

© 2021

Neil Raj Balchan

ALL RIGHTS RESERVED

UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

RESISTANCE TO RATTLESNAKE VENOMS IN AN  
EASTERN COLORADO RODENT COMMUNITY

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

Neil Raj Balchan

College of Natural and Health Sciences  
School of Biological Science

May 2021

This Thesis by: Neil Raj Balchan

Entitled: *Resistance to rattlesnake venoms in an eastern Colorado rodent community*

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

Accepted by the Thesis Committee:

---

Stephen Mackessy, Ph.D., Chair

---

Lauryn Benedict, Ph.D., Committee Member

---

Mark Thomas, Ph.D., Committee Member

Accepted by the Graduate School

---

Jeri-Anne Lyons, Ph.D.  
Dean of the Graduate School  
Associate Vice President of Research

## ABSTRACT

Balchan, Neil Raj. Resistance to rattlesnake venoms in an eastern Colorado rodent community. Unpublished Master of Science Thesis, University of Northern Colorado, 2021.

The grasslands of eastern Colorado are inhabited by two species of rattlesnakes, the Prairie Rattlesnake (*Crotalus viridis*) and the Desert Massasauga Rattlesnake (*Sistrurus tergeminus edwardsii*). An array of rodent species, both native and introduced, also occupy these grasslands, and serve as a varied prey base for the previously listed rattlesnakes. Predator-prey interactions in this system gain an additional level of complexity due to the presence of venoms, a chemical arsenal possessed by both rattlesnakes to incapacitate their prey. Rodents in other systems have demonstrated resistance to snake venoms, and there is potential for a similar dynamic to be present in eastern Colorado. This project aimed to characterize resistance to Prairie Rattlesnake and Desert Massasauga Rattlesnake venoms in a suite of wild rodents, using two field sites to better understand the role of co-occurrence of predator-prey partners. Resistance to venoms was explored using *in-vivo* techniques (LD<sub>50</sub> assays on test populations of wild-collected rodents) and *in-vitro* assays (serum metalloprotease inhibition). Rodent serum was further analyzed using affinity chromatography to isolate potential venom-resistance proteins. Results provide a community-level view of venom resistance and indicate that resistance to venoms is variable across predator-prey species pairings. Additionally, this study characterized the diet of the Prairie Rattlesnake, a taxon for which little dietary data has been collected. The Prairie Rattlesnake occupies a broad latitudinal distribution, spanning a climatic gradient, and is

believed to be a dietary generalist. We analyzed prey remains from preserved museum specimens and compiled a list of prey items consumed. This dataset was further used to detect feeding variation as a result of latitude, ontogeny, and seasonality. No apparent feeding differences occurred as a result of latitude, a strong signature of dietary ontogeny was absent in the dataset, and snakes did not exhibit prolonged foraging in warmer regions. These studies elucidate the trophic biology of snakes on their ecosystems, an area of study that at present remains poorly understood. Results indicate that rattlesnakes have the potential to exert tremendous selective pressures upon the prey communities they interact with, and thus may impact prey species over evolutionary timescales. Understanding the underpinnings of snake foraging and resistance to snake venoms allows us to contextualize better the role of snakes in natural systems.

## ACKNOWLEDGMENTS

This research thesis could not have been completed without the generous support of many individuals, groups, and organizations. First, I thank Dr. Stephen Mackessy for his unfaltering support, mentorship, enthusiasm, and friendship throughout my Master's program at the University of Northern Colorado. I also thank committee members Dr. Lauryn Benedict and Dr. Mark Thomas for guidance and input throughout the course of this research project.

I thank graduate student lab mates A.E. Nash, Emily Grabowsky, Tanner Harvey, Eric Januszkiewics, and Kaleb Hill for ongoing support, humor, and the maintenance of a lab "family" dynamic. A special thanks to lab mate Cara Smith for working alongside me tirelessly on many of these projects, as our research questions often dovetail into one another's. Additionally, conversations with Cara have challenged me to think about ideas critically and have always provided a welcomed and refreshing viewpoint. Lab undergraduate students Parker Gottsch, Keira Lopez, Forrest Tappy, and Alex Roberts were instrumental to much of this work, and were largely responsible for seamlessly workflow in the lab. I extend a special thanks to University of Central Arkansas undergraduate student Calvin Vick for always being willing to drop anything at hand and visit for a brief "research sabbatical". Calvin has been instrumental as a sounding board for all of my errant ideas and has been incredibly eager to participate in my research. Dr. Kaye Holman was often the glue that held our lab group together at scientific meetings and in the field, and her overwhelming support has been very much appreciated.

Many individuals were instrumental in the field, assisted with sample collection, or otherwise facilitated aspects of this research. While this list is not exhaustive by any means, I thank the following individuals for their assistance with various tasks: Tim Warfel, Hunter Johnson, Karen Green, Andrew Dubois, Lauren McGough, Aaron Short, Joe Ehrenberger, Sean McLaughlin, Bryon Shipley, Drew Olson, Braeli Hardt, Christine Williams, Kevin Urbanek, Hayley Urbanek, Micha Hanbury, Tasha Leong, Joe Atkinson, Dr. Todd Castoe, Richard Orton, Zack, Dr. Drew Schield, Blair Perry and Dr. Corey Roelke.

Dietary data could not have been collected without access to natural history collections, and I thank the following institutions and individuals for generously allowing access to preserved rattlesnake specimens: University of Northern Colorado, Sternberg Museum of Natural History (Curtis Schmidt), Sam Noble Museum (Dr. Cameron Siler and Jessa Watters), Brigham Young University (Dr. Allison Whiting), University of Wyoming (Dr. Beth Wommack and Dr. Matthew Carling), and University of Texas at Arlington (Gregory Pandelis).

Funding for this research, and for presentation of this research at meetings, was provided by the UNCO School of Natural and Health Sciences, Society for the Study of Amphibians and Reptiles, International Herpetological Symposium, UNCO Graduate Student Association, Kansas Herpetological Society, Center for North American Herpetology, and the California Academy of Sciences. Without this financial support, I would have been unable to carry out much of the sampling and research travel required for this project.

Finally, my greatest thanks go to my family, Kris, Neelam, and Ravi Balchan, who always encouraged my need to leave no stone or log unturned. I dedicate this thesis to my father, Kris Balchan, who lost his fight with cancer before he could see this project come to completion.

## TABLE OF CONTENTS

### CHAPTER

I.	INTRODUCTION.....	1
	Background.....	1
	Physiology of Venom Resistance .....	7
	Colorado Rattlesnake Ecology.....	11
	Study System.....	15
	Specific Aims – Chapter II: Prairie Rattlesnake Diet Ecology.....	17
	Specific Aims – Chapter III: Venom Resistance in Colorado Rodents.....	18
II.	RANGEWIDE FEEDING ECOLOGY OF THE PRAIRIE RATTLESNAKE (CROTALUS VIRIDIS).....	21
	Abstract.....	21
	Introduction.....	22
	Methods.....	28
	Results.....	30
	Discussion.....	37
	References.....	48
III.	RESISTANCE OF RODENT PREY TO PRAIRIE RATTLESNAKE (CROTALUS VIRIDIS) AND DESERT MASSASAUGA RATTLESNAKE (SISTRURURUS TERGEMINUS EDWARDSII) VENOM IN AN EASTERN COLORADO GRASSLAND HABITAT.....	60
	Abstract.....	60

Introduction.....	61
Methods.....	70
Results.....	74
Discussion.....	86
References.....	102
IV CONCLUSIONS.....	112
COMPLETE REFERENCE LIST.....	117
APPENDIX	
A. INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM.....	140

## LIST OF TABLES

	PAGE
Table 2.1. Museum collections investigated, listing full and abbreviated collection name, collection location, and number of specimens examined.....	29
Table 2.2. Stomach contents of analyzed <i>Crotalus viridis</i> specimens, including frequency (n), proportion of total prey items, and proportion of identifiable items within classes Aves, Mammalia and Reptilia.....	31
Table 3.1. Snake venom metalloproteinase activities of three rattlesnake populations. Each population is represented by a pooled venom sample from three adult individuals from the same field site.....	75
Table 3.2. Contingency table of inhibitory effect (percent inhibition) of rodent serum (mean $\pm$ standard deviation) against snake venom metalloproteinase activity of three rattlesnake venoms.....	76
Table 3.3. Contingency table of intraperitoneal median lethal dose (IP LD <sub>50</sub> ) of rattlesnake venoms against tested rodent populations.....	85
Table 3.4. Hypothetical patchwork dynamic across species and sites where interacting pairings may exhibit differing adaptation outcomes. Red rectangles suggest the snake as the locally adapted partner in the interaction and green rectangles suggest the rodent as the locally adapted partner in the interaction. Black rectangles indicate pairings where interactions have not been determined. These dynamics represent a snapshot in evolutionary time and may change considerably as arms-race dynamics occur.....	99

## LIST OF FIGURES

	PAGE
Figure 1.1. Venom proteomes of A) <i>Crotalus viridis</i> (Lincoln Co.) B) <i>Crotalus viridis</i> (Weld Co.) and C) <i>Sistrurus tergeminus edwardsii</i> (Lincoln Co.), depicting major toxin groups present in venoms of respective populations. (Mackessy, unpubl. data).....	8
Figure 1.2. Reverse-phase high performance liquid chromatography chromatogram of representative <i>Crotalus viridis</i> venom proteome. Major toxin groups are indicated below by colored boxes, and corresponding mechanisms of resistance are indicated where they are known. Adapted from Holding et al. 2016.....	13
Figure 1.3. Map of study sites and select present species, indicating 1) Weld Co. and 2) Lincoln Co. field sites. Both <i>Crotalus viridis</i> (photo: David Nixon) and <i>Sistrurus tergeminus edwardsii</i> (photo: Tyler Carlson) are present at the Lincoln Co. field site, but only <i>Crotalus viridis</i> is present at the Weld Co. field site. Various rodents, including but not limited to <i>Peromyscus maniculatus</i> (photo: Danny Poet), <i>Mus musculus</i> (photo: Milos Andera), <i>Microtus pennsylvanicus</i> (photo: Daniel Cadieux), and <i>Dipodomys ordii</i> (photo: Jim Zapp) are present at both field sites. Orange overlay represents the geographic distribution of <i>Sistrurus tergeminus edwardsii</i> in Colorado.....	16
Figure 2.1. Proportion of prey classes represented in <i>Crotalus viridis</i> A) stomach and B) hindgut contents, including percent of total hindgut contents and frequency (n).....	33
Figure 2.2. Prey classes consumed by <i>Crotalus viridis</i> as a function of body size (snout-vent length). Data depicted represent a combination of stomach contents (n = 76) and hindgut contents (n = 267) recovered from all specimens.....	34
Figure 2.3. Seasonal distribution of prey consumed by <i>Crotalus viridis</i> . Data depicted represent combined stomach (n = 67) and hindgut contents (n = 162) recovered from all specimens with known collection dates.....	35
Figure 2.4. Latitudinal distribution of prey classes consumed by <i>Crotalus viridis</i> . Data depicted represent stomach contents (n = 70) and hindgut contents (n = 186) recovered from all specimens with known collection latitudes.....	36

Figure 3.1. Map of study sites and select present species, indicating 1) Weld Co. and 2) Lincoln Co. field sites. Both <i>Crotalus viridis</i> (photo: David Nixon) and <i>Sistrurus tergeminus edwardsii</i> (photo: Tyler Carlson) are present at the Lincoln Co. field site, but only <i>Crotalus viridis</i> is present at the Weld Co. field site. Orange overlay represents the geographic distribution of <i>Sistrurus tergeminus edwardsii</i> in Colorado (based on Hammerson, 1999).....	71
Figure 3.2. Dose-response curves for venoms from two populations of Prairie Rattlesnake venoms against NSA <i>Mus musculus</i> .....	78
Figure 3.3. Dose-response curves for venoms from three populations of rattlesnakes against Weld County <i>Peromyscus maniculatus</i> .....	79
Figure 3.4. Dose-response curves for venoms from three populations of rattlesnakes against Lincoln County <i>Peromyscus maniculatus</i> .....	80
Figure 3.5. Dose-response curves for Weld County <i>Dipodomys ordii</i> against two populations of rattlesnake venoms. Both curves are depicted here but overlay each other completely.....	81
Figure 3.6. Dose-response curves for venoms from two populations of rattlesnakes against Lincoln County <i>Dipodomys ordii</i> .....	82
Figure 3.7. Dose-response curve for Weld Co. Prairie Rattlesnake venom against Weld County <i>Onychomys leucogaster</i> .....	83
Figure 3.8. Dose-response curve for Weld Co. Prairie Rattlesnake venom against Weld County <i>Mus musculus</i> .....	84
Figure 3.9. SDS-PAGE gels (A) with <i>Onychomys leucogaster</i> (OL) serum fractions and (B) <i>Dipodomys ordii</i> (DO), <i>Neotoma floridana</i> (NF), and <i>Peromyscus maniculatus</i> (PM) serum fractions. MW, molecular weight standards. FT, flow-through serum at beginning of elution containing material unbound to matrix. RP, retained serum proteins eluted from affinity chromatography matrix.....	86

## CHAPTER I

### **Introduction**

#### **Background**

Ecosystems, and the biotic elements within these systems, have the potential to be influenced by various selective pressures. Abiotically, environmental factors and anthropogenic developments and structures can exert pressures that result in evolutionary change in species behavior (Luther and Derryberry, 2012), morphology (Cook and Saccheri, 2013), or even physiology (Dunson, 1969). Conversely, biotic factors such as interspecific interactions can result in pressures that drive evolutionary change in interacting partners. Predators may dictate the way their prey moves across a landscape (Bergman et al., 2006), influence how prey forages (Nachappa et al., 2011) and even alter prey group dynamics (Brierley and Cox, 2010). While predators frequently impact the behavior of their prey, due to prey acquisition typically being a physical and behavioral process, predation can select for additional adaptations, including various chemical, physiological, and other innovations. For example, birds exerted predation pressures on their Monarch Butterfly prey, resulting in evolved sequestration of distasteful and toxic alkaloids in the butterfly (Brower and Calvert, 1985). In this way, predation has the potential to modify the inner workings of a prey animal over evolutionary time, resulting in physiological adaptations to cope with predatory pressures. A similar system influences

interactions involving venomous snakes, where the chemical arsenal that is snake venom can apply unique pressures to species that frequently incur bites from snakes.

