

The last sample location took place in the northern interior of the Sutter Buttes. The Sutter Butte mountains are a circular formation 10 miles (16km) in diameter near the cities of Live Oak to the east, and Colusa to the west. Its approximate center is located at 39°15’25 N, 121°49’01 W. The Buttes were created approximately 1.6 million years ago by a volcanic upheaval, taking place in the Northern California Sacramento River valley. Elevations range from 85 feet to 2000 feet. The periphery of the mountains is primarily private agriculture land including olive, grape and almond orchards. Sampling took place in Sutter Butte State Park, on the north side of the Buttes, in a region called Peace Valley.

This blue oak savannah also has a dominant understory of non-native grasses and Yellow Star Thistle. The land is used primarily for cattle grazing within Peace Valley.

Field Techniques

Snakes were caught and classified as either "Sutter Butte" snakes, or "Sierra Nevada" snakes depending on the sampling location. All venom samples were extracted from snakes near the site of capture. Only snakes that could be safely captured without disruption of habitat were considered for sampling. Snakes were placed into a breathable, nylon bag using snake tongs and snake hooks after capture (Figure 4a). They were placed in the shade for a period of time no exceeding 30 minutes while sampling materials were prepared. For venom extraction, the snake's head was held down with the snake hook to allow the capturer to grab behind the head. Snakes were encouraged to bite through a latex glove stretched over the collection container (Antonio 2007) (Figure 4b). The venom was then placed into a sterile, 1.5 mL plastic vial, and stored in a cooler containing dry ice for transport to the lab where the samples were placed in long-term storage at 20°C.

Figure 4: A) Snake tongs, snake hook and nylon sack for the handling of *C. o. oreganus*. B) Biohazard collection cup used for venom collection with a latex glove stretched over the top.

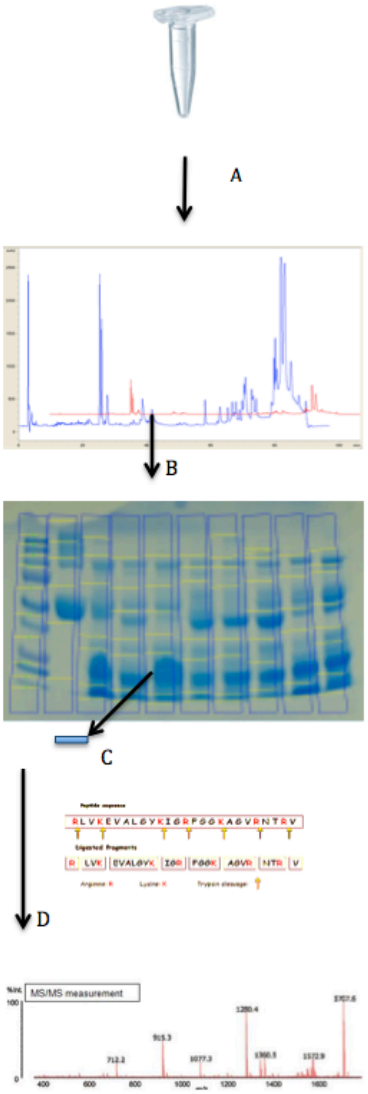
Upon completion of the venom extraction, addition measurements were taken for each snake. A 600g or 5kg Pesola scale was used to measure the weight of the snake in the nylon bag. The scales were tared with the nylon bag so that it did not contribute to the measurement. The snout-vent length of each snake was measured using a flexible measuring tape for length, total length, and circumference taken at the widest part of the snake. The sex of each snake was determined by gently inserting a sexing probe inside the vent of the snake, posteriorly (Antonio 2007) . The hemipenes of *C. o. oreganus* extend caudally. If the probe could be inserted into the snake, hemipenes were present, and the snake was deemed male. If the probe could not be inserted the snake was deemed female.

For identification purposes, Passive Integrated Transponders (PIT) tags (Biomark, 5x12.5mm) were inserted into snakes that were longer than 30 cm Snout Vent Length and not pregnant.. The PIT tag needle was inserted according to Herpetology Lab

Guidelines (Rice et al. 2006). Upon completion of data collection, all snakes were returned to their place of capture.

Venom Processing

All crude venom samples were evaporated to dryness in a Speed-Vac centrifuge and stored at -20°C. Between 1.5 - 2.5 mg of the crude dry sample was weighed on an analytical balance and dissolved in nanopure water to the concentration of 10mg/mL. Samples were vortexed for 30 seconds to ensure all soluble proteins were dissolved. Insoluble material was removed by centrifugation in an Eppendorf centrifuge for 10 minutes at 16873 g-force. 100 µL of crude venom was separated by High Pressure Liquid Chromatography (HPLC) using an Agilent Liquid Chromatography system fitted with a Dionex 218 MR C18 Reversed Phase 4.6x 250mm column with initial conditions of 5% of solution B (99.9% acetonitrile with 0.1% Trifluoroacetic acid or TFA) and 95% solution A (0.1% TFA in nanopure water) at 1ml/min (Figure 5).



Rattlesnake venom is extracted and processed for LC-MS

A) Venom proteins are passed through an HPLC column made to separate proteins.

B) For each peak, a fraction is collected and put in a micro-centrifuge tube. The tubes are later processed for gel electrophoresis.

Each fraction is processed and loaded into a gel lane. Proteins are separated by size during SDS-PAGE.

Each fraction often contains multiple proteins represented by different bands.

C) Individual gel bands are excised from their lane and digested using a Pierce In-Gel tryptic digestion procedure.