Snake venoms are a complex mixture largely comprised of proteins, peptides, and enzymes that work in concert to function as a biochemical weapon (Mackessy, 2010). Venoms serve snakes a variety of actual and potential functions including prey acquisition (Daltry et al., 1996), maintenance of oral hygiene (Stiles et al., 1991), accelerated digestive capability (Thomas and Pough, 1979) and defensive weaponry (Ang et al., 2014). With this diversity of functions considered, the greatest factor driving the evolution of snake venoms is likely to be their use in prey acquisition, and we see this recapitulated across taxa via locally adapted venoms (Barlow et al., 2009). Because venoms are so intimately linked to the organism into which they will be injected, it becomes advantageous that these organisms find a way to remain competitive in the face of this predatory innovation. Indeed, many organisms have evolved strategies to negate the function of venoms of their respective snake predators, and these evolved strategies will be referred to here collectively as venom resistance.

Venom resistance mechanisms are not solely isolated to snake prey species. As described previously, venoms function as a potent antipredator innovation, because they provide snakes a unique way to repel or subdue an assailant in fractions of a second with minimal physical contact (Ferraz et al., 2019). An array of different animals prey upon snakes, including various birds (Webb and Whiting, 2005), small mammals (Jansa and Voss, 2011), and even other snakes (Maritz et al., 2019). One consistent means by which a snake can defend itself in all of these predatory events is by using venom as a defensive mechanism, and if a predator includes snakes as a significant part of its diet, it will likely suffer from envenomation on a routine basis. This strong evolutionary pressure to survive envenomation has resulted in a variety of snake predators

developing resistance to venoms. For example, the Virginia Opossum (*Didelphis virginianus*) exhibits exceptionally high levels of resistance to the venoms of sympatric North American pitvipers (Werner and Vick, 1977). The Honey Badger (*Mellivora capensis*) is a formidable predator of co-occurring African snakes and displays a great deal of resistance to venoms of both elapid and viperid snakes that occupy its distribution (Drabeck et al., 2015). Perhaps most famously, the Egyptian Mongoose (*Herpestes ichneumon*) exhibits tremendous resistance to the venoms of co-occurring viperid, elapid and atractaspidid snakes (Bdolah et al., 1997). These examples emphasize the independent evolution of venom resistance across a variety of locales and in a variety of species. Although not the focus of this thesis, resistance to snake venoms is a vital adaptation for predators of venomous snakes to possess, and the widespread presence of resistant predators reaffirms this idea.

Defensive purposes may have weaker selective impacts on the composition of venoms, as a venom succeeds in punishing an antagonist as long as it can provide enough pain to ward off that attacker (Ferraz et al., 2019). However, venom in the majority of snake taxa may not serve a primary role in defense, as bites from most species may not inflict immediate and severe pain to an antagonist (Ward-Smith et al., 2020). Conversely, venom compositions must be much more fine-tuned to subdue prey quickly and effectively. For example, one venom toxin may prove highly lethal against mammalian prey, while being functionally nontoxic to lizards and birds (Modahl et al., 2018). As a result, venoms are evolved to shut down a prey animal effectively, and this makes them the source of significant selective pressure for a population of prey animals. Prey organisms may develop relatively pointed ways to resist venoms as a result, and this often occurs through the modification of serum proteins that bind to injected venom toxins (Goetz et al., 2019).

The reciprocal evolution of prey-adapted venoms in snakes countered by venom resistance mechanisms in prey may have the potential to result in a co-evolutionary arms race (ex. Brodie and Brodie 2015). The Red Queen Hypothesis (Van Valen, 1973) describes coevolutionary dynamics between interacting organisms, and one tenet of this hypothesis revolves around the concept of evolutionary arms races. These arms races are widespread in the natural world and tend to be particularly common in parasite-host (Turko et al., 2018) and predator-prey (Brodie and Brodie, 2015) systems. A well-studied example of an arms race from a predator-prey interaction occurs between the Common Garter Snake (*Thamnophis sirtalis*) and Rough-Skinned Newt (*Taricha granulosa*) in coastal western North America (Brodie and Brodie, 2015). In this example, the newt prey produces a potentially neurotoxic tetrodotoxin, while the snake predator possesses resistance to this toxin. An arms race occurs here, and both newts and snakes increase their respective toxin production or toxin resistance over evolutionary time in an attempt to outcompete the other (Brodie and Brodie, 2015). At any given time and in any given location, either the newt or the snake may be “winning” this evolutionary race, but the dynamic nature of evolution means that both partners must continue their evolutionary trajectories to remain competitive (Brodie and Brodie, 2015).

This same scenario unfolds in venomous snakes and co-occurring rodents, where venoms are functionally pitted against inhibitory serum proteins in an arms-race dynamic. Once again, resistance to snake venoms in rodents is apparent in an array of species, and one sees this physiological innovation in Woodrats (*Neotoma spp.*; Perez et al., 1979), California Ground Squirrels (*Otospermophilus beecheyi*; Poran et al., 1987) and Fox Squirrels (*Sciurus niger*; Pomento et al., 2016), among other rodent species in response to various sympatric predatory viperid snakes. Interestingly, we also see circumstances where resistance to snake venoms is not

present. Notably, the Cape Ground Squirrel (*Xeris inauris*) of southern Africa apparently lacks resistance to the venoms of several co-occurring predatory snakes that feed on this squirrel (Phillips et al., 2012).

It is important to note that resistance transcends simple interspecific interactions and can be influenced by additional variables such as geography (Pomento et al., 2016) or even ontogeny (Heatwole et al., 1999). While venom resistance may be present in a snake/prey pair, the strength of that resistance can vary considerably across the landscape, reflecting which partner is “winning” the evolutionary arms race in space and time. In general, resistance to venom can be expected to be greater in areas where snakes and prey actively co-occur, as selection can be expected to be stronger with increased pressure from snake predation (Pomento et al., 2016). We can extend this idea to snake/prey pairings that are geographically disparate and expect that snakes and prey from the same site should typically generate greater resistance dynamics than a snake/prey pairing from disparate locations, resulting from a lack of interaction between partners over evolutionary time.

Ontogeny may also impact resistance through various means, both from the perspective of the snake and of the prey. North American pitvipers are well-characterized in exhibiting a strong ontogenetic shift in venom biochemistry (Mackessy, 1988), with juvenile venoms typically being more lethal toward ectothermic prey, and adult venoms being suited to endothermic prey. This compositional shift in venom phenotype is generally correlated with dietary ecology, reflecting a shift from primarily lizard predation to rodent predation in many rattlesnake taxa (Taylor, 2001; Mackessy et al., 2003; Glaudas et al., 2008). As such, one may expect lizards to exhibit increased resistance mechanisms to a juvenile venom phenotype, as this venom presumably exerts greater pressure upon them in comparison to the adult venom

phenotype. Conversely, rodents in the same location may show greater resistance mechanisms to the adult venom phenotype. While selection should push for these outcomes, these two venom phenotypes should inherently be more toxic to their respective prey type, thus complicating our understanding of the lethality of venom phenotypes against their targeted prey items.

Ontogeny of prey species may also be a significant factor influencing venom resistance across the lifetime of both an individual predator and individual prey organism. It is expected that certain prey species may interact differently with their environment at different life stages. An example of this is the defenseless and immobile nature of an altricial baby bird versus the comparatively well defended and highly mobile adult bird (Naef-Danzer and Gruebler, 2016). In this example, we might expect relaxed selection on the snake to subdue the baby bird quickly, but strong selection on the snake to subdue the adult quickly. This may be reflected in venom composition, with differential production of venom components showing greater effectiveness toward prey at relevant life stages. Resistance to venoms may change in a single prey organism over its lifetime, and this change may correlate with dramatic ontogenetic developments. This concept has been characterized in a system using the American Bullfrog (*Lithobates catesbeianus*) as a prey species and challenging it with venoms of the predatory Copperhead (*Agkistrodon contortrix*) and Cottonmouth (*Agkistrodon piscivorus*) pitvipers (Heatwole et al., 1999). In this system, bullfrog tadpoles demonstrate low levels of resistance to the venoms of both vipers, but resistance to venoms increases considerably once the tadpoles metamorphose into frogs. Ontogenetic changes in venom resistance may occur for a variety of reasons, ranging from changes in organism physiology over developmental time to differences in pressure exerted on organismal life stages by predatory venomous snakes. Overall, it is apparent that the

development and maintenance of venom resistance is complex from ecological and evolutionary points of view.

### **Physiology of Venom Resistance**

At the physiological level, resistance to venoms can be conferred via a variety of mechanisms. Venoms themselves are a complex mixture of proteins, peptides, and enzymes individual activities that work in concert to incapacitate a prey item (Mackessy, 2010). As each venom component functions differently, it generally is not feasible for resistance to evolve to an entire venom. Rather, animals evolve resistance to dangerous components in a venom.

Rattlesnake venom is generally comprised of several important and abundant constituents (Figure 1.1), including phospholipase A2s, serine proteases, metalloproteases, lectins, L-amino acid oxidases, bradykinin-potentiating peptides, disintegrins and cysteine-rich secretory proteins. Of these important venom molecules, corresponding resistance mechanisms in rodents have been identified for only a few toxin families (Holding et al. 2016). While resistance to many of these venom molecules may be present, the mechanisms by which these molecules are resisted remains largely unknown.

Resistance can generally occur in four ways in an organism: 1) venom inhibitors are present, 2) physiological targets are biochemically altered, 3) toxins are redirected and are no longer effective and 4) repeated exposure leads to acquired immunity. These four avenues to venom resistance arise and occur in very different ways and ultimately work differently in how they negate venoms from functioning. The first class of resistance mechanisms, venom inhibitors, is comprised of proteins that circulate in an animal's body that bind to and inhibit the function of circulating venom components.

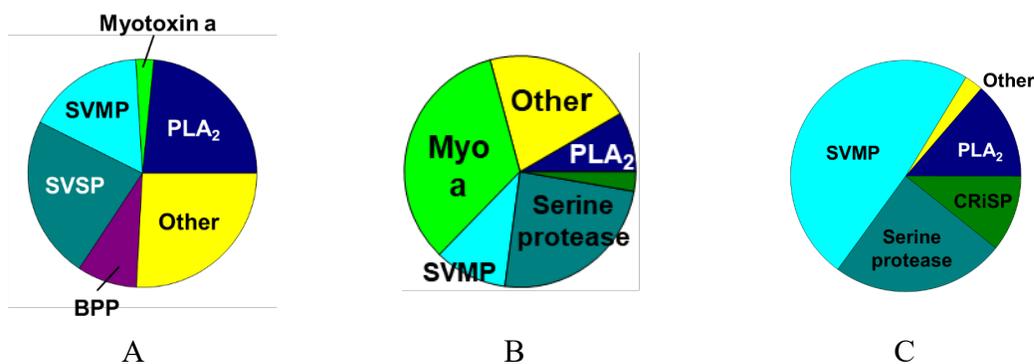


Figure 1.1 Venom proteomes of A) *Crotalus viridis* (Lincoln Co.) B) *Crotalus viridis* (Weld Co.) and C) *Sistrurus tergeminus edwardsii* (Lincoln Co.), depicting major toxin groups present in venoms of respective populations. (Mackessy, unpubl. data).

To date, several serum proteins have been identified that bind to snake venom metalloproteases and phospholipases (e.g., Perez et al., 1979; Perales et al., 2005; Gibbs et al., 2020), but it is possible that serum proteins exist to bind other venom proteins as well. Specific toxin inhibitors are widespread and have been identified in various mammals (Voss and Jansa, 2012) and reptiles (Perales et al., 2005). In the case of mammals, venom inhibitors almost certainly evolved due to selective pressures imparted by venomous predators (Poran et al., 1987). With squamate reptiles, venom inhibitors may be selected for as resistance mechanisms to an organism's own venom (Perales et al., 2005).

Altered targets are the second means by which an organism can acquire resistance to a snake venom. Certain venom components function by targeting specific receptors, and an alteration of these receptors that allows for regular physiological function while preventing venom from binding effectively neutralizes these components (Barchan et al., 1992). Altered targets appear to be relatively uncommon in venom-resistant organisms, though several examples have been identified in various taxa. The muscular nicotinic acetylcholine receptor is one

example of an identified altered target. This receptor, mediating signal transmission from nerves to muscles, is targeted by elapid snake venom alpha-neurotoxins (Neumann et al., 1989). Experiments conducted on several taxa known to be resistant to elapid venoms have demonstrated strong resistance to binding of venom alpha neurotoxins (Barchan et al., 1992, 1995; Takacs et al., 2004). Interestingly, there are at least two different biochemical mechanisms allowing for this particular target alteration, and convergence in the alteration at this modified receptor is observed in various species (Drabeck et al., 2015). As a second example of target alteration, we see modification of the blood coagulation protein von Willebrand Factor. This protein is targeted by snake venom C-type lectins and has been modified in a group of *Didelphis* opossums to inhibit binding of this lectin (Jansa and Voss, 2011). The opossums possess yet another altered target in the way of their alpha-1-proteinase inhibitor, which serves as an inhibitor of endogenous proteases but surprisingly is not deactivated by the venom of crotaline snakes (Catanese and Kress, 1993). This would suggest that inhibition against snake venom metalloproteases is also conferred by this serum protein, but further work is needed to characterize this resistance against snake venom metalloproteases. Altered targets frequently occur in other toxin resistant systems and may represent a useful and efficient route to circumvent toxicity in a variety of contexts (Feldman et al., 2012).

Redirected toxins represent a unique way to cope with envenomation, and this mechanism of resistance has been demonstrated only in Grasshopper Mice (*Onychomys* spp.) that feed on venomous Bark Scorpions (*Centruroides* spp.), becoming envenomated in turn (Rowe and Rowe, 2006; Rowe and Rowe, 2008). While scorpion venoms often induce extreme pain, Grasshopper Mice display reduced pain responses compared to domestic mice (Rowe and Rowe, 2008). Grasshopper Mice achieve this reduced pain response by the scorpion toxin

binding to a previously untargeted nociceptor, inducing analgesia and blocking the effects of other pain-inducing venom components in scorpion venom (Rowe et al., 2013). Redirected toxins represent a novel way to adapt resistance to pain-inducing venom components, but it is unlikely that this mechanism could be used to confer resistance against degradative or toxic components. While this mechanism of resistance has been very useful in facilitating scorpion predation by Grasshopper Mice, it is not expected to be commonly occurring in snake-rodent interactions.

Finally, acquired immunity occurs in an organism after repeated exposures to a venom, and this does not represent an evolved resistance mechanism to cope specifically with envenomation. While acquired immunity certainly confers protection against envenomation, an organism would require multiple sublethal doses to gain this resistance. While this is unlikely in a prey animal, this route to resistance may be easier to develop in predators of snakes. Mammals that feed on snakes may expose themselves to venom, both via sublethal bites and potentially even consumption of venom glands (Almeida-Santos et al., 2000; Begg et al., 2003). However, it appears that laboratory mice mount an innate immune response via mast cell activation when challenged with snake venom, and these mast cells release carboxypeptidase A, which further protects against the systemic impacts of venom, representing yet another mechanism that may be beneficial to resist against the degradative impacts of venom (Metz et al., 2006). Innate immunity, without corresponding coevolution, may, as a result, be of importance in some contexts involving envenomation.

Of the four resistance mechanisms described above, venom inhibitors and altered targets are likely the most important in the context of a prey rodent. Venom inhibitors and altered targets represent two mechanisms that occur over evolutionary time and can be expected to be present as

endogenous mechanisms in a prey rodent. They should protect against envenomation upon the first bite from a snake and do not require any prior exposure to venom in that individual's lifetime. Redirected toxins should not be prevalent in the context of snake-rodent predation, as snake venom toxins are not optimized for pain induction. Rather, degradation and toxicity resulting from envenomation may not effectively be countered by redirected toxins. Acquired immunity can also be ruled out as a primary mechanism of resistance in wild rodents, as it is not expected that rodents will gain repeated sublethal exposures to venom prior to receiving a potentially lethal dose of venom in a snakebite.

### **Colorado Rattlesnake Ecology**

The eastern plains of Colorado are inhabited by two species of rattlesnakes: the larger and broadly distributed Prairie Rattlesnake (*Crotalus viridis viridis*) and the diminutive and range-restricted Desert Massasauga Rattlesnake (*Sistrurus tergeminus edwardsii*) (Hammerson, 1999). Both of these snakes belong to the pitviper subfamily Crotalinae, united by the presence of a thermosensitive loreal pit organ used in prey detection (Chen et al., 2012). While these two rattlesnakes have many similarities, they interact with their environment very differently from an ecological standpoint.

The Prairie Rattlesnake is a medium sized rattlesnake broadly distributed across the Great Plains, from southern Canada to northern Mexico (Klauber, 1956; Hammerson, 1999). In Colorado, it occurs across the entirety of the eastern plains region and can be quite abundant in some areas. Prairie Rattlesnake diet is poorly characterized, but the snake is known to consume various mammalian, reptilian, and avian prey species (Hammerson, 1999). A study on an ecologically similar taxon with close phylogenetic affinities, the Great Basin Rattlesnake (*Crotalus oreganus lutosus*), revealed an ontogenetic diet shift from lizards to endothermic prey

that is likely also present in Prairie Rattlesnakes (Glaudias et al., 2008). While predictions on the feeding ecology of the Prairie Rattlesnake are based on anecdotal information (Klauber, 1956) and comparisons with related taxa can be made, further study is needed to characterize the diet of this species.

Though the diet of the Prairie Rattlesnake remains poorly characterized, other aspects of this snake's biology are well understood (Dawson, 2018, unpub. data). Venom composition of the Prairie Rattlesnake has been well characterized (Smith et al., unpub. data; Saviola et al., 2015). Prairie Rattlesnake venoms appear to fall into two classes: 1) a highly proteolytic type, present in southern Colorado, and 2) a highly myotoxic type, present in northern Colorado (Smith et al., unpubl.; Figure 1.2). The primary difference between these two venom phenotypes is a difference in the abundance of myotoxin a, a small peptide resulting in muscle necrosis, and snake venom metalloproteases, enzymes resulting in protein degradation (Mackessy, 2010a). These two venom types align with the Type I vs. Type II venom dichotomy present in rattlesnakes on a more general level, where a Type I venom exhibits high metalloprotease activity and a Type II venom exhibits low metalloprotease activity (Mackessy, 2010b). It has been suggested that Type I venoms may be optimized for increased digestion of prey items, while Type II venoms are optimized for rapid immobilization and incapacitation of prey (Mackessy, 2010b). As such, one can expect these two venom types to function differently in an ecological context and interact with prey in different ways.

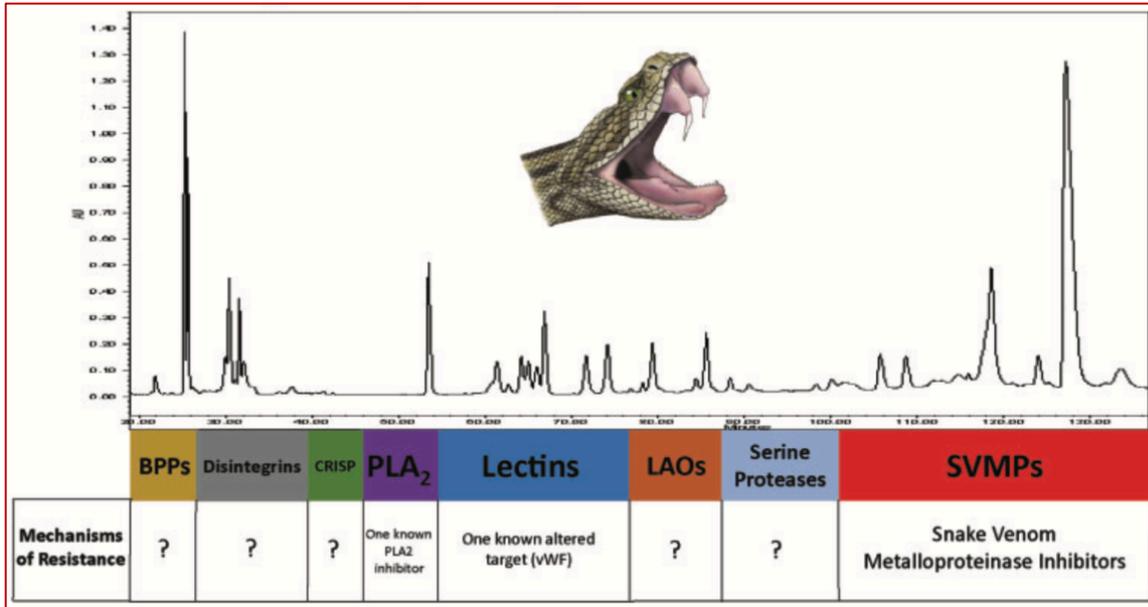


Figure 1.2. Reverse-phase high performance liquid chromatography chromatogram of representative *Crotalus viridis* venom proteome. Major toxin groups are indicated below by colored boxes, and corresponding mechanisms of resistance are indicated where they are known. Adapted from Holding et al. 2016.

The Desert Massasauga is a diminutive rattlesnake occurring in SE Colorado, extreme SE Arizona, New Mexico and parts of west Texas and barely extending into northern Mexico (Hammerson, 1999). In Colorado, the Desert Massasauga occurs as a disjunct population and is present in the southeastern part of the state (Wastell and Mackessy, 2011). Considerable work has been done on the Colorado population to characterize the natural history and ecology of this snake (Mackessy, 2005, 2017; Wastell and Mackessy, 2011, 2016). In addition to ecological studies, investigators have put considerable effort into understanding the diet of the Massasauga (Holycross and Mackessy, 2002). Desert Massasaugas in all studied populations, including the Colorado population, demonstrate a strong shift from primarily lizard predation as juveniles to the inclusion of rodents as a large portion of adult diets. Given the small maximum size of adult

Massasaugas, rodents consumed by this species must have small body sizes, and this is evident based on the rodent species found as stomach contents in diet studies (Holycross and Mackessy, 2002). This strong ontogenetic shift in diet aligns with other rattlesnake species and suggests that adult venoms should be better optimized for rodent prey than juvenile venoms.

Venom biochemistry of the Desert Massasauga has also been well studied, and the proteome of this species from the southeast Colorado population is well characterized (Sanz et al., 2006; Pahari et al., 2007; Mackessy, 2017). These snakes are characterized by having venoms comprised of primarily phospholipase A<sub>2</sub>, serine proteinases, and snake venom metalloproteinases. This combination of venom constituents results in a degradative Type I venom, resulting in hemorrhage and tissue degradation in envenomed prey items (Figure 2). Venoms of southeast Colorado Massasaugas have been found to be more toxic to naïve lizards (LD<sub>50</sub> = 0.39 mg/kg) than to a murine model (LD<sub>50</sub> = 0.60 mg/kg), indicating that some level of taxon specificity may be present (Gibbs and Mackessy, 2009). Broader study into the genus *Sistrurus* suggests that diet may be an important factor contributing to venom compositional differences among taxa (Sanz et al., 2006).

In summary, the differing diets and venom compositions of the two rattlesnake species mean that they will interact with their ecosystem differently. The larger Prairie Rattlesnake will be able to prey on rodents too large for the Desert Massasauga to consume, and the two snakes are also likely to segregate in their microhabitat and microenvironmental preferences. Additionally, Prairie Rattlesnakes with Type I venom can be expected to interact with prey differently on a biochemical level than those with Type II venom. These divergent venom phenotypes result in differing selective pressures on prey from the same source of predation, and thus prey may evolve differently among sites dependent on the venom phenotype of the Prairie

Rattlesnakes present at a site. Consequently, these differences among taxa should be considered when creating predictions for how ecological interactions may unfold, given the complex nature of such interactions.

### **Study System**

The eastern plains of Colorado provide an ideal setting to test venom resistance in a multi-predator and multi-prey system. Here, the predatory Prairie Rattlesnake and Desert Massasauga are present in some regions, and both feed on a variety of rodents. The eastern plains are inhabited by a diverse community of rodent species (Armstrong et al., 2011), including but not limited to: Deer Mouse (*Peromyscus maniculatus*), House Mouse (*Mus musculus*), Meadow Vole (*Microtus pennsylvanicus*), Northern Grasshopper Mouse (*Onychomys leucogaster*), Ord's Kangaroo Rat (*Dipodomys ordii*), Plains Pocket Mouse (*Perognathus flavescens*), and the Eastern Woodrat (*Neotoma floridana*). This combination of multiple predators and an array of prey species allows for exploration into the presence and distribution of resistance to venoms across a food web and allows for exploration of how feeding ecology factors into the presence of resistance. Additionally, geography can be expected to influence levels of resistance as a result of the sympatry and allopatry of populations that it creates, and this can be explored by incorporating multiple study sites.

To understand the impact of geography on venom resistance, two field sites on the eastern Plains were chosen (Figure 1.3). The first site is located in Weld County, Colorado (hereafter referred to as Weld) and the second in Lincoln County, Colorado (hereafter referred to as Lincoln). As a result of the geographic positioning of these two study sites, two concepts of interest arise. First, both sites are inhabited by the Prairie Rattlesnakes, but rattlesnakes at Weld have Type II venom (high myotoxin expression), while rattlesnakes at Lincoln have Type I

venom (high metalloprotease expression). This allows for exploration into resistance of rodents to two different venom phenotypes produced by the same predator. Second, only the Lincoln site is inhabited by the Desert Massasauga Rattlesnake. This allows us to test for resistance to Massasauga venoms in the same species of rodents, where they are either sympatric or allopatric with Massasaugas. The aforementioned rodents are present at both field sites and can allow for thorough testing of hypotheses based on feeding ecology and geography.

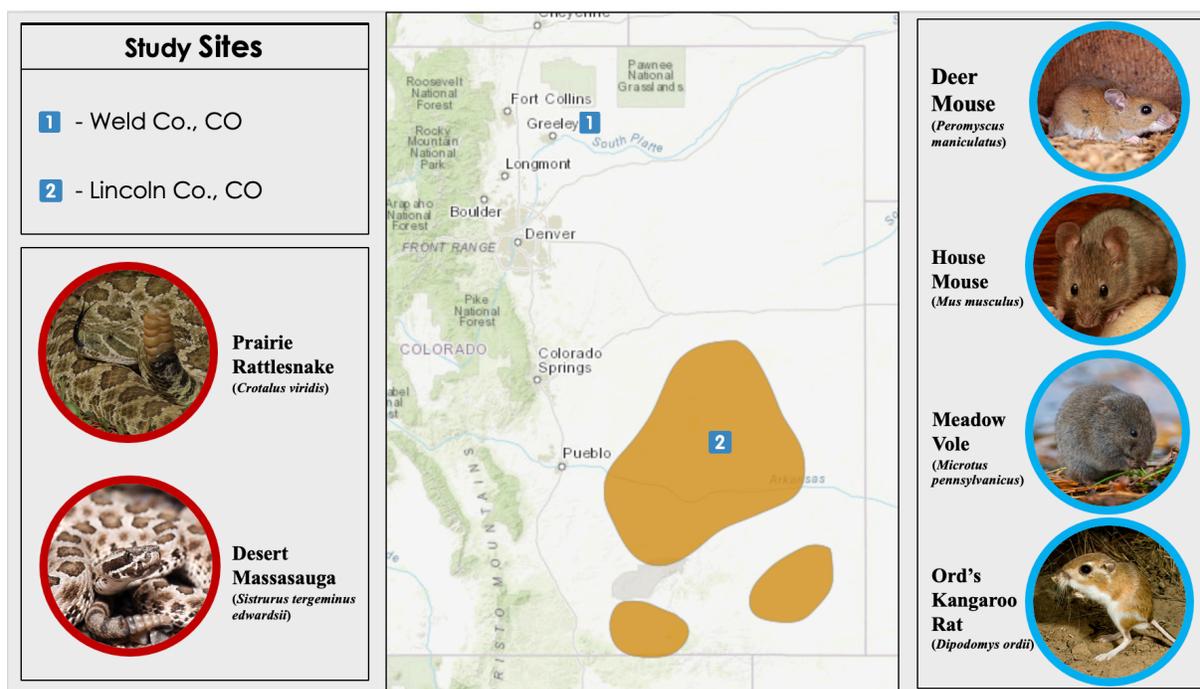


Figure 1.3. Map of study sites and select present species, indicating 1) Weld Co. and 2) Lincoln Co. field sites. Both *Crotalus viridis* (photo: David Nixon) and *Sistrurus tergeminus edwardsii* (photo: Tyler Carlson) are present at the Lincoln Co. field site, but only *Crotalus viridis* is present at the Weld Co. field site. Various rodents, including but not limited to *Peromyscus maniculatus* (photo: Danny Poet), *Mus musculus* (photo: Milos Andera), *Microtus pennsylvanicus* (photo: Daniel Cadieux), and *Dipodomys ordii* (photo: Jim Zapp) are present at both field sites. Orange overlay represents the geographic distribution of *Sistrurus tergeminus edwardsii* in Colorado.

## **Specific Aims – Chapter II: Prairie Rattlesnake Diet Ecology**

A knowledge of feeding ecology is critical in forming predictions regarding how resistance will be distributed across a rodent community, because one can expect a snake to exert the greatest selective pressure on the species it consumes with the greatest frequency.

Ontogenetic shifts in diet can also suggest which prey types rattlesnake venoms are optimized for at different ontogenetic stages. Additionally, broadly distributed organisms can be expected to exhibit dietary variation across their range due to local differences in prey species as a result of environmental factors. As such, it is important that the range-wide diet ecology of a species is taken into consideration when formulating predictions on its ecological interactions.