D) Digested peptides are separated and analyzed by LC-MS/MS for identification

Figure 5: Summary of venom separation and identification procedure

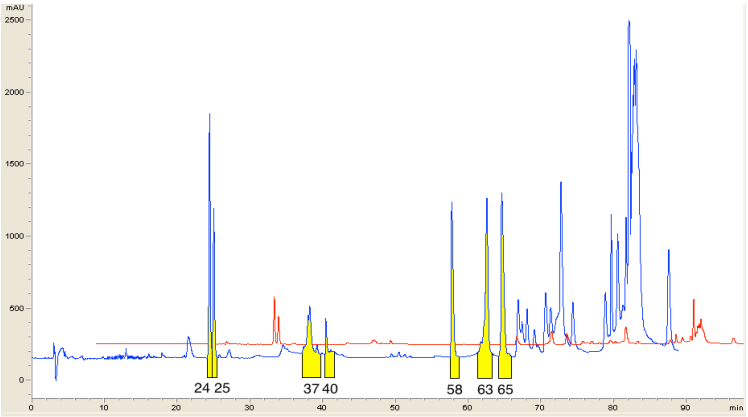
Crude venom was followed by a linear gradient of 5% solution B for 10 minutes, 5-15% B over 20 min, 15-45% B over 120 min and 45-70% B over 20 min (Lomonte et al. 2012). The protein elution was monitored by UV with a Diode Array Detector (DAD).

Protein elution was monitored at wavelengths of 215 nm and 245 nm. Peptide bonds present in proteins absorb in the wavelength range of 190-230 nm (Lomonte et al.

2012), therefore the absorbance at 215 nm gives an estimate of the abundance of amino acids at a given time. Protein abundance is proportional to peak size on the chromatogram (Lomonte et al. 2012). Thus, proteins are separated polarity, and using the abundance of peptide bonds in the protein, assigned a relative size peak based on the abundance of protein at a given time. Each peak was integrated and a percent area was calculated as a percentage of the total sample. This process was done for each venom sample and a chromatogram generated.

Chromatogram data were analyzed by visually inspecting the output from each crude venom sample for similarities and differences in the retention times of the proteins separated by liquid chromatography. Retention times and relative abundance of each peak were compiled for statistical analysis. Peaks were chosen for statistical analysis based on the criteria that they were well resolved from other peaks and showed a broad pattern of similarity between all individuals. The percent area of the selected peaks was analyzed for differences in relative abundance between site locations and sex. Peaks representing elution times of 23 minutes, 25 minutes, 37 minutes, 40 minutes, 58 minutes, 63 minutes and 65 minutes in characteristic pattern (Figure 6) were selected for futher analysis due to consistent peak separation.

Specimen 2



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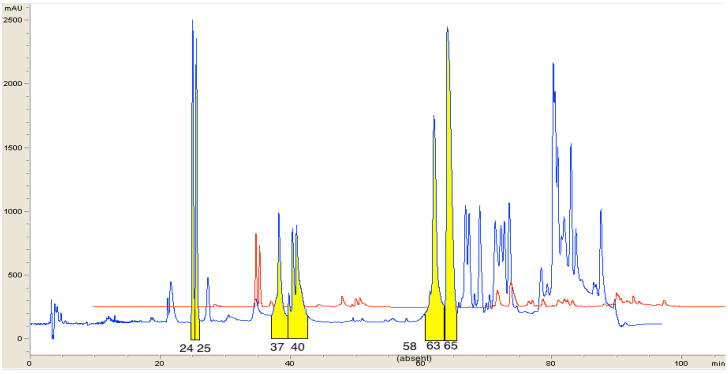

Specimen 40

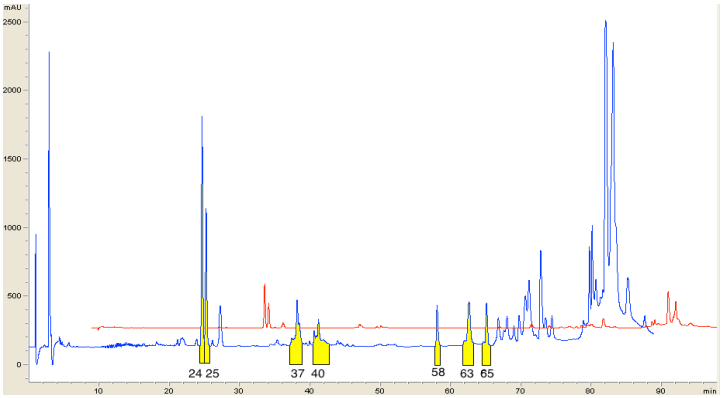

Figure 6. Three chromatograms from three *C. o. oreganus* individuals. Highlighted area under peaks used for quantifying differences for statistical significance.

Peaks such as 37 and 40 that seemed to comprise a "peak complex" were grouped together as a single protein for statistical analysis. Research on the myotoxin family of proteins suggests that these peaks are isomers of the same protein (O'Keefe et al. 1996)

A two-way ANOVA was run for each of the seven peaks to test whether there was significant variation between locations and sex for each represented protein. The dependent variable was the relative proportion of sample under the respective peak, and the independent variables were the location (Sutter Buttes or Sierra Nevada) and sex. The peptides from these peaks were analyzed from six different samples to ensure the differences being statistically analyzed were for the same proteins.

In-Solution Digestion

Based on the two-way ANOVA of the fractions previously mentioned, the five peaks that were selected for analysis were collected for in-solution digestion so the proteins within each fraction could be identified. Protein identification was necessary because peaks eluting at the same time from different samples are not necessarily the same compounds, so identifications were made to confirm that the peaks corresponded with the same proteins in each sample. Six specimens had fractions coinciding with peaks 37 and 40 collected off the HPLC column and digested according to the procedure described by Thermo-Fisher (2013).

The collected HPLC fractions were evaporated under vacuum on the Speed-Vac until only the solid protein remained. They were suspended in 100 μL nanopure water and their pH adjusted to 7 using 0.5M NAOH. This was necessary because the TFA from

the HPLC solutions would produce conditions too acidic for trypsin digestions to be viable. For each fraction, 100 μL of protein solution was combined with 125 μL of 50 mM NH_4HCO_3, 25 μL of 200 mM Dithiothreitol (DTT), and 250 μL trifluoroethanol. This mixture was heat treated for 20 minutes in a Perkin Elmer DNA Thermocycler 480 at 95°C. Samples are briefly centrifuged to remove any condensation from cap.

25 μL of 630 mM iodoacetamide is added and the tubes are incubated in the dark at room temperature for 30 minutes. To a new tube, 25 μL of the original mixture are added to 205 μL of 50mM NH_4HCO_3, 10 μLof 125 mM $CaCL_2$ and 10 μL of trypsin working solution. This mixture is heated to 55°C for 30 minutes in the thermocycler. 15 μL of neat formic acid is added to each tube and vortexed. 100 μL of solution from each tube is placed in the 96 well-plate for LC-MS analysis. The well plate is covered with foil and the instrument is set to inject 50 μL onto the 2.1 x 150mm C18 column. Solvent A (0.1% formic acid in water) and Solvent B (0.1% formic acid in acetonitrile) are used to run the sample through the column. The gradient used for this procedure starts at 2% Solvent B with a linear gradient over 32 minutes to 60% Solvent B. It is bumped up to 80% for 8 minutes and a linear gradient back to 2% Solvent B for the next injection. Peptide mass spectra were submitted to database search against National Center for Biotechnology Information (NCBI) using Mascot algorithm and Xcaliber software.

In-Gel Digestion

I performed In-Gel digestion for two venom samples (one from the Sutter Buttes and one from the Sierra Nevada foothills) to obtain molecular weight data from proteins.

Fractions for all peaks visible on the chromatogram were collected . This provides additional information for protein identification not available from HPLC and other "ground-truthing" procedures alone. The collected HPLC fractions for this venom sample were further separated using sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS- PAGE). All fractions were evaporated to dryness using a Speed-Vac. Residual peptides were stored at -20 °C until further analysis could be conducted. The dehydrated elutant from each fraction was resuspended in 750 μL of 1x running buffer, to a concentration of 6.67 mg/mL. Using a micropipetter, 37.5 μL of resuspended venom was placed into a .5 mL centrifuge tube along with 12.5 μL of sample prep cocktail (250mM Tris, 40% glycerol, 4% SDS, 0.08% bromophenol blue, 400mM DTT, 4mM EDTA, pH 6.8) for a total of 50 μL of total fluid. Samples were briefly vortexed to ensure the mixture was homogenized. Each sample was then placed in a Perkin-Elmer 480 Thermocycler for 5 minutes at 95°C allowing the SDS to bind to the polypeptides in the venom proteins at a constant weight ratio (Guzzetta 2001)**.** These samples were briefly centrifuged to remove fluid from the cap, and 25μL total fluid was added to lanes 2-12 from one of each of the fractions. 10 μL of Dual-Color Precision standard was injected into the first lane for molecular weight reference. Once loaded, samples ran on 1mm 12% polyacrylamide BioRad gels for 45 minutes, or until the bands reached the bottom of the gel, at a voltage of 200 with constant amps. Gels were then stained using Simple Stain overnight so that the bands could be made visible. Gels were rinsed twice with nanopure water, and destained with nanopure water and a Kim-Wipe for about an hour until the protein bands were visible. Once protein bands were clearly identifiable, gels were photographed using a light box with a digital camera and analyzed using Imageaid

software. Molecular weights for the protein bands was determined based on comparisons to the Precision Dual Color standards (BioRad) in lane one. Once photographed, gels were placed on a clean glass plate, and bands were assigned a specific identification number based on the venom sample, fraction time, gel lane and band location. Bands were excised from the gel using a sterile razor blade. They were diced into eighths, stored in 1.5mm centrifuge tubes and properly labeled. Gel pieces were stored at +4°C until digestion could commence.

Gels bands were digested according to the Pierce In-Gel Digestions instruction manual (Figure 7). The purpose of digestion is to cleave the peptide bonds present in peptides of each gel band. Without first cleaving the peptides into their amino acid substituents, identifications of the peptides could not be made in later steps.

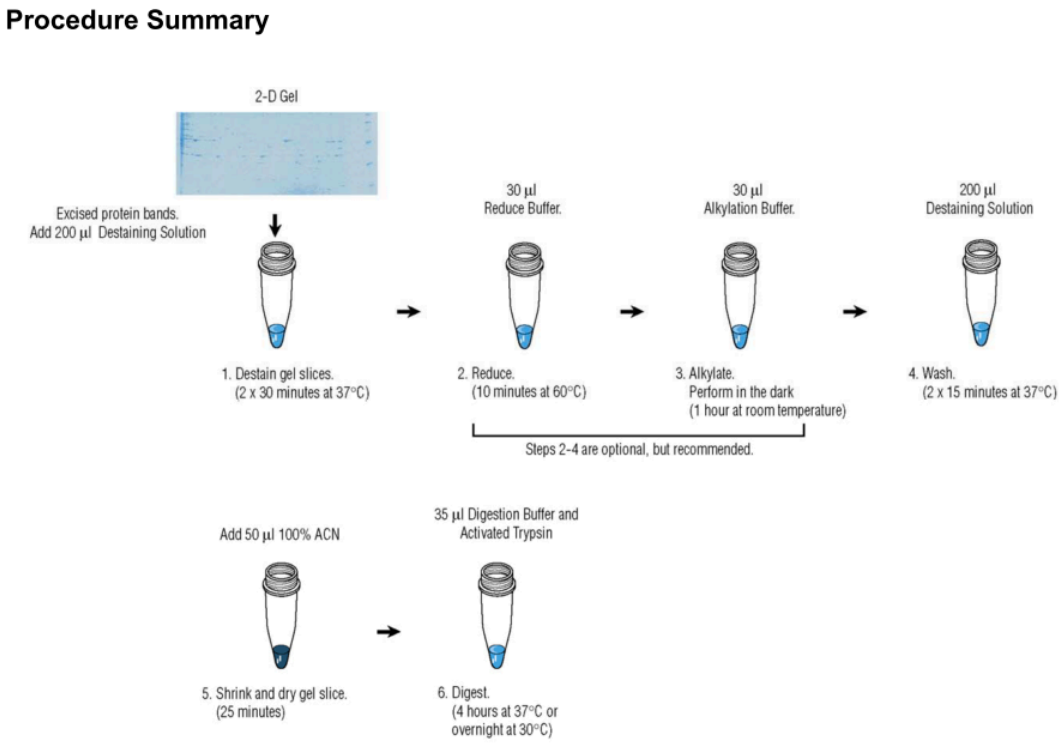

Figure 7. Pierce In-Gel Tryptic digestion (Thermo Scientific, 2010)

Upon completion of the digestion procedure, vials were centrifuged for one minute to ensure all gel fragments were forced to the bottom and out of the way. 100 µL of fluid was drawn off the gel pieces and placed in a 96 well plate for analysis. 50 µLs from each well was injected into a Dionex 218 TP C18 Reversed Phase 2.1 x 150mm 300Â 5µ column and a LCQ Advantage ThermoFinnigan electo-spray ionization liquid chromatography instrument using the same HPLC solvent gradient conditions as described for the In-Solution procedure.