However, dietary data for the Prairie Rattlesnake is lacking, and this information is a vital component of Prairie Rattlesnake natural history. While investigators have explored Prairie Rattlesnake diet on a small scale in discrete populations (Rothe-Groleau et al., pers. comm.), an investigation on the range-wide feeding ecology of this species has not been conducted. Diet studies in other wide-ranging rattlesnake taxa (e.g., Glaudas et al., 2008; Schuett et al., 2016) have revealed differences in proportion of prey type taken based on location of the snake within its distribution. The same trend is expected to be upheld for the Prairie Rattlesnake, which spans the greatest latitudinal range of any rattlesnake species. Dietary differences among geographic regions or populations may also explain venom variation, and divergent venom phenotypes are present in Prairie Rattlesnakes, thus it is important that diet across the range is characterized. We aim to explore the following hypotheses:

- H1     The Prairie Rattlesnake will display dietary ontogeny.
- P1     Snakes will display an ontogenetic diet shift from reptile to mammal prey as they increase in body size.

- H2 Seasonality will impact foraging duration across the geographic distribution of the Prairie Rattlesnake.
  - P1 Snakes from northern regions will show a cessation in feeding over winter months.
  - P2 Snakes from southern regions will contain prey items later into the fall and earlier in the spring than northern snakes and may feed during the winter months.
- H3 Diet of the Prairie Rattlesnake will vary latitudinally throughout its distribution.
  - P1 The proportion of lizards consumed by snakes will increase moving southward in the geographic distribution.

Range-wide diet ecology of the Prairie Rattlesnake was explored, using preserved specimens in natural history collections. Specimens were analyzed for stomach and hindgut remains via two small incisions and prey remains were identified to greatest level of taxonomic resolution using a variety of available characteristics. Trends in diet were also explored, based on snake sex, size, and collection location. The resulting data provide a well-rounded image of Prairie Rattlesnake diet across the expansive distribution inhabited by this species.

### **Specific Aims – Chapter III: Venom Resistance in Colorado Rodents**

Venom resistance has been studied by numerous investigators, but few have done so in a system with multiple predator and prey species. Using a complex system, one can explore the role of selective pressures on the presence and distribution of venom resistance across rodent species. As rodent species are preyed upon at different frequencies by co-occurring rattlesnakes, it is expected that selection pressures will impact levels of resistance among rodent species in the system. Additionally, the implementation of two field sites allows for exploration of geographical questions, namely how prevalent resistance is in mismatched rodent-snake pairs due to discrepancies in distributions.

The first set of aims for this chapter are to characterize venom resistance based on feeding ecology predictions:

- H1 Predation frequency exerted on rodents by rattlesnakes will influence the level of venom resistance present.
  - P1 Smaller rodent species should exhibit venom resistance to both rattlesnake species.
  - P2 Larger rodents should exhibit stronger resistance to the larger Prairie Rattlesnake
  - P3 Given the high level of conservation of venom constituents across rattlesnake taxa, resistance should be present to both snake species at some level in all rodent species.
- H3 Co-occurrence should affect rodent resistance to snake venoms: Sympatric rodent-snake pairings should show greater resistance than allopatric rodent-snake pairs.
  - P1 Rodents should have greater resistance to their local snake population, as that population exerts far greater selective pressure than a distant population of the same species

To complicate this idea, only the Prairie Rattlesnake is present at both field sites and it exhibits divergent venom types at these two sites. This difference in venom composition between sites further complicates predictions, as divergent venom phenotypes work to subdue prey in different fashions. The Desert Massasauga is absent from the Weld site, and this absence allows for effectively testing how release from Massasauga predation impacts resistance to that venom in rodents from the Weld site.

Studying venom resistance at this broad scale allows for exploration into the roles of selective forces on the development and maintenance of resistance. Resistance will be evaluated via a variety of methods, including whole organism assays and serum-based assays. Additional affinity column and proteomic techniques will be used to identify serum proteins conferring resistance in rodents to venoms. The final analysis will provide a community level view of

venom resistance in a naturally-occurring food web. I will explore the roles of geography and predation pressures on the presence and distribution of venom resistance by studying two predatory snakes and a suite of interacting rodents at two geographically distant field sites.

CHAPTER II  
RANGEWIDE FEEDING ECOLOGY OF THE PRAIRIE  
RATTLESNAKE (*CROTALUS VIRIDIS*)

**Abstract**

Studies of diet are paramount to our understanding of an organism's interactions with its ecosystem. Snakes serve as important predators in their respective communities, though little is known about the dietary habits of most species. We describe the diet of the broadly distributed pitviper, the Prairie Rattlesnake (*Crotalus viridis*), using preserved specimens from natural history collections. Dietary samples were collected from across the species' range from specimens collected throughout the year. The examination of 449 specimens resulted in recovery of 79 prey boli from the stomachs of 76 individuals and hindgut remains from 267 individuals. Mammals were found to comprise the vast majority of Prairie Rattlesnake diet (87.3% of prey boli and 65% of hindgut remains), with lizards (7.7% of prey boli and 6.4% of hindgut remains) and birds (5.3% of prey boli and 1.9% of hindgut remains) comprising minor dietary components. A weak ontogenetic shift in diet was present, with lizards decreasing in prey frequency as snake body length increased. Rodents were consumed by rattlesnakes of all sizes. Strong seasonality was present in feeding, with snakes containing prey from April 18<sup>th</sup> to November 3<sup>rd</sup>, but lacking food boli and prey remains outside of this interval. Our data suggest that the Prairie Rattlesnake is a mammal specialist, preying primarily on small rodents

throughout its lifetime across its distribution. Lizards are consumed by small snakes, and birds by larger snakes, but with much less frequency than rodent prey for either size group. Dietary characterization of snakes is important in understanding their roles in ecosystem interactions, specifically regarding how snakes fit into their respective food webs. Additionally, diet may serve as a main driver for venom variation, and proper understanding of diet can inform venom variation research.

### **Introduction**

Organisms interact with their environments in various ways, and to characterize their role in an ecosystem fully requires a near-complete understanding of these many factors. Species can exert forces upon their ecosystem that significantly modify the environment's capability to provide ecosystem service, such as Beavers modifying an environment's waterways through the construction of dams (Wohl, 2013), and they can impact habitat suitability for other taxa by providing additional shelter, as is the case with Prairie Dog towns (Ceballos et al., 1999). Species also interact with their ecosystems on a trophic level and can be biologically important as predator (Kittle et al., 2008) and/or as prey species (Maerz et al., 2005). It is in this trophic context that some organisms may be especially important, as their complete removal from a system may result in drastic alteration of trophic dynamics or entire ecosystem collapses (Bundy et al., 2009). To mitigate adverse impacts to trophic dynamics in ecosystems, it is important that investigators explore the trophic roles of all biotic components of an ecosystem.

Beyond the functionality of trophic systems, understanding an organism's feeding habits allows us to characterize better the natural history of that species. Dietary studies can address questions about activity patterns (Prenter et al., 2013), habitat usage (Wasko and Sasa, 2009) and morphology (Smith, 2014) of organisms. One can also glean unexpected insight from diet

studies, such as discovering novel means of seed dispersal by animals that incidentally consume seeds with their prey items (Reiserer et al., 2018).

Snakes serve as model systems for a variety of questions and have been used as subjects to study movement ecology (Wastell and Mackessy, 2011), thermal ecology (Webb and Shine, 1998), and general habitat usage (Blouin-Demers and Weatherhead, 2001). Snakes also serve as a unique and important group for dietary study, as their physiology presents many novel freedoms and challenges when compared to other vertebrates (Secor, 2003; Lignot et al., 2005; Glaudas et al., 2018). Additionally, snakes are one of the only vertebrate predators specialized in eating whole prey, with very few examples present of species able to circumvent this constraint (but see Jayne et al., 2002). Being gape-limited predators (Forsman, 1996), snakes must interact with their faunal communities differently than predators that can effectively tear prey apart and consume pieces of larger animals. This obligate consumption of whole prey may even have developmental impacts on the snake, with prey size potentially affecting a snake's mouth morphology throughout development (Smith, 2014). Snakes are also unique in their trophic interactions in that they are ectotherms, and as such are considerably more efficient than endotherms in converting consumed energy into biomass (Pough, 1980).

Because of these differences and the opportunities that snakes pose, a variety of species have been studied in a diet ecology context, ranging from broadly distributed generalist species (Rodriguez-Robles, 2002) to specialists with more restricted distributions (Avila-Villegas et al., 2007). Dietary studies have occurred on many species of rattlesnakes (Mackessy, 1988; Dugan and Hayes, 2002; Glaudas, 2008; Webber et al., 2016) due to their ease of study, abundance in their respective ecosystems, and the presence of many preserved specimens in natural history collections. Investigators have generally done well in investigating diet across the ranges of

broadly distributed species (Glaudas, 2008), but for snakes with very large distributions, diet studies often focus in on a particular geographical region (e.g., Goetz et al., 2016). This narrow scope in geographic region can effectively mask dietary differences resulting from geographic variation, seasonality, sexual dimorphism and ontogeny, and as a result it is critical that investigators sample across a species' range when making conclusions on general feeding ecology.

Geographic variation can impact feeding ecology of a species widely, as prey items that are locally abundant in some regions may be absent in others. As a result, an organism may prey almost entirely on a single prey species in one region, and almost exclusively on another elsewhere (Kross et al., 2016; Platt et al., 2009). Because of this potential for drastic diet differences across a landscape, one cannot draw conclusions on an organism's overall feeding ecology based on sampling from a single area within its range. Geographic differences in species compositions can also result in local adaptation that can directly impact feeding ecology, and this is exemplified in rattlesnake-populated systems by cases of venom-resistant prey populations (Poran et al., 1987).

Seasonality also has the potential to influence feeding ecology greatly, with potential for food types and amounts consumed changing drastically depending on time of year (Thompson et al., 2015). Many snakes are unique among predatory animals in that they have the ability to undergo non-feeding states for prolonged periods of time, often corresponding with prey unavailability or seasonally unsuitable periods (Secor and Diamond, 1998; O'Donnell et al., 2004). While this is often the case in temperate climates, snakes in warmer areas may not face the same constraints and consequently may be able to feed year-round (Dugan and Hayes, 2002). The interplay between geographic variation and seasonality becomes particularly apparent with

broadly distributed species, where seasons may be harsh in part of the distribution yet milder in other regions. For this reason, species ranging across varied climatic conditions can function dramatically differently across their range, and thus characterization of diet in all climatic regions becomes critical.

While extrinsic factors like geography and seasonality certainly impact feeding across a species' range, individual dependent factors can also impact feeding. Sexual dimorphism has the potential to result in highly divergent feeding ecology between sexes (Pearson et al., 2002), where males and females may effectively partition niches within their environment. By doing this, intersexual competition becomes reduced and an area may be able to support a higher density of individuals of a single species. While sexual niche partitioning in feeding ecology has been noted in many snake taxa (e.g., Shine et al., 2002), it does not appear to be common among rattlesnakes (Dugan and Hayes, 2002). In general, sexual niche partitioning should be most commonly expected in species with significant morphological divergence between sexes.

Finally, ontogeny is an important factor that influences the diets of many organisms, as shifts in diet often correspond to growth and developmental changes. Ontogenetic dietary shifts have been documented in an array of taxa and coincide with various developmental milestones (Mackessy, 1988; Essner et al., 2014) or simply change over the organism's lifetime concurrent with growth (Ford and Hampton, 2009). In many rattlesnake taxa, a marked ontogenetic dietary shift is apparent where juveniles feed primarily on ectothermic prey and gradually shift to endothermic prey as adults, due largely to the size and availability of lizard and mammalian prey in their environments (Webber et al., 2016; Holycross and Mackessy, 2002). This ontogenetic shift is recapitulated in venom ontogeny, with young rattlesnakes generally exhibiting a higher toxicity venom that gradually shifts towards a more degradative venom into maturity (Mackessy

et al., 2018; Mackessy, 1988). It is hypothesized that this shift in venom phenotype facilitates quick prey immobilization when snakes are young but favors improved digestion of larger prey items as snakes grow in size (Mackessy, 2010). While this trend is generally upheld throughout rattlesnake clades, notable exceptions do exist, namely situations where an ontogenetic venom shift occurs without corresponding change in diet (Dugan and Hayes, 2002), and those where an ontogenetic dietary shift occurs without a corresponding change in venom phenotype (Mackessy et al., 2003). It is evident that ontogenetic dietary shifts are important in rattlesnake taxa, and sampling should incorporate all age classes to account for these shifts.

The Prairie Rattlesnake (*Crotalus viridis* Rafinesque, 1818) is a medium-sized pitviper broadly distributed across the North American Great Plains, ranging from southern Canada to northern Mexico (Hammerson, 1999). This rattlesnake has served as a model species for an array of questions ranging from ecological (Shipley et al., 2013) to behavioral (Saviola et al., 2012; Clarke et al., 1996) to genetic (Schield et al., 2019). Across its broad geographic range, the Prairie Rattlesnake generally inhabits grassland habitats, but can occur in in rockier, arid habitats or more mesic environments. Additionally, the rattlesnake ranges over a wide climatic gradient, with extreme winters at its northern range limits and less pronounced seasonality in the south. Across this large distribution, there is much potential for variation in prey species availability. Notably, a clinal increase in lizard species abundance occurs moving southward through the rattlesnake's range, and one might expect this to impact diet.

Venom variation in the Prairie Rattlesnake is well characterized, with previous studies characterizing the venom proteome (Saviola et al., 2015) and more recent investigations seeking to understand distribution-wide trends in venom variation (Smith et al., in prep.). Venom appears to vary latitudinally across this snake's range, with a more toxic phenotype present in northern

populations and a more degradative phenotype present in southern populations (Smith et al., in prep.). Diet has been shown to impact snake venom composition significantly (Mackessy, 1988; Daltry et al., 1996), perhaps serving as a selective force far exceeding defensive (Ward-Smith et al., 2020) and digestive pressures (Thomas and Pough, 1979). As a result, it is crucial that the diet of an organism is well characterized when attempting to understand variation in venom composition or to make sense of toxin abundance variation in a venom.

While the Prairie Rattlesnake is easily accessible, occupies a large range in North America, and has been the subject of relatively intense study, a thorough range-wide characterization of the dietary ecology of this species has not been conducted. General descriptions of this snake's diet indicate that it feeds on various small mammals, lizards, and birds (Hammerson, 1999). Much like other rattlesnakes, Prairie Rattlesnakes do not appear to have a propensity to eat other snakes or to cannibalize, but at least a single case exists to suggest that they may consume conspecifics under unique circumstances (Gloyd, 1933).