Peptide mass spectra were submitted to database search against National Center for Biotechnology Information (NCBI) using Mascot algorithm and Xcaliber software. One complete venom sample had all possible proteins identified using this method. This

allowed there to be molecular weight data in conjunction with the protein identifications for the peaks that came off the HPLC. Using this information, a more complete table of the *C. o. oreganus* venom profile could be constructed to help identify intraspecific differences and similarities between other subspecies and species of rattlesnakes.

CHAPTER III

Results

Field Collection Success

Between June 2011 and October 2012, 53 *C. o. oreganus* were sampled from Northern California, in the Sutter Buttes State Park and the three locations within the Sierra Nevada foothills; Chico, Loma Rica, and Forest Ranch, Ca. A total of 19 snakes were sampled from the Sutter Buttes. A total of 34 Sierra Nevada *C. o. oreganus* were sampled from the BCCER, Forest Ranch, Humboldt road, Chico and Saddleback ranch, Loma Rica (Figure 8). Venom was initially processed using RP-HPLC for a general comparative analysis of their venom protein chromatograms. Mature (>70 cm) rattlesnakes were encountered the most frequently, and yielded the best venom samples (Figure 8). Sub-adult (45-70 cm) were successfully sampled from both sites, but far fewer snakes were encountered in this size class. Juvenile (<45 cm) snakes were also collected with the intent of being incorporated into the study, but no usable venom samples were obtained from juvenile snakes in the Sutter Buttes (Figure 8).

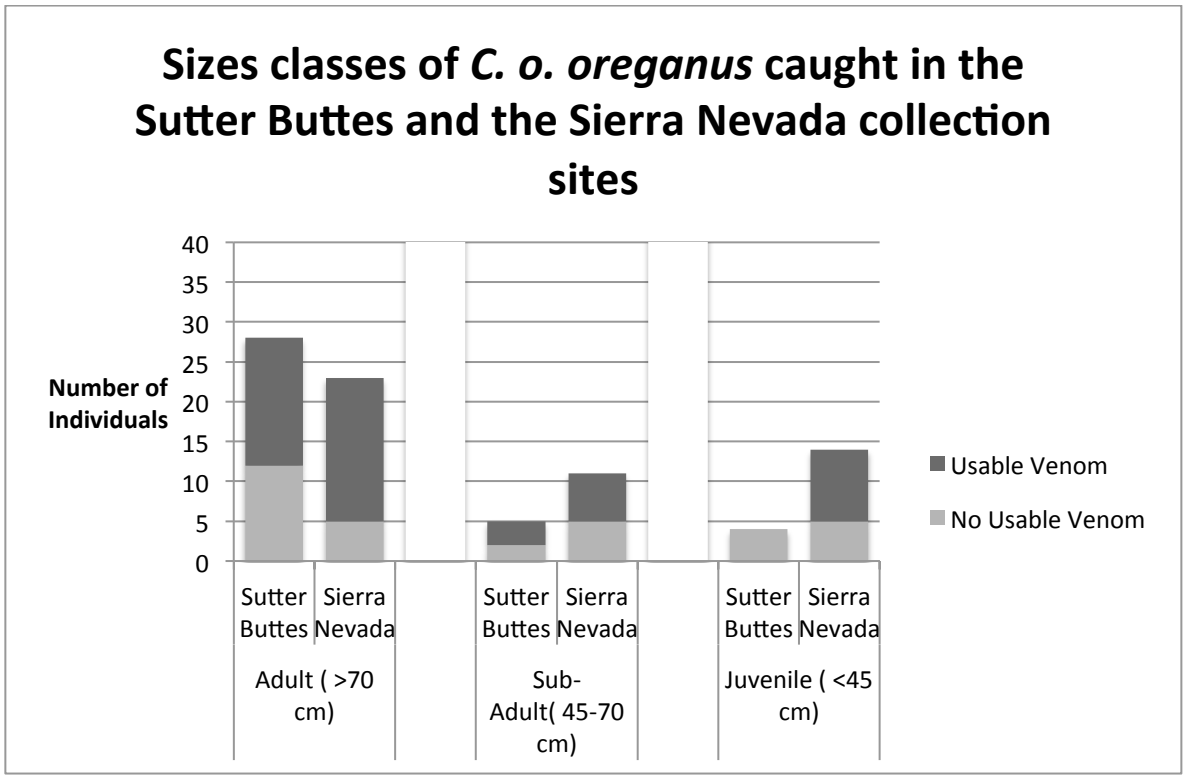

Figure 8. Total number of *C. o. oreganus* caught, measured and sexed including those that no usable venom could be extracted.

The discrepancy between collecting demographic information from the snakes and usable venom samples is due to an initial learning curve for rattlesnake venom collection and the increased danger to both handler and snake associated with handling juvenile snakes. These smaller snakes are more delicate and have a greater potential for handler error than larger sub-adults and adults. The smaller head and lack of well-defined "neck" made venom extraction from juveniles more difficult. As a result, few samples were successfully collected from juvenile snakes and the only juvenile snake venom samples with sufficient volume to analyze were from the Sierra Nevada sites making comparison between locations impossible.

Therefore, only adult and sub-adult snakes were used for statistical analysis of between site variation.

Venom profile

A venom profile was constructed for one *C. o. oreganus* adult female from the Sierra Nevada (Table 1). This was performed in order to obtain a more broad toxicological profile for this subspecies. Currently, no venom proteom database exists for C. o. oreganus, but MS/MS sequences were compared to proteins obtained from other species using National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). The main families of venom proteins identified were myotoxin, flavin monoamine oxidase, phospholipase, vascular endothelial growth factor (VEGF) toxin, cysteine-rich secretory proteins (CRISP) and peptidase.