While the prey items indicated above are certainly consumed by Prairie Rattlesnakes, this information provides only a foundation and leaves much to be desired. Limited investigation has occurred to characterize the diet of this species at the population level, with population studies having been conducted in Kansas, USA (Rothe-Groleau et al., in prep.) and Alberta, Canada (Hill et al., 2001). While studies like these are the first step in understanding the diet of this species, they do not allow us to draw broad conclusions about how diet is impacted by environmental parameters and geography, or how diet may serve as a driver for venom variation. To address this lack of information, we aim to characterize the diet of the Prairie Rattlesnake across its range by analyzing stomach and hindgut contents from preserved specimens in natural history collections.

- H1 An ontogenetic diet shift will be present in the Prairie Rattlesnake.
  - P1 Snakes will display a dietary shift from ectothermic (lizard) to endothermic (mammalian) prey
- H2 Seasonality will impact the duration of Prairie Rattlesnake foraging.
  - P1 Northerly snakes will have a reduced foraging period.
  - P2 Southerly snakes will have an increased foraging period and may feed opportunistically throughout the winter months.
- H3 Latitude will impact Prairie Rattlesnake diet composition.
  - P1 Southerly snakes will eat a greater proportion of lizards compared to northerly snakes.

### **Methods**

Natural history collections at seven institutions containing fluid-preserved Prairie Rattlesnake specimens were visited for specimen examination (Table 2.1). Specimens were considered usable if accompanying data did not indicate that animals were captive or were held in captivity prior to preservation. We sampled specimens of both sexes of all sizes and from across the entirety of the species' distribution. Before sampling for prey remains, snakes were sexed via presence or absence of hemipenes, and snout-vent length (SVL) and tail length (TL) were recorded by measuring snakes with a soft metric tape measure. Snakes that were deemed too fragile to handle without damage or those felt to represent important voucher specimens were omitted from sampling.

Table 2.1. Museum collections investigated, listing full and abbreviated collection name, collection location, and number of specimens examined.

Collection Name (Abbreviation)	Location	# specimens examined
University of Northern Colorado Museum of Natural History (UNC-MNH)	Greeley, Colorado	189
Sternberg Museum of Natural History (FHSM)	Hays, Kansas	178
Monte L. Bean Life Science Museum (BYU)	Provo, Utah	35
Sam Noble Oklahoma Museum of Natural History (OMNH)	Norman, Oklahoma	21
Amphibian and Reptile Diversity Research Center (UTA)	Arlington, Texas	18
University of Wyoming Museum of Vertebrates (UWYMV)	Laramie, Wyoming	10

Prey remains were sampled via inspection of stomach and hindgut contents. Inspection of stomach contents was conducted by making a small (2-6 cm) midventral incision through ventral scales of the snake. Once located, an additional incision was made through the stomach wall to determine if a food bolus was present. If present, direction of ingestion of the prey item was recorded (inferred from orientation in the stomach), and the bolus was removed for identification to greatest taxonomic resolution possible (typically to genus or species level). Following identification, food boli were returned to the stomach of the specimen, or stored separately, depending on preferences of collections curators.

Hindgut remains were sampled via inspection of the snake's intestinal tract. A small incision (2-6 cm) was made in the ventral surface of the snake anterior to the cloaca, and intestines were inspected for the presence of prey/fecal remains. If detected, prey remains were removed from the hindgut and placed in a 2 ml screw-cap tube filled with 70% ethanol. Prey remains were identified to greatest level of taxonomic resolution by inspection under a dissecting

microscope. Identifying features including but were not limited to hair, feathers and keratinized scales (further analyzed under a dissecting microscope to determine avian or reptilian identity) were used to determine type and number of prey consumed. Prey item classes were plotted against various individual correlates including snake snout-vent length, date of collection, and collection latitude to infer trends present in predation.

## Results

### Stomach Contents

A total of 449 *Crotalus viridis* specimens were examined at six natural history collections (Table 2.1). Of the specimens studied, stomach contents were recovered from 76 snakes (16.9%), and hindgut contents were recovered from 267 snakes (59.5%). Three snakes contained two prey items each in the stomachs, with one of these snakes containing prey from two different taxonomic classes. Stomach and hindgut contents are discussed separately throughout, because while each can represent different prey items, they may also represent the same prey over time, and thus combining these two in discussion may overrepresent samples.

A variety of prey items were recovered from the stomachs of preserved Prairie Rattlesnakes. Identified prey in rattlesnake stomachs comprised three taxonomic classes: Aves, Mammalia and Reptilia (Table 2.2). Of these prey classes, Mammalia was the best represented, comprising 87.3% of rattlesnake stomach contents. Mammalian prey items were primarily small rodents, with *Chaetodipus hispidus*, *Dipodomys ordii*, and *Microtus pennsylvanicus* and *Peromyscus* spp. being particularly well represented. A single Cottontail Rabbit (*Sylvilagus* spp.) was recovered from the stomach of a Prairie Rattlesnake. The rabbit was a juvenile and was retrieved from a large (841mm SVL) adult male rattlesnake.

Table 2.2. Stomach contents of analyzed *Crotalus viridis* specimens, including frequency ( $n$ ), proportion of total prey items, and proportion of identifiable items within classes Aves, Mammalia and Reptilia.

Prey Taxon	$n$	Percentage of total (% within class)
Aves	5	6.3
Unidentified bird	5	6.3 (100.0)
Mammalia	69	87.3
<i>Chaetodipus hispidus</i>	3	3.8 (4.3)
<i>Cynomys ludovicianus</i>	1	1.3 (1.4)
<i>Dipodomys ordii</i>	7	8.9 (10.1)
<i>Microtus pennsylvanicus</i>	4	5.1 (5.8)
<i>Microtus</i> spp.	1	1.3 (1.4)
<i>Onychomys leucogaster</i>	2	2.5 (2.9)
<i>Perognathus flavescens</i>	1	1.3 (1.4)
<i>Peromyscus maniculatus</i>	1	1.3 (1.4)
<i>Peromyscus</i> spp.	5	6.3 (7.2)
<i>Sylvilagus</i> spp.	1	1.3 (1.4)
Unidentified rodent	43	54.4 (62.3)
Reptilia	5	6.3
<i>Aspidoscelis sonorae</i>	1	1.3 (20.0)
<i>Plestiodon multivirgatus</i>	1	1.3 (20.0)
<i>Plestiodon obsoletus</i>	2	2.5 (40.0)
<i>Sceloporus consobrinus</i>	1	1.3 (20.0)
Totals	79	100.0

Five lizards were recovered from the stomachs of Prairie Rattlesnakes in this study (Table 2.1): one Many-Lined Skink (*Plestiodon multivirgatus*), two Great Plains Skinks (*Plestiodon obsoletus*), one Prairie Lizard (*Sceloporus consobrinus*) and one Sonoran Spotted Whiptail (*Aspidoscelis sonorae*). Lizards represented only 6.3% of all prey items recovered from Prairie Rattlesnake stomachs, forming only a small part of this species' diet. Avian prey items were also recovered as stomach contents, with five recovered food boli being identifiable as birds.

## **Hindgut Contents**

Of the 449 specimens analyzed, 267 snakes contained hindgut contents that could be identified for further analysis (Figure 2.1A). Seventy-one (27%) of the hindgut samples were unidentifiable to a prey item class, appearing to lack hair, scales, feathers or chitin plates. These samples could represent aggregations of material that were not derived from a consumed prey item, such as detached pieces of intestinal lining or fragments of uric acid. Additionally, some unidentifiable samples may consist of debris consumed with a prey item that had since digested. In at least one case, hindgut remains consisted solely of arthropod fragments (beetle fragments), and we presume this to have been incidentally ingested or potentially to have entered the stomach post-mortem from a rupture in the snake's body wall.

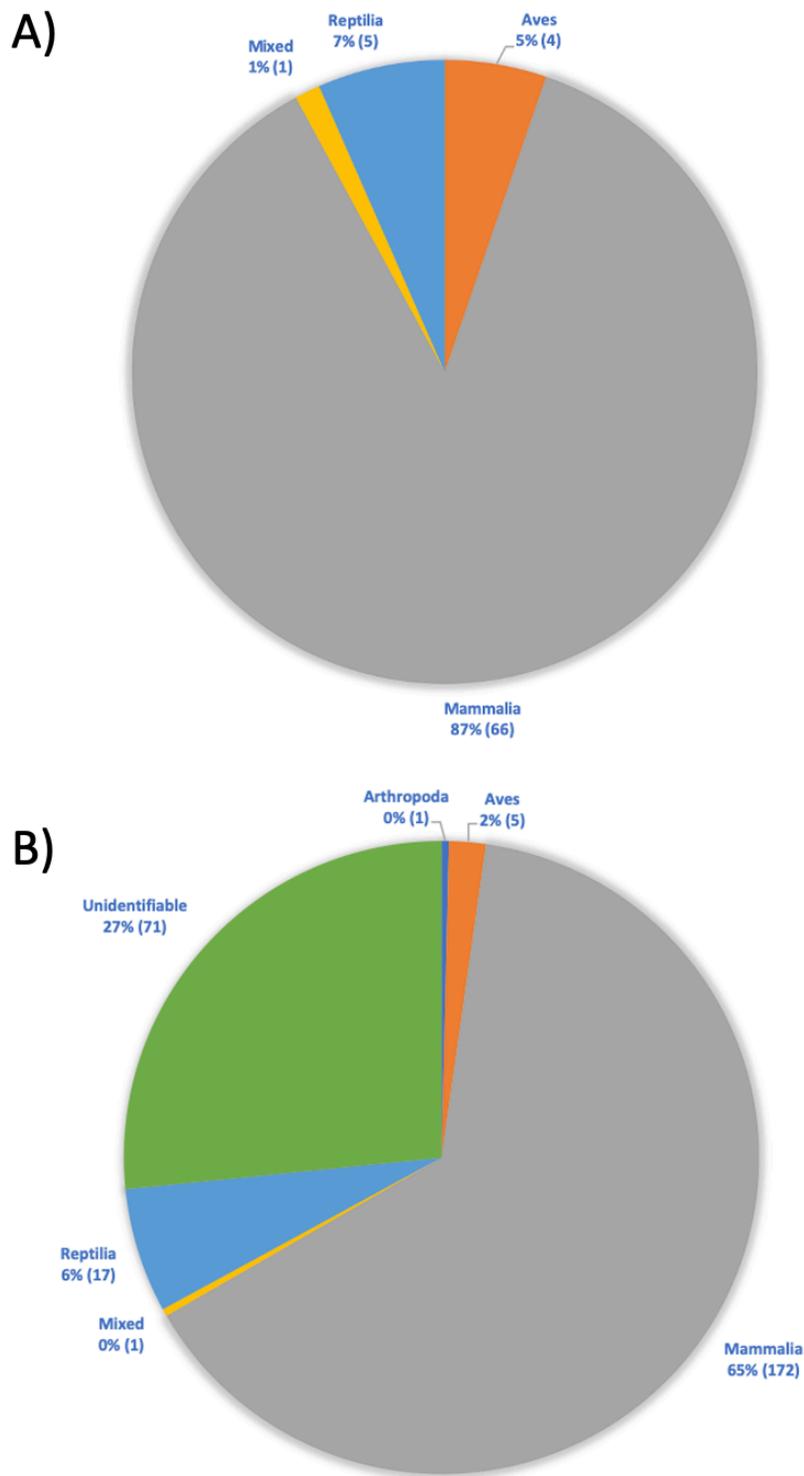


Figure 2.1. Proportion of prey classes represented in *Crotalus viridis* A) stomach and B) hindgut contents, including percent of total hindgut contents and frequency (n).

Overall, hindgut remains comprised primarily mammals (65% of hindgut contents), and fewer lizard (6% of contents) and bird (2% of contents) remains. A single hindgut sample contained both mammal and avian remains, and this accounts for the mixed sample noted in Figure 2.1. Because a prey item spends a considerably longer time in the hindgut of a snake than in the stomach, hindgut analysis allows for more information to be extracted from each animal. Additionally, the material collected in the hindgut may represent a different prey item than that present in the stomach, and this was verified by the presence of at least four samples with mismatched stomach and hindgut content, and the single sample with mixed material in the hindgut.

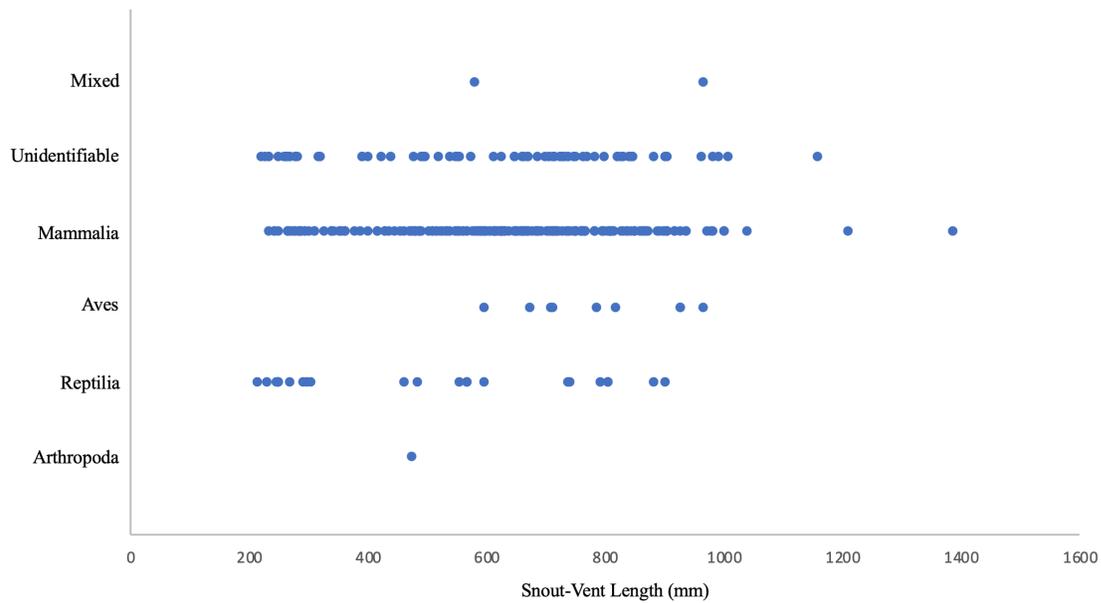


Figure 2.2. Prey classes consumed by *Crotalus viridis* as a function of body size (snout-vent length). Data depicted represent a combination of stomach contents (n = 76) and hindgut contents (n = 267) recovered from all specimens.

The majority of prey items consumed belonged to class Mammalia and were predominately small rodents. Mammal prey were consumed by snakes of all body lengths (Figure 2.2), ranging from 236mm-1391mm. Lizards (class Reptilia) were the second most commonly consumed prey item, having been preyed on by snakes ranging in length from 217mm-904mm. Birds formed the last most frequently preyed upon group and were only consumed by snakes with body lengths between 600mm-970mm. A single arthropod was extracted from the stomach of a snake, but we believed this animal to represent an accidental occurrence given the possibility to scavenging beetles interacting with roadkill. A large subset of hindgut contents contained material that was unassignable to prey items, and these were present in snakes of all sizes. Two individuals contained multiple prey items of differing prey classes within them, a 584mm and 970mm long individual.

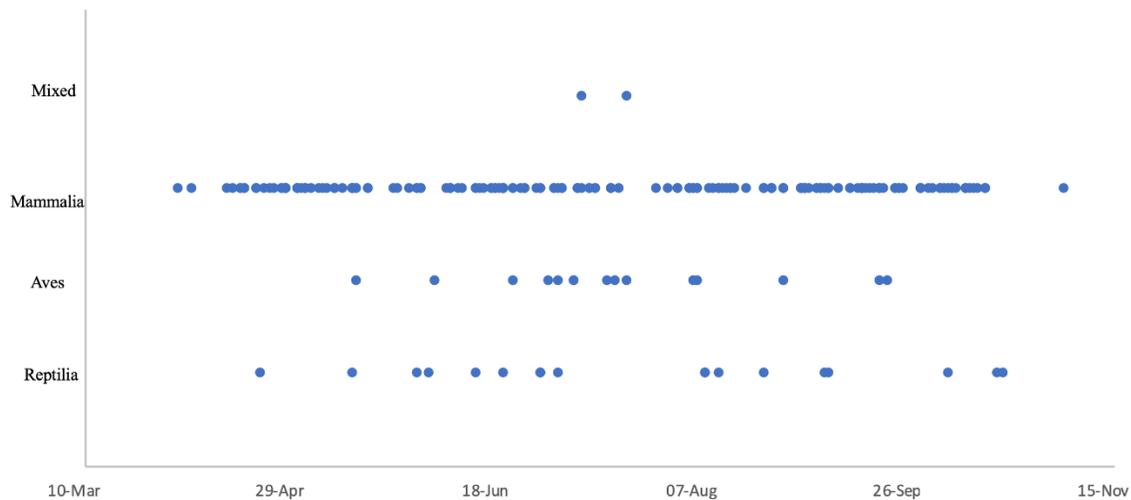


Figure 2.3. Seasonal distribution of prey consumed by *Crotalus viridis*. Data depicted represent combined stomach (n = 67) and hindgut contents (n = 162) recovered from all specimens with known collection dates.

Again, mammals comprised the majority of prey items taken by snakes, and they had the widest date interval of predation among all prey classes (Figure 2.3). Mammalian prey was consumed throughout the active season, from the beginning of April to the beginning of November. Reptilian prey (lizards) followed a similar pattern, but were slightly more constrained in span, being present in snakes from the end of April until the end of October. Finally, avian prey taken fell within the most constrained window, with bird prey items present in snakes from mid-May until the end of September. Two snakes both containing multiple prey items of mixed classes were samples in July. Specimens collected throughout the entirety of the year were present in this dataset, and distribution of prey items does not reflect a lack of sampling outside of the months depicted.

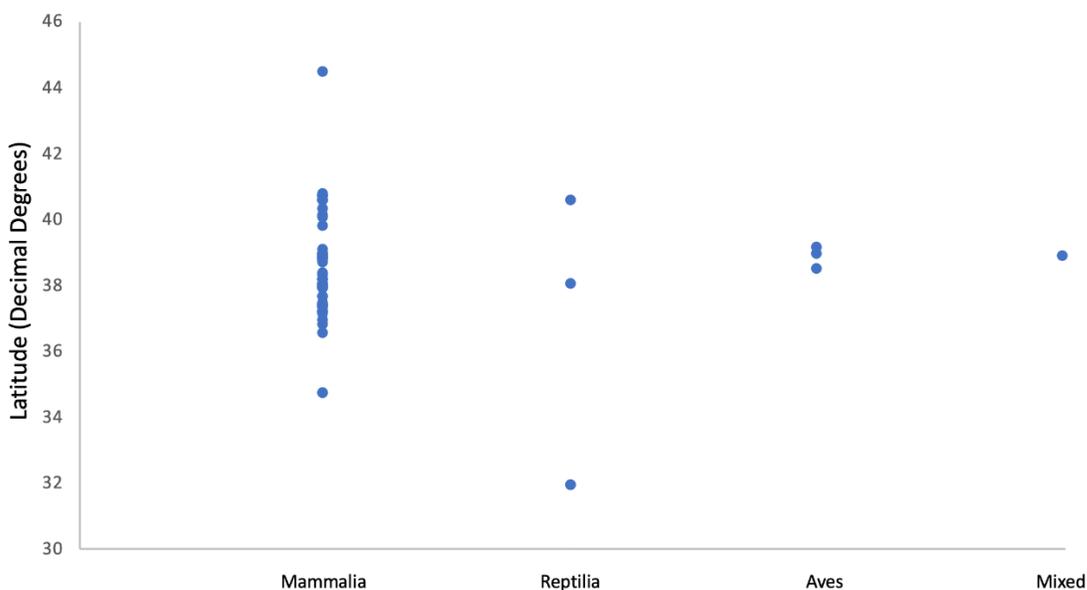


Figure 2.4. Latitudinal distribution of prey classes consumed by *Crotalus viridis*. Data depicted represent stomach contents (n = 70) and hindgut contents (n = 186) recovered from all specimens with known collection latitudes.

Because of the poor collection location associated with many of the sampled individuals, latitudinal analyses included only the subset of animals with usable data. Mammalian prey was consumed across a wide latitudinal breadth, with the northernmost prey item in the study set being a mammal. Mammalian prey ranged across a latitudinal distribution from 34.7 to 44.4 decimal degrees. Because of poor locality resolution, only three lizard samples could be plotted, at 31.9, 38.0 and 40.5 decimal degrees, with the latter representing the lowest latitude of any prey item present in the dataset. Avian prey items clustered in latitude, with records ranging from 38.4 to 39.1 decimal degrees. A single mixed record of mammalian and avian prey was present at a latitude of 38.8 decimal degrees.

### **Discussion**

We found a broad array of prey species (over 16 species) distributed among four animal classes. Rodents were by far the most abundant prey group consumed, followed by lizards and small birds. Prey items were recovered from snakes of all body lengths and a broad sampling of individuals throughout the active season was present in the specimens analyzed. This pattern of dependence on mammalian prey is consistent with results of more limited studies on the Prairie Rattlesnake (Hill et al., 2001) and on other species of wide-ranging, larger bodied rattlesnakes (i.e., Clark, 2000; Loughran et al., 2013; Gren et al. 2016)

#### *Prey Items*

The majority of mammalian prey items consumed comprised small rodents, with *Chaetodipus hispidus*, *Microtus pennsylvanicus* and *Peromyscus* spp. being particularly well represented and *Dipodomys ordii* being the most commonly taken prey species represented by stomach contents. These rodents all represent species that are locally abundant in their respective

environments and may be preyed upon most frequently as a result of this local abundance, and this presumably varies among regions and across temporal scales. Small rodents like the aforementioned species present little danger to a predatory rattlesnake, but also provide less caloric benefit than a larger prey item. A snake may consume greater numbers of small rodent prey as opposed to fewer meals of larger rodents, and this may be a favorable strategy in the case of small rodent abundances. A large number of additional unidentified rodents were removed from snake stomachs ( $n = 43$ ), and the majority of these samples likely belong to one of the four aforementioned taxa. Further work is needed to characterize these currently unidentified samples and provide better resolution into the taxonomic breadth of the Prairie Rattlesnake's diet, though overall, the role of locally abundant small rodents cannot be understated for Prairie Rattlesnake foraging.

A single Black-tailed Prairie Dog (*Cynomys ludovicianus*) was recovered from the stomach of a large adult Prairie Rattlesnake. Prairie Rattlesnakes have an affinity for dog towns, as the burrow systems provide suitable shelter, thermal insulation, and hibernacula (Fitzgerald et al., 2013). In fact, prairie dog towns may bolster reptile abundances in general, as well as improve the robustness of biological communities as a whole (Shiple et al., 2008; Shipley and Reading, 2006; Davis and Theimer, 2003). As a result of this tight ecological interface with Prairie Dogs, one would expect Prairie Rattlesnakes to encounter them either in an offensive or defensive context. Prairie Dogs exhibit strong agonistic behavior against rattlesnakes, suggesting that rattlesnakes may be perceived as threatening predators (Loughry, 1987). While an adult Black-tailed Prairie Dog may be too large for all but the largest Prairie Rattlesnake to consume, juvenile and subadult Prairie Dogs represent a suitable meal for adult rattlesnakes. Given the abundance of biomass as a whole in the vicinity of a prairie dog town (Shiple and Reading,

2006), it is possible that Prairie Rattlesnakes continue to exert the greatest predation on smaller rodents because of their heightened biomass here. Additionally, given the strong agonistic tendencies of Prairie Dogs (Loughry, 1987), rattlesnakes may incur retaliation during these predatory events if directed against Prairie Dogs.

The Northern Grasshopper Mouse (*Onychomys leucogaster*) was represented in this diet dataset by two consumed individuals (Table 2.2). While this rodent is similar to other rodents in its small body size, it is quite different from an ecological standpoint. The Grasshopper Mouse is one of a few carnivorous rodents, and while the mainstay of its diet is invertebrates, the Grasshopper Mouse will opportunistically take larger prey items. Notably, its diet includes reptiles surpassing its own size (Sherbrooke, 1991), making the Northern Grasshopper Mouse a formidable prey item. As an adaptation to a predatory lifestyle, the Grasshopper Mouse possesses a much stronger bite than that of comparably sized rodents (Williams et al., 2009) and could pose harm to a rattlesnake in retaliation against a predation attempt. Additionally, Grasshopper Mice may have endogenous predation defenses lacking in other rodents. Resistance to scorpion venoms has been well characterized in Grasshopper Mice (Rowe et al., 2013; Rowe and Rowe, 2008), an adaptation that in turn allows the mice to prey upon scorpions without consequences. Evidence suggests that Northern Grasshopper Mice may also possess strong resistance to pitviper venoms, making them a more difficult prey item to subdue effectively (Balchan et al., unpub. data; Chapter 3). Consequently, Grasshopper Mice represent a well-defended prey item via both physical and physiological defense mechanisms. While well defended, Northern Grasshopper Mice still appear as a prey item of the Prairie Rattlesnake, indicating that their defenses are not entirely effective in avoiding predation.

A single Plains Pocket Mouse (*Perognathus flavescens*) was recovered from the stomach of a juvenile rattlesnake from Lincoln County, Colorado. With adult body masses ranging from 6.9 to 11.5 grams (Hibbard and Beer, 1960), Plains Pocket Mice represent one of the smallest rodents present in their respective communities and may be an ideal prey item for young Prairie Rattlesnakes. Pocket Mice are relatively defenseless, nocturnally active, and potentially abundant in their environments, making them a prey species easily capitalized upon by small rattlesnakes. In support of this idea, Pocket Mice were found to comprise a considerable proportion of prey items consumed by the Desert Massasauga Rattlesnake (Holycross and Mackessy, 2002), a small grassland specialist.

Cottontail Rabbits (*Sylvilagus* spp.) are frequently preyed upon by larger rattlesnake species as they represent a sizable meal and can be abundant in an ecosystem. Cottontails have been found in the diets of the Northern Pacific Rattlesnake (*Crotalus oreganus oreganus*; Fitch and Twinning, 1946), Great Basin Rattlesnake (*C. o. lutosus*; Glaudas et al., 2008), Red Diamond Rattlesnake (*C. ruber*; Dugan and Hayes, 2002) and Timber Rattlesnake (*C. horridus*; Clark, 2002; Reinert et al., 2011), illustrating the importance of this prey item to various medium and large sized rattlesnakes. Some rattlesnake species may take this a step further, preferentially preying on rabbits to maximize energetic intake per feeding event, as has been suggested for *Crotalus atrox* (Loughran et al., 2013). Prairie Rattlesnakes would indeed maximize energetic intake by feeding on large prey items, and opportunity for this is certainly present with Cottontails. Surprisingly, only a single Cottontail was recovered from stomach contents of Prairie Rattlesnake specimens. While additional rabbit remains are likely present in hindgut contents, it was somewhat unexpected that lagomorphs were not better represented in the diet of the Prairie Rattlesnakes we sampled. It is possible that an abundance of small prey, and therefore

more frequent meals, is a preferred foraging strategy over sporadic large meals, and hence rabbits may not be consumed as frequently in environments where small rodent abundances exist. In any event, rabbits do comprise part of the Prairie Rattlesnake's diet, but my data suggest that they are infrequently taken. Further sampling is needed to understand more fully the role of rabbits in Prairie Rattlesnake diets.

Lizards are an important component of viper diets, and they are represented in the diets of tropical (Daltry et al., 1998), arid (Sivan et al., 2013) and temperate (Canova and Gentili, 2008) viper taxa. Among North American pitvipers, a strong inclusion of lizards as a dietary component is observed, with many species perhaps relying on them as a prominent prey source (Holycross and Mackessy, 2002). Rattlesnakes generally display dietary ontogeny, largely as a function of gape limitations. Lizards are heavily favored as prey at small body sizes, and as the snake grows, diet shifts to larger, and as a result endothermic, prey species (Klauber, 1956; Mackessy, 1988; Gren et al., 2016). Rattlesnakes appear to feed on lizards with varying degrees of frequency, with some species (*S. t. edwardsii*) consuming large quantities of lizards (Holycross and Mackessy, 2002) and others (*C. o. lutosus*) only occasionally taking lizard prey (Glaudas et al., 2008). A total of five lizards were recovered from the stomachs of Prairie Rattlesnakes of all size classes in this study, representing 6.3% of prey items collected from rattlesnake stomachs and 6% of prey remains collected from hindgut contents. This lack of reliance on lizards may be a result of abundant rodents, particularly a prevalence of small rodents that can be preyed on by neonate and juvenile Prairie Rattlesnakes. Alternatively, a lack of lizard prey may reflect a lack of lizards, or lack of accessible lizards in the environment the snake inhabits. In Colorado alone, lizard abundances increase moving southward across the state (Lambert and Reid, 1981), and this latitudinal trend is upheld throughout the geographic

distribution of the Prairie Rattlesnake. Lizards were found to be largely absent in the diets of Northern Pacific Rattlesnakes (*C. o. oregonus*) in two populations, likely due to a combination of these reasons (Macartney, 1989; Wallace and Diller, 1990). Overall, stomach and hindgut contents do not appear to indicate a particularly high proportion of lizards in the diet of Prairie Rattlesnakes.