Table 1: Venom profile of *C. o. oreganus*, sampled from the Sierra Nevada. Proteins are grouped together based on the gel bands they were initially excised prior to tryptic digestion. The molecular weight for each band derived from the Precision-Plus molecular weight standard is shown under "SDS-PAGE M.W.", rounded to the nearest kilodalton (kD). Molecular weights of the same protein gathered from Swiss Protein Database are provided under "Theoretical M.W." rounded to the nearest kilodalton. MS/MS Derived Sequence shows the peptide sequence that matched the records in the database, allowing it to be assigned to a related protein. M/Z is the mass to charge ratio for that peptide sequence.

Elution Time (min)	**Observed MW (kDal)**	**Theoretical MW (kDal)**	**Identified Protein**	**MS/MS- Derived Sequence**	**m/z**
Elution Time (min)	**Observed MW (kDal)**	**Theoretical MW (kDal)**	**Identified Protein**	**MS/MS- Derived Sequence**	**m/z**
37	7	5	Crotamine-01	K.ICIPPSSDFGK	610.3
40	8	5	Myotoxin-01	TVICLPPSSDFGK	1363.69
61	13	15	Phospholipase A2	K.TDIYSYSWK.R	581.98
	13	16	Vascular endothelial growth toxin	R.EMLVSILDEYPSE	755.68
	13	26	Catrin-1	R.IGCAAAYCPSSK.Y	643.06
64	26	27	Piscivorin	R.SVNPTASNMLK	589.49
67	38	26	Thrombin-like enzyme bilineobin	R.NSEHIAPLSLPSS	773.41
69	25	26	Thrombin-like enzyme gyroxin	R.LNKPVSYSEHIAP	1002.63
72	26	28	Serine protease catroxase	R.LDRPVSNSEHIAP	987.37
	11	14	CHH-B subunit beta	K.FCTQQHTGGHLV	592.7
74	58	59	L-amino acid oxidase	R.ETDYEEFLEIAK	744.1
78	21	22	Zinc metalloproteinase disintegrin	R.YVELFIVVDHGM	605.69

Proteins from Table 1 were initially separated by SDS-PAGE under reducing conditions. The descending order of the Elution Time reflects the actual time the proteins eluted from the HPLC column. Certain peaks were not identified including those peaks eluting prior to minute 37. The relatively minute quantities represented by these proteins in the corresponding chromatogram of Figure 9 made their detection difficult using this technique.

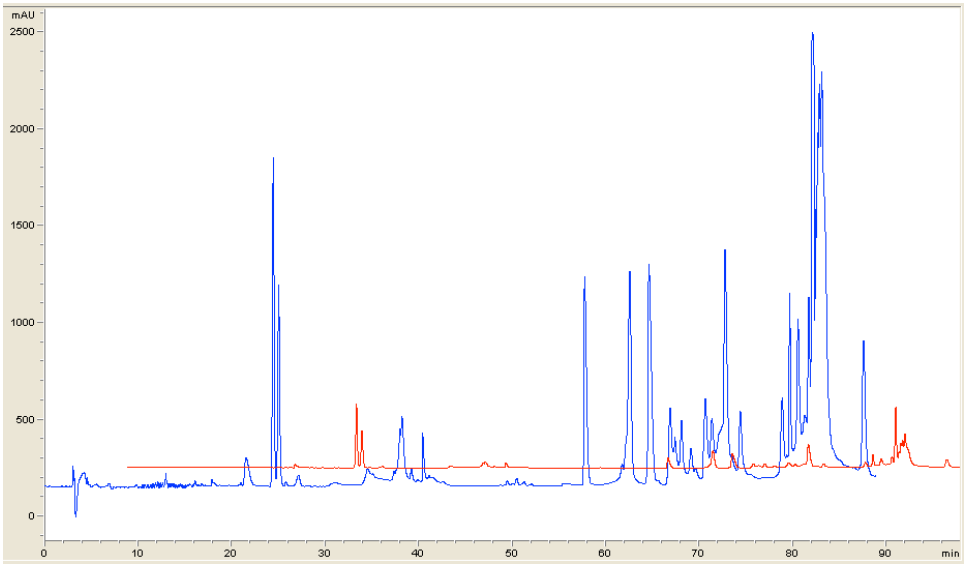

Figure 9: Chromatogram for Specimen 2 from the Sierra Nevada corresponding to Table 1.

Elution times from Table 1 correspond to the peaks present in Figure 9. My protein identification technique required that peaks be well-resolved and relatively abundant. More sensitive techniques and instrumentation as well as additional background knowledge of venom protein could provide a better yield for protein identification.

Comparative proteomics

Chromatograms were collected from the Agilent instrument in the CSU, Chico Physical Sciences building and compared with one another to find patterns within the crude venom signature for each snake. The latter portion of the chromatogram has poorer resolution due to the increased number and temporal overlap of eluted peptides. For this reason, peaks after 67 minutes were not considered for comparison analysis. Peaks with elution times of 24, 25, 37, 40, 58, 64 and 67 minutes were consistent among the majority of snakes (Figure 9).

The 2-way ANOVA provided information on how well correlated the relative peak abundance was between sexes and between locations. No positive correlations were found for peaks eluting at time 24, 25, 58 and 67, indicating there is no significant difference between location and sex for these peaks. Peaks that showed the same characteristic shape at times 37, 40 and 64 did however show statistically significant differences with respect to location (Table 2). The relative area under peak 64 showed significance for both location and sex, though no correlations between the two (Table 2).

Table 2: Summary results of a two-way ANOVA using SPSS. Percent area of specific peaks as a function of sex and location. Significance was chosen for values with P <.05. Asterisk denotes statistical significance at P <0.05.