Birds appear in the diets of many rattlesnake taxa (Dugan and Hayes, 2002; Webber et al., 2016) and are likely taken, at least opportunistically, by most rattlesnake species. To underscore the value of birds as a prey item, Prairie Rattlesnakes exhibit a modified prey handling strategy when subduing avian prey (Hayes, 1992). While rodents are typically envenomed and released, birds are held by a rattlesnake until they are subdued, demonstrating that rattlesnakes are able to modify prey handling mode to suit prey type (Hayes, 1992). This would suggest that birds comprise an important dietary component such that their loss following envenomation has selected for a strike-and-hold feeding mechanism. Five birds were retrieved from rattlesnake stomachs in this study, none of which were identifiable to genus or species level. Birds are likely preyed upon opportunistically, as they may be unaware of rattlesnakes when landing or foraging on the ground. Little evidence exists to indicate that rattlesnake species actively hunt birds or change their foraging strategies to improve ambush specifically for birds (Nowak et al., 2015). In particular, ground nesting birds are expected to be at elevated risk of predation, as their prolonged presence on the ground raises their likelihood of encountering a Prairie Rattlesnake, and they also leave olfactory cues that snakes can detect. Additionally, various rattlesnakes have been observed preying upon nestling birds (i.e., Savarino-Drago and Ruvalcaba-Ortega, 2019).

## Ontogeny

The general ontogenetic dietary shift in rattlesnakes describes an affinity for ectothermic prey items (primarily lizards) early in the snake's life and shifting to endothermic prey (primarily rodents) into maturity (Mackessy et al., 2003; Gren et al., 2016). This shift may be highly pronounced in some taxa (Holycross and Mackessy, 2002), or quite muted in others (Dugan and Hayes, 2012). Ontogenetic changes in diet are believed to be a force selecting for venom ontogeny in rattlesnakes (Mackessy, 2010) but may be more meaningful in promoting venom changes in some lineages than in others (Mackessy et al., 2003). Overall, it is believed that this lizard to mammal switch is an important factor maintaining ontogenetic venom changes, and that diet is an important contributor in selecting for snake venom composition (Barlow et al., 2009).

We do not see evidence for a strong ontogenetic dietary shift in the Prairie Rattlesnake, but a weak shift might be present (Figure 2.2). Reptilian and mammalian prey items were found in snakes of all sizes, starting at approximately the same body lengths. There does appear to be a cessation of reptilian prey after ca. 900 mm SVL, though this may be an artifact of sampling, where a lack of large snakes has been surveyed from southern regions with increased lizard abundances. Lizards have considerably smaller body masses than co-occurring mammals, and as such, they can be expected to be less favorable prey items as snakes increase in body length. Given the size limitations of lizard prey, we expect a reduction in lizard prey items as a whole with increased snake length. That trend does appear to be upheld, and the largest snakes in the prey item dataset are found to contain mammal remains. While it does appear that a weak ontogenetic shift from lizards to mammals may be present in this context, this is not an exclusionary shift, and rather lizard predation decreases with increasing body length, while mammal predation remains consistent.

A noticeable commencement of avian predation begins at ca. 600mm SVL. It seems reasonable that predation on birds would not occur until snakes have grown considerably, as birds pose additional handling challenges when compared to terrestrial prey in that they cannot be envenomated and released as mammalian prey frequently are due to their ability to fly a great distance (Hayes, 1992). Avian prey can potentially be viewed as becoming accessible after a body size threshold is reached and remaining available after this point. The exception to this may be nestling birds, which are unable to fly away or otherwise evade predation by smaller rattlesnakes (Savarino-Drago and Ruvalcaba-Ortega, 2019), but these remains may be poorly represented in diet studies due to a lack of feathers, hindering identification of prey remains. Further sampling is needed to quantify diet ontogeny in the Prairie Rattlesnake better, but initial data appear to indicate relatively weak dietary ontogeny compared to other taxa.

### **Seasonality**

The Great Plains region spanning from southern Canada to northern Mexico encompasses a broad climatic gradient with extreme temperatures in the north, and less dramatic variances in the south. Overall, the region is characterized by defined seasonality, and winters across the plains are thermally challenging for endotherms. As a result, Prairie Rattlesnakes hibernate throughout the winter in underground refugia (Gardiner et al., 2013). These hibernation periods are generally characterized as non-feeding bouts, but some coastal rattlesnakes will feed throughout cooler times of the year provided temperatures allow for activity (Dugan and Hayes, 2002). Temperatures in the southern reaches of the Prairie Rattlesnake's range may allow for limited winter activity and for the potential to feed. With this comes the risk of thermal instability, and a snake caught in unsuitable temperatures for digestion with a food bolus may suffer from regurgitation or mortality due to decaying prey in the gastrointestinal tract. As such,

the risks associated with winter feeding may outweigh potential energetic gains from securing a meal at this time.

Prey items collected from snakes in this study (Figure 2.3) indicate that *Crotalus viridis* do not feed through the winter months. Stomach contents were present in snakes from mid-March to mid-November throughout all years but were absent for those collected outside of this interval. These start and end dates roughly coincide with den site egress and ingress and suggest that snakes are not actively feeding while sheltering in the den during inclement weather. Many of these snakes collected during the winter appeared to be the byproduct of den-eradication efforts, as evidenced by notes associated with their collection, and one would not expect a hibernating individual to be actively foraging. Our dataset provides no evidence that Prairie Rattlesnakes feed year-round, even at their southern range limits, as has been observed with other congeners that experience a release from strong seasonality. For example, the Red Diamond Rattlesnake (*Crotalus ruber*) of southern California and the Baja California Peninsula appears to experience a release from a cessation of winter-feeding due winter temperatures being sufficient to allow for foraging and digestion at times (Dugan and Hayes, 2002). While the Prairie Rattlesnake certainly inhabits climatic regions in the southern extent of its distribution where this is feasible, it does not appear that this occurs, at least not with frequency.

### **Latitudinal Effects on Diet**

Wide-ranging species can be expected to display dietary differences across their distributions that may coincide with local prey availability (ex. Sinclair and Zeppelin, 2002). The Prairie Rattlesnake spans a broad latitudinal distribution, reaching from southern Canada to northern Mexico (Hammerson, 1999), and a strong climatic gradient is apparent between these two extremes. Moving southward on this latitudinal gradient, conditions become less severe for

snakes, with higher winter temperatures in southern regions. With these milder winters, snakes may be able to maintain longer active periods in southern regions, as they are not forced to shelter in thermally insulative refugia for an extended time period as in northerly regions. As such, there is potential for feeding earlier in the spring and later into the fall for snakes in southern regions, as a result of differences in seasonality.

In addition to increased activity periods, prey availability may be considerably different across this climatic gradient. Squamate species richness and abundance increases with movement toward the equator (McCain 2010), and lizards may thus be more available as a prey item for Prairie Rattlesnakes in southern parts of their range. Conversely, small mammals become far more available in northern regions relative to lizards, and this may result in greater proportions of mammalian prey items in the diets of northern rattlesnakes.

Mammalian prey items are the most important prey class for Prairie Rattlesnakes, and mammal predation occurred across a broad latitudinal distribution (Figure 2.4). This widespread consumption of mammalian prey indicates that mammals are the most important prey class consumed regardless of latitudinal distribution. Reptilian prey items, namely lizards, were poorly represented in the dataset, and the three lizard prey with accompanying locality information come from mid and low latitudes in the rattlesnake's range (Figure 2.4). With increased sampling of snakes, particularly those at northern and southern distributional extremes, a latitudinal shift in the abundance of lizard prey might be better supported. Avian prey items ( $n = 9$ ) were also limited in occurrence, and these appear to cluster in mid latitudinal regions (Figure 2.4). This apparent distribution likely reflects sampling bias, as the majority of snakes in the current dataset come from mid-latitude regions within their range. Avian prey are likely consumed at similar levels across regions, as grassland birds are present throughout the range of the Prairie

Rattlesnake. As birds may represent an opportunistic prey item rather than an intentionally targeted one (Nowak et al. 2015), one would expect similar predation frequencies across the snake's distribution. Further sampling should focus on snake specimens from northern and southern regions to represent range wide trends better.

To understand fully the role an organism plays within its ecosystem, and as a basis from which to generate scientific questions, understanding an organism's diet is a critical aspect of its biology. For venomous snakes in particular, diet may be the defining factor from which venom composition and geographic variation arise and are maintained. *Crotalus viridis* has an array of anecdotal and short note observations surrounding its trophic interactions within a particular environment (i.e., Chepsongol and Burkett, 2013), but investigators have yet to study adequately the diet of this broadly distributed snake in any systematic fashion. This study provides the first quantification of Prairie Rattlesnake diet across its broad distribution, using a robust sample size of preserved specimens. We find that *C. viridis* is largely a mammal specialist, with the majority of prey being small rodents. In addition, the Prairie Rattlesnake feeds on lizards and birds, and weak dietary ontogeny may be present, with a decline in lizard predation as snake body length increases. Seasonality does impact foraging in the Prairie Rattlesnake, and we failed to find animals with prey items during winter months. Additional sampling is needed to strengthen the trends that are apparent in this study, and further work will incorporate additional collection and specimens into this diet analysis.

Snake diet can inform a variety of ecological and evolutionary questions and also hold real-world implications for understanding venom variation in medically important taxa. With venoms being highly variable across landscapes, it becomes critical that we understand all factors that influence venom compositional differences, with perhaps the most important factor

being diet. Further studies should consider the dietary ecology of a broad sampling of snakes, as these organisms possess several unique adaptation and constraints as predators, resulting in trophic interactions within their ecosystems that may be radically different from those of other predatory animals.

### References

- Avila-Villegas, H., Martins, M., and G. Arnaud. 2007. Feeding ecology of the endemic rattlesless rattlesnake, *Crotalus catalinensis*, of Santa Catalina Island, Gulf of California, Mexico. *Copeia* 1:80-84.
- Barlow, A., Pook, C. E., Harrison, R. A., and W. Wüster. 2009. Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution *Proceedings of the Royal Society B* 276:2443-2449.
- Sinclair, E. H., and T. K. Zeppelin. 2002. Seasonal and spatial differences in diet in the western stock of Steller Sea Lions. *Journal of Mammalogy* 83:973-990.
- Blouin-Demers, G., and P. J. Weatherhead. 2001. Habitat use by Black Rat Snakes (*Elaphe obsoleta obsoleta*) in fragmented forests. *Ecology* 82:2882-2896.
- Bundy, A., Heymans, J. J., Morissette, L., and C. Savenkoff. 2009. Seals, cod and forage fish: a comparative exploration of variations in the theme of stock collapse and ecosystem change in four Northwest Atlantic ecosystems. *Progress in Oceanography* 81:188-206.
- Canova, L., and A. Gentili. 2008. Diet of the asp viper (*Vipera aspis*) in woodland habitats of the Po plain (NW Italy). *Acta Herpetologica* 3:175-178.
- Ceballos, G., Pacheco, J., and R. List. 1999. Influence of Prairie Dogs (*Cynomys ludovicianus*) on habitat heterogeneity and mammalian diversity in Mexico. *Journal of Arid Environments* 41:161-172.

- Chepsongol, R. M., and D. W. Burkett. 2013. *Crotalus viridis* (Prairie Rattlesnake). Diet. *Herpetological Review* 44:520-521.
- Clark, R. W. 2002. Diet of the Timber Rattlesnake, *Crotalus horridus*. *Journal of Herpetology* 36:494-499.
- Clarke, J. A., Chopko, J. T., and S. P. Mackessy. 1996. The effect of moonlight on activity patterns of adult and juvenile Prairie Rattlesnakes (*Crotalus viridis viridis*). *Journal of Herpetology* 30:192-197.
- Daltry, J. C., Wüster, W., and R. S. Thorpe. 1996. Diet and snake venom evolution. *Nature* 379:537-540.
- Davis, J. R., and T. C. Theimer. 2003. Increased Lesser Earless Lizard (*Holbrookia maculata*) abundance on Gunnison's Prairie Dog colonies and short-term responses to artificial prairie dog burrows. *American Midland Naturalist* 150:282-290
- Dugan, E. A., and W. K. Hayes. 2002. Diet and feeding ecology of the Red Diamond Rattlesnake, *Crotalus ruber* (Serpentes: Viperidae). *Herpetologica* 68:203-217.
- Essener, R. L. Jr., Patel, R., and S. M. Reilly. 2014. Ontogeny of body shape and diet in Freshwater Drum (*Aplodinotus grunniens*). *Transactions of the Illinois State Academy of Science* 107:27-30.
- Fitch, H. S., and H. Twining. 1946. Feeding habits of the Pacific Rattlesnake. *Copeia* 1946:64-71.
- Fitzgerald, K. T., Shipley, B. K., Newquist, K. L., Vera, R., and A. A. Flood. 2013. Additional observations and notes on the natural history of the Prairie Rattlesnake (*Crotalus viridis*) in Colorado. *Topics in Companion Animal Medicine* 28:167-76.

- Ford, N. B., and P. Hampton. 2009. Ontogenetic and sexual differences in diet in an actively foraging snake, *Thamnophis proximus*. *Canadian Journal of Zoology* 87:254-261.
- Forsman, A. 1996. Body size and net energy gain in gape-limited predators: a model. *Journal of Herpetology* 30:307-319.
- Gardiner, L., Somers, C., Martino, J., Parker, D., and R. Poulin. 2013. Balancing the dumbbell: summer habitats need protection in addition to winter dens for northern snake communities. *The Journal of Wildlife Management* 77:975-982.
- Glaudas, X., Glennon, K. L., Martins, M., Luiselli, L., Fearn, S., Trembath, D. F., Jelic, D., and G. J. Alexander. 2018. Foraging mode, relative prey size and diet breadth: A phylogenetically explicit analysis of snake feeding ecology. *Journal of Animal Ecology* 88:757:767.
- Glaudas, X., Jezkova, T., and J. A. Rodríguez-Robles. 2008. Feeding ecology of the Great Basin Rattlesnake (*Crotalus lutosus*, Viperidae). *Canadian Journal of Zoology* 86:723-734.
- Gloyd, H. K. 1933. An unusual feeding record for the Prairie Rattlesnake. *Copeia* 1933:98.
- Goetz, S. M., Petersen, C. E., Rose, R. K., Kleopfer, J. D. and A. H. Savitzky. 2016. Diet and foraging behaviors of Timber Rattlesnakes, *Crotalus horridus*, in eastern Virginia. *Journal of Herpetology* 50:520-526.
- Gren, E. C. K., Kelln, W., Travis, Z. D., Fox, G., Person, C., and W. K. Hayes. 2016. Diet and venom ontogeny in insular and high-altitude populations of the Southern Pacific Rattlesnake (*Crotalus oreganus helleri*). *Toxicon* 117:108.
- Hammerson, G. A. 1999. Amphibians and Reptiles in Colorado. Second edition. University Press of Colorado, Niwot, Colorado.