Peak	Location		Sex		Location x Sex	
	F-value	P-value	F-value	P-value	F-value	P-value
24	0.382	0.539	0.065	0.800	1.213	0.276
25	2.110	1.530	0.725	0.399	0.769	0.385
37	6.107	***0.017**	0.010	0.920	0.896	0.340
40	5.725	***0.021**	0.006	0.941	0.404	0.528
58	1.807	0.185	0.217	0.643	1.388	0.244
64	11.653	***0.001**	6.498	***0.014**	0.575	0.452
67	0.752	0.390	0.063	0.803	1.975	0.166

Based on these results, and P- values of less than .05 for these tests, we can be >95% confident that the abundance of proteins represented by peaks 37, 40 and 64 are statistically significantly different between the Sutter Buttes and the Sierra Nevada. We can also be >95% confident that the abundance of proteins represented by peak 64 is significantly different between males and females. Figure 10 and Figure 11 show actual abundances of the analyzed peaks and the error within the sample.

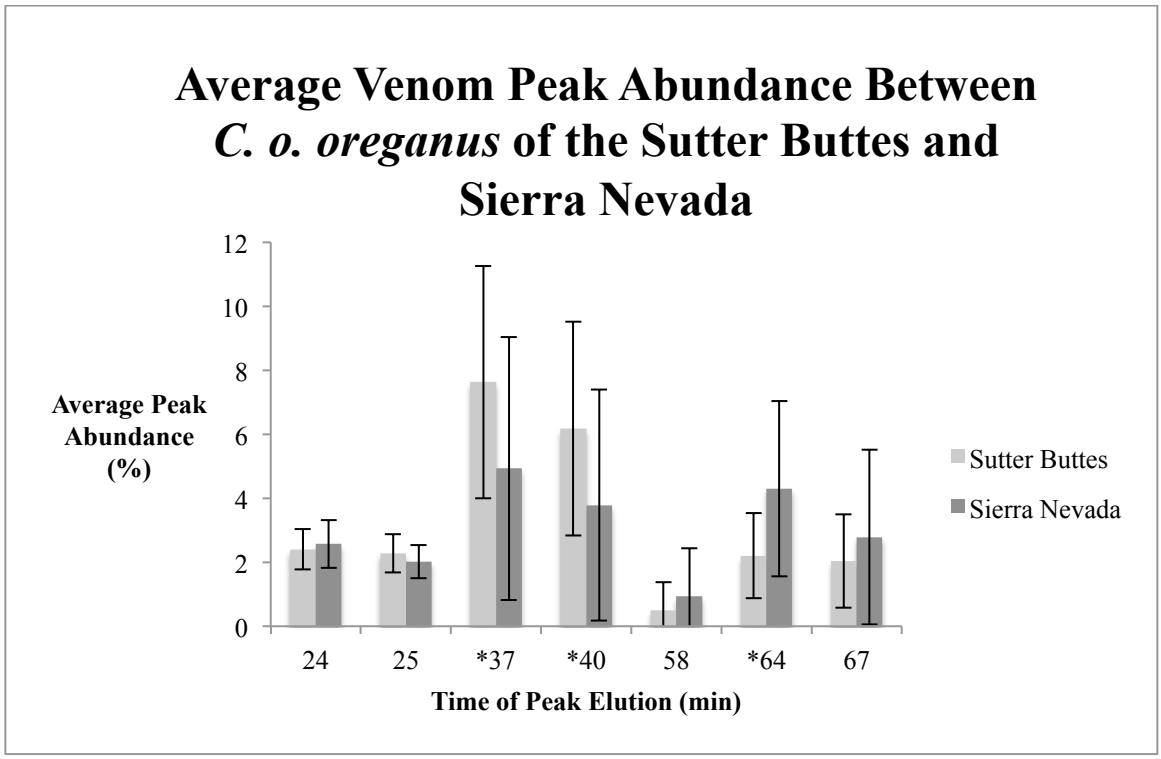

Figure 10: Average Peak Abundance Between Sutter Butte and Sierra Nevada *C. o. oreganus.* Astericks denotes significance.

Each of the peaks analyzed were compiled so that the averages between locations and sexes could be examined (Figure 10 and 11). Figure 10 shows that the Sutter Buttes snakes have greater average peak abundance for peak 37 and 40, whereas Sierra Nevada snakes have greater average peak abundance for peak 64. These peaks are significantly different as shown by the 2-way ANOVA. The error bars reflect the variations in quantity of each respective protein from snake to snake.

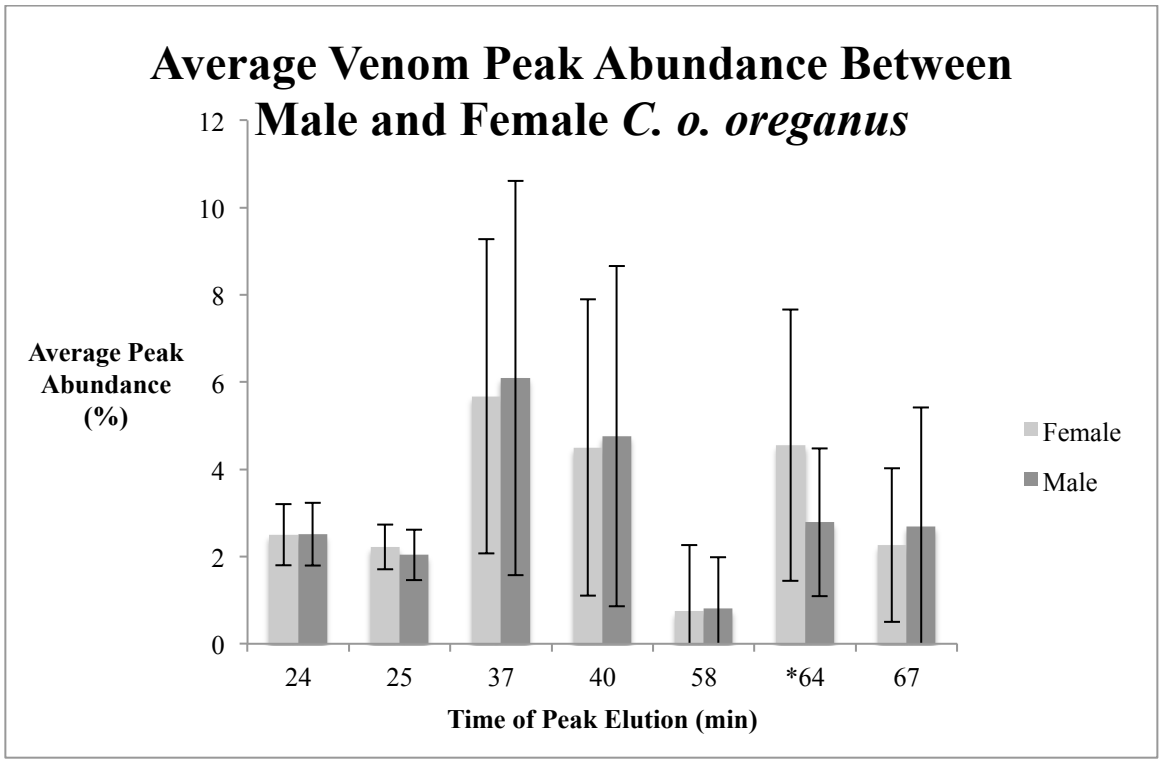

Figure 11: Average Peak Abundance Between Male and Female *C. o. oreganus* of the Sutter Buttes and Sierra Nevada. Asterisk denotes significance.

Figure 11 shows that females had greater average peak abundance for peak 64. This was the only significant difference between males and females as shown by the 2-way ANOVA. Additional identifications were done for two peaks (peaks 37 and 40) to give greater certainty that the peaks being compared are indeed representing the same proteins. HPLC runs were redone for five individuals and peaks 37 and 40 were collected to be identified using LC-MS/MS (Table 3).

Table 3: Protein identification for peaks 37 and 40 from four individuals. Used ensure peaks represented the same proteins in different snakes. Identifications were made using Xcaliber software, and the "top match" protein for each specimen is represented first.

Specimen	2	36	57	72	74
Peak 37	Crotamine-02	Crotamine-02	Crotamine-01	Crotamine-01	Crotamine-02
		Crotamine-01	Crotamine-02	Crotamine-02	Crotamine-01
Peak 40	Myotoxin A	No Data	Myotoxin A	Myotoxin A	Myotoxin A

For all five specimens, elution times correlated with the same proteins. As shown by specimen 36 and 57, the top hit proteins were different for these two individuals, but the same proteins were present with high probability nonetheless. This "ground truthing" was an important step to ensure that the positive correlations found from the statistical test were indeed for the same proteins for different snakes. Additional "ground truthing" was not completed for additional peaks, but given the regularity of elution times for the proteins demonstrated by Table 3, it was assumed that identifications made in Table 1 are valid for this subspecies. Peak 37 was identified as Crotamine, peak 40 was identified as Myotoxin A. Peaks 24, 25 and 58 were not identified due to low relative abundance in all specimens. Peak 64 was identified as Piscivorin and Peak 67 was identified as Thrombin-like enzyme bilineobin.

In order to test whether the variation between the Sutter Buttes and Sierra Nevada shown by significant differences for peaks 37, 40 and 64 could be found between *any* two

populations, samples from the Sierra Nevada were split into four populations and examined for statistically significant differences in location and sex. Snakes in the Sierra Nevada were grouped into the following populations with the corresponding number of individuals sampled from that location: Loma Rica (6), Humboldt Road (13), Cohasset (8) and the BCCER (5). No statistically significant differences were found among any of the Sierra Nevada populations.

CHAPTER IV

Discussion

The purpose of this research was to characterize venom composition of *Crotalus oreganus oreganus* and to compare venom composition from the geographically isolated populations in the Sutter Buttes to populations in the Sierra Nevada. Previous studies examining venom variation within the western rattlesnake complex (O'Keefe et al. 1996, Jurado et al. 2007, Mackessy 2010, Sunagar et al. 2014) have shown that rattlesnake venom protein variation can evolve relatively rapidly, allowing for even recent anthropogenic changes to have a potential effect (Nakashima 1995, Biardi et al. 2006, Mackessy 2008, Biardi and Cross 2010, Calvette et al. 2012,). This in addition to evidence of the Sutter Buttes peculiar location and topography causing isolation of other organisms (Rodgers 1953, Stebbins 2003, Anderson 2004), lead to the prediction that the *C. o. oreganus* in the Sutter Buttes would show differences in venom composition from those inhabiting regions of the Sierra Nevada.

Instrument availability and sensitivity forced several assumptions in research including the extrapolation of identification of venom proteins from a few individuals to all chromatogram data for all individuals for the use of statistical comparisons.. Another assumption involved identifying proteins by matching peptide sequences previously identified from other *Crotalus* and *Agkistrodon* species. No venom protein sequencing has been done for *C. o. oreganus*. The peptides sequences were exact matches from Crotalus and Agkistrodon venom proteins that were previously described, suggesting there is a high level of venom protein conservation. It is assumed that the peptides

matched between Crotalus oreganus oreganus and these other species are the same or very similar protein for the purposes of identification. Lastly, multi-peak complexes, such as those associated with Crotamine and Myotoxin, were grouped together as one peak for the purposes of statistical analysis, based on the research of O'Keefe et al. (1996), who studied this same phenomena and found that these proteins occur as complexes of isomers that exhibit multi-peak chromatographic patterns due to cis/trans variations.

Venom proteins identified using LC-MS/MS were consistent with findings from other researchers (Jurado et al. 2007, Mackessy 2010). A study by Jurado et al. (2007), which compiled similar data for a different subspecies of western rattlesnake shows similar proteins from what is shown in Table 4.

Table 4: Venom proteins present in *C. o. helleri* from Jurado et al. 2007.

Spot #	Protein identified/organism	Matched peptide(s)	Mascot score	Theoritical MW (kDal)/pI	Observed MW (kDal)/pI
1	Catrocollastatin/*Crotalus atrox*	SGSQCGHGDCCEQCK VIGLAYVCSMCEPK	53 20	70.4/5.03	62/5
2	L-Amino acid oxidase/*Crotalus adamanteus*	NNPGLLEYPVKPSEEGK LNEFSQENENAWYFIK	98 83	59.02/6.30	65/6
		YILDKYDTYSTK VIEIQQNDRQ	64 56		
3	Catroxase I/*Crotatus atrox*	TLCAGILEGGK LDRPVSNSEHIAPLSLPSSPPSVGSVCR	28 38	29.3/7.0	26/5
4	Catrin/*Crotatus atrox*	YBYFYVCQVCPAGNIIGK QMQSDCFAICFCQNK MEWYPEAAANAER CGENIYMSPVPIK SVNPTASNMLK	51 38 82 49 64	27.5/7.5	24/6
5	Hemorrhagic Toxin II HT-2/*Crotalus ruber ruber*	VHEIVNFINEFYR GASLCIMRPGLTPGR FLDQULPQCILNK TRVHEIVNFINEFYR	45 50 31 23	23.3/7.26	20/6.5
6	Hemorrhagic Toxin II HT-2/*Crotalus ruber ruber*	VHEIVNFINEFYR FLDQULPQCILNK	41 25	23.3/7.26	20/6.5
7	Phospholipase A2 alpha/*Crotalus adamanteus*	CCFVHDCCYGK SLVQFETLIMK YWLFPPK	35 42 56	14.4/5.06	14/5

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C. o. helleri venom has high metalloproteinase activity in the form of proteins such as catrocollastatin and hemorrhagic toxins. Noticeably absent from this (which?)

venom profile are proteins from the myotoxin family. Despite not being mentioned by Jurado et al. (2007), myotoxin in *C. o. helleri* venom has been shown to be present in large quantities (Mackessy 2010). This comparative approach to venom proteinomics can show how certain proteins are conserved among the western rattlesnake subspecies. Proteins commonly conserved for all subspecies include L-amino acid oxidase, Catrin, metalloproteinases and phospholipase A2 (Jurado et al. 2007, Calvete et al. 2009, Mackessy 2010, Sunagar et al. 2014).

Mackessy (2010) investigated a common Vipirid "trade-off" trend in the western rattlesnake complex whereby snakes either have high levels of digestive metalloproteinases and low levels of toxic components including serine proteases and phospholipase A2 (Type 1) or low levels of digestive components and high levels of toxins (Type 2). Mackessy (2008) suggested that elevation contributes to variation in venom composition in that snakes living in higher elevation regions with greater fluctuations in temperature generally express Type 1 venoms to better facilitate digestion. Populations of *C. o. oreganus* that have been studied show one of the highest levels of digestive metalloproteinase activity, and highest LD50 in the western rattlesnake complex (Biardi et al. 2006, Mackessy 2010). Given that this venom has a high digestive component and low levels of toxicity, it follows this Mackessy's proposed "trade-off" trend with Type 1 venom. In both populations that I sampled, the highest relative abundance of proteins are suspected metalloproteinases (Figure XH Sunagar et al. 2014). Coupled with the smaller peaks

associated with serine proteases and phospholipase A2, the findings for this research are consistent with the Type 1 venom trend described by Mackessy (2010).

Proteins in the myotoxin family are common to the western rattlesnake complex (Aird et al. 1988, Biardi et al. 2006, Mackessy 2008, Mackessy 2010, Sunagar et al. 2014). In most subspecies, myotoxins are present in large quantities and are associated with the immobilization of prey (Mackessy 2010, Jurado et al. 2014). Conservation of myotoxin in *C. oreganus*, regardless of the Type 1 or Type 2 venom dichotomy, suggests that immobilization of prey is extremely important (Mackessy 2008, Mackessy 2010, Sunagar 2014). Similar to the findings of other researchers, venom collected from *C. o. oreganus* contained a significant proportion of myotoxin. The pattern of venom composition of *C. o. oreganus* in the Sutter Buttes containing a greater proportion of proteins associated with the myotoxin family (peaks 37 and 40) than populations in the nearby Sierra Nevada Foothills suggests variations in selective pressures between the two regions.

Anderson (2004) suggested that the Sutter Buttes are an inland island, and providing numerous examples of isolated plant and animal species to illustrate his point. He highlights anomalous pockets of organisms remaining in the Sutter Buttes whose disjunct populations beg the question: How did they become isolated and are other species isolated in the Sutter Buttes? Results from venom profiles of *C. o. oreganus* offer additional support this hypothesis of isolation of the fauna and flora of the Sutter Buttes. Statistically significant variation exists between venom protein composition of the *C. o. oreganus* population in the Sutter Buttes and their geographically closest neighboring

populations in the Sierra Nevada, indicates that that there is reduced connectivity and gene flow between *C. o. oreganus* populations in the Sutter Buttes and Sierra Nevada. Significant variation was not present among the three populations in the Sierra Nevada even though these populations are more geographic dispersed. The isolation between the Sutter Buttes and Sierra Nevada populations could be on a time scale of millions of years associated with geologic history of the formation of the Buttes ~1.6 million years ago. Alternatively, the isolation could established 10's or 100's of thousands of years associated with the ebb and flow of ice age glaciers, historically maintained through natural cycles of flooding in the Sacramento Valley since the last glacial retreat and currently maintained by anthropogenic modifications of the landscape between the Sutter Buttes and similar habitats in the nearby foothills of the Sierra Nevada and Coast Range. Future researchers interested in isolation and local adaptation of venom composition of *C. o. oreganus* in the Sutter Buttes might look at the prey in the Sutter Buttes versus prey in the Sierra Nevada. A slightly different prey base could provide clues as to why the Sutter Buttes population has more myotoxins than the Sierra Nevada populations. Another option that would help to clarify the level of difference between the Sutter Buttes and Sierra Nevada would be to collect more samples to conduct a more robust comparison among populations in the Sierra Nevada. The statistical analysis within the Sierra Nevada comparison comprised only 32 individuals broken into four different populations. More individuals from each of these populations would provide a better answer to the question of whether protein composition differences found between the Sutter Buttes and Sierra Nevada populations are unique.

This research is beneficial because it helps accomplish two goals: (1) it contributes to knowledge of venom proteins in rattlesnakes which have medical significance for antivenin production and (2) provides a greater understanding of the relationship between the Sutter Buttes and its surroundings. With a more complete understanding of the Sutter Buttes, connectivity for potentially sensitive populations of organisms could be protected through increased awareness.

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