- Hayes, W. 1992. Prey-handling and envenomation strategies of Prairie Rattlesnakes (*Crotalus v. viridis*) feeding on mice and sparrows. *Journal of Herpetology* 26:496-499.
- Hibbard, E. A., and J. R. Beer. 1960. The Plains Pocket Mouse in Minnesota. *Flicker* 32:89-94.
- Hill, M. M. A., Powell, G. L., and A. P. Russell. 2001. Diet of the Prairie Rattlesnake, *Crotalus viridis viridis*, in southeastern Alberta. *Canadian Field-Naturalist* 115:241-246.
- Holycross, A., and S. P. Mackessy. 2002. Variation in the diet of *Sistrurus catenatus* (Massasauga), with emphasis on *S. c. edwardsii* (Desert Massasauga). *Journal of Herpetology* 36:454-464.
- Jayne, B. C., Voris, H. K., and P. K. L. Ng. 2002. Snake circumvents constraints on prey size. *Nature* 418:143.
- Kittle, A. M., Fryxell, J. M., Desy, G. E., and J. Hamr. 2008. The scale-dependent impact of Wolf predation risk on resource selection by three sympatric ungulates. *Oecologia* 157:163-175.
- Kross, S. M., Barbour, R. P., and B. L. Martinico. 2016. Agricultural land use, Barn Owl diet, and vertebrate pest control implications. *Agriculture, Ecosystems & Environment* 223:167-174.
- Lambert, S., and W. H. Reid. 1981. Biogeography of the Colorado herpetofauna. *The American Midland Naturalist* 106: 145-156.
- Lignot, J. -H., Helmstetter, C., and S. M. Secor. 2005. Postprandial morphological response of the intestinal epithelium of the Burmese Python (*Python molurus*). *Comparative Biochemistry and Physiology, Part A* 141:280-291.

- Loughran, C. L., Nowak, E. M., Schofer, J., Sullivan, K. O., and B. K. Sullivan. 2013. Lagomorphs as prey of Western Diamond-Backed Rattlesnakes (*Crotalus atrox*) in Arizona. *The Southwestern Naturalist* 58:502-505.
- Loughry, W. J. 1987. Harassment by Black-Tailed Prairie Dogs. *Behaviour* 103:27-48.
- Macartney, J. M. 1989. Diet of the Northern Pacific Rattlesnake, *Crotalus viridis oregonus*, in British Columbia. *Herpetologica* 45:299-304.
- Mackessy, S. P. 1988. Venom ontogeny in the Pacific Rattlesnakes *Crotalus viridis helleri* and *C. v. oregonus*. *Copeia* 1:92-101.
- Mackessy, S. P., Leroy, J., Mociño-Deloya, E., Setser, K., Bryson, R. W., and A. J. Saviola. 2018. Venom ontogeny in the Mexican Lance-Headed Rattlesnake (*Crotalus polystictus*). *Toxins* 10:271.
- Mackessy, S. P., Williams, K., and K. Ashton. 2003. Characterization of the venom of the Midget Faded Rattlesnake (*Crotalus oregonus concolor*): a case of venom paedomorphosis? *Copeia* 4:769-782.
- Mackessy, S.P. 2010. The evolution of venom composition in the Western Rattlesnakes (*Crotalus viridis* sensu lato): toxicity versus tenderizers. *Toxicon* 55:1463-1474.
- Maerz, J. C., Karuzas, J. M., Madison, D. M., and B. Blossey. 2005. Introduced invertebrates are important prey for a generalist predator. *Diversity and Distributions* 11:83-90.
- Nowak, E. M., Schuett, G. W., Theimer, T. C., Sisk, T. D., and K. Nishikawa. 2015. Does short-term provisioning of resources to prey result in behavioral shifts by rattlesnakes? *Wildlife Management* 79:357-372

- O'Donnell, R. P., Shine, R., and R. T. Mason. 2004. Seasonal anorexia in the male Red-Sided Garter Snake, *Thamnophis sirtalis parietalis*. *Behavioral Ecology and Sociobiology* 56:413-419.
- Pearson, D., Shine, R., and R. How. 2002. Sex-specific niche partitioning and sexual size dimorphism in Australian Pythons (*Morelia spilota imbricata*). *Biological Journal of the Linnean Society* 77:113-125.
- Platt, S. G., Rainwater, T. R., Leavitt, D. J., and S. M. Miller. 2009. Diet of Barn Owls (*Tyto alba*) in Northern Belize. *Southwestern Naturalist* 54:104-107.
- Poran, N. S., Coss, R. G., and E. Benjamini. 1987. Resistance of California ground squirrels (*Spermophilus beecheyi*) to the venom of the Northern Pacific rattlesnake (*Crotalus viridis oregonus*): A study of adaptive variation. *Toxicon* 25:767-777.
- Pough, F. H. 1980. The advantages of ectothermy for tetrapods. *American Naturalist* 115:92-112.
- Prenter, J., Weldon, C. W., and P. W. Taylor. 2013. Age-related activity patterns are moderated by diet in Queensland Fruit Flies *Bactrocera tryoni*. *Physiological Entomology* 38:260-267.
- Reinert, H., MacGregor, G., Esch, M., Bushar, L., and R. Zappalorti. 2011. Foraging ecology of Timber Rattlesnakes, *Crotalus horridus*. *Copeia* 2011:430-442.
- Reiserer, R. S., Schuett, G. W., and H. W. Greene. 2018. Seed ingestion and germination in rattlesnakes: overlooked agents of rescue and secondary dispersal. *Proceedings of the Royal Society B* 285:20172755.
- Rodriguez-Robles, J. A. 2002. Feeding ecology of North American Gopher Snakes (*Pituophis catenifer*, Colubridae). *Biological Journal of the Linnean Society* 77:165-183.

- Rowe, A. H., and M. P. Rowe. 2008. Physiological resistance of grasshopper mice (*Onychomys* spp.) to Arizona Bark Scorpion (*Centruroides exilicauda*) venom. *Toxicon* 52:597-605.
- Rowe, A., Xiao, Y., Rowe, M., Cummins, T., and H. Zakon. 2013. Voltage-gated sodium channel in Grasshopper Mice defends against Bark Scorpion toxin. *Science* 342:441-446.
- Savarino-Drago, A., and I. Ruvalcaba-Ortega. 2019. A new bird nest predator: Mexican Dusky Rattlesnake (*Crotalus triseriatus*) predation on Sierra Madre Sparrow (*Xenospiza baileyi*) nestlings. *Wilson Journal of Ornithology* 131:663-666.
- Saviola, A. J., Chiszar, D., and S. P. Mackessy. 2012. Ontogenetic shift in response to prey-derived chemical cues in Prairie Rattlesnakes *Crotalus viridis viridis*. *Current Zoology* 58:549-555.
- Saviola, A. J., Pla, D., Sanz, L., Castoe, T. A., Calvete, J. J., and S. P. Mackessy. 2015. Comparative venomomics of the Prairie Rattlesnake (*Crotalus viridis viridis*) from Colorado: identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom CroFab®. *Journal of Proteomics* 121:28-43.
- Schild, D. R., Card D. C., Hales N. R., Perry, B. W., Pasquesi, G. I. M., Blackmon, H., Adams, R. H., Corbin, A. B., Smith, C. F., Ramesh, B., Demuth, J. P., Betrán, E., Tollis, M., Meik, J. M., Mackessy, S. P., and T. A. Castoe. 2019. The origins and evolution of chromosomes, dosage compensation, and mechanisms underlying venom regulation in snakes. *Genome Research* 29:590-601.
- Secor, S. M. 2003. Gastric function and its contribution to the postprandial metabolic response of the Burmese Python, *Python molurus*. *Journal of Experimental Biology* 206:1621-1630.

- Secor, S. M., and J. Diamond. 1998. A vertebrate model of extreme physiological regulation. *Nature* 395:659-662.
- Sherbrooke, W. 1991. Behavioral (predator-prey) interactions of captive Grasshopper Mice (*Onychomys torridus*) and horned lizards (*Phrynosoma cornutum* and *P. modestum*). *American Midland Naturalist* 126:187-195.
- Shine, R., Reed, R. N., Shetty, S., and H. G. Cogger. 2002. Relationships between sexual dimorphism and niche partitioning within a clade of sea-snakes (Laticaudinae). *Oecologia* 133:45-53.
- ShIPLEY, B. K., READING, R. P., and B. J. MILLER. 2008. Capture rates of reptiles and amphibians on Black-Tailed Prairie Dog (*Cynomys ludovicianus*) colonies and on uncolonized prairie in Colorado. *Western North American Naturalist* 68:245-248.
- ShIPLEY, B. K., and R. P. READING. 2006. A comparison of herpetofauna and small mammal diversity on Black-Tailed Prairie Dog (*Cynomys ludovicianus*) colonies and non-colonized grasslands in Colorado. *Journal of Arid Environments* 66:27-41.
- ShIPLEY, B. K., CHISZAR, D., FITZGERALD, K. T., and A. J. SAVIOLA. 2013. Spatial ecology of Prairie Rattlesnakes (*Crotalus viridis*) associated with Black-Tailed Prairie Dog (*Cynomys ludovicianus*) colonies in Colorado. *Herpetological Conservation and Biology* 8:240-250.
- SIVAN, J., KAM, M., HADAD, S., DEGEN, A. A., ROZENBOIM, I., and A. ROSENSTRAUCH. 2013. Temporal activity and dietary selection in two coexisting desert snakes, the Saharan Sand Viper (*Cerastes vipera*) and the Crowned Leafnose (*Lytorhynchus diadema*). *Zoology* 116:113-117.
- SMITH, M. T. 2014. Induction of phenotypic plasticity in rattlesnake trophic morphology by diet manipulation. *Journal of Morphology* 275:1339-1348.

- Strickland, J. L., Smith, C. F., Mason, A. J., Schield, D. R., Borja, M., Castañeda-Gaytán, G., Spencer, C. L., Smith, L. L., Trápaga, A., Bouzid, N. M., Campillo-García, G., Flores-Villela, O. A., Antonia-Rangel, D., Mackessy, S. P., Castoe, T. A., Rokyta, D. R., and C. L. Parkinson. 2018. Evidence for divergent patterns of local selection driving venom variation in Mojave Rattlesnakes (*Crotalus scutulatus*). *Scientific Reports* 8:1-15.
- Thomas, R. G., and F. H. Pough. 1979. The effect of rattlesnake venom on digestion of prey. *Toxicon* 17:221-228.
- Thompson, I. D., Wiebe, P. A., Mallon, E., Rodgers, A. R., Fryxwell, J. M., Baker, J. A., and D. Reid. 2015. Factors influencing the seasonal diet selection by Woodland Caribou (*Rangifer tarandus tarandus*) in boreal forests in Ontario. *Canadian Journal of Zoology* 93:87-98.
- Wallace, R. L., and L. V. Diller. 1990. Feeding ecology of the rattlesnake, *Crotalus viridis oregonus*, in Northern Idaho. *Journal of Herpetology* 24:246-253.
- Ward-Smith, H., Arbuckle, K., Naude, A., and W. Wüster. 2020. Fangs for the memories? A survey of pain in snakebite patients does not support a strong role for defense in the evolution of snake venom composition. *Toxins* 12:201.
- Wasko, D. K., and M. Sasa. 2009. Activity patterns of a neotropical ambush predator: spatial ecology of the Fer-De-Lance (*Bothrops asper*, Serpentes: Viperidae) in Costa Rica. *Biotropica* 41:241-249.
- Wastell, A. R., and S. P. Mackessy. 2011. Spatial ecology of the Desert Massasauga Rattlesnake (*Sistrurus catenatus edwardsii*) in southeastern Colorado: habitat and resource utilization. *Copeia* 2011:29-37.

- Webb, J. K., and R. Shine. 1998. Using thermal ecology to predict retreat-site selection by an endangered snake species. *Biological Conservation*, 86:233-242.
- Webber, M. M., Jezkova, T., and J. A. Rodriguez-Robles. 2016. Feeding ecology of Sidewinder Rattlesnakes, *Crotalus cerastes* (Viperidae). *Herpetologica* 72:324-330.
- Williams, S. H., Peiffer, E., and S. Ford. 2009. Gape and bite force in the rodents *Onychomys leucogaster* and *Peromyscus maniculatus*: does jaw-muscle anatomy predict performance? *Journal of Morphology* 270:1338-1347.
- Wohl, E. 2013. Landscape-scale carbon storage associated with Beaver dams. *Geophysical Research Letters* 40:3631-3636.

## Appendix

## Specimens examined

UNC-MNH 80, 82, 84, 204, 210, 271, 300, 301, 302, 343, 379, 383, 400, 401, 491, 512, 525, 537, 538, 574, 613, 624, 629, 670, 683, 724, 729, 731, 732, 746, 756, 782, 792, 793, 809, 822, 855, 856, 870, 883, 887, 888, 889, 895, 911, 914, 918, 925, 936, 981, 986, 989, 1008, 1035, 1050, 1052, 1053, 1054, 1055, 1056, 1057, 1058, 1059, 1060, 1061, 1062, 1063, 1070, 1071, 1072, 1073, 1077, 1078, 1079, 1080, 1083, 1084, 1085, 1086, 1087, 1088, 1097, 1102, 1103, 1107, 1108, 1110, 1466, 1490, 1491, 1533, 1577, 2238, 2366, 3915, 4054, 4061, 4073, 4074, 4078, 4101, 4127, 4130, 4132, 4142, 4146, 4176, 4183, 4212, 4225, 4285, 4393, 4401, 4451, 4459, 4460, 4464, 4472, 4487, 4488, 4492, 4497, 4504, 4514, 4515, 4516, 4533, 4553, 4554, 4558, 4589, 4593, 4594, 4597, 4600, 4603, 4605, 4627, 4629, 4630, 4634, 4637, 4640, 4642, 4652, 4666, 4683, 6020, 6031, 6093, 6113, 6154, 6285, 6301, 6302, 6594, 6684, 6705, 6730, 6766, 6767, 6815, 7723, 7732, 8006, 8068, 8468, 8481, 8501, 8598, 8599, 8957, 9105, 9540, 9547, 9555, 9567, 9575, 9589, 9590, 9599, 9601, 9602, 9603, 9604, 9605, 9615, 9616, 9617

FHSM 351, 425, 440, 441, 442, 443, 444, 473, 474, 475, 477, 478, 566, 701, 755, 779, 1400, 1401, 1402, 1674, 2012, 2379, 2509, 2579, 2652, 2653, 2654, 2655, 2656, 2692, 2739, 2922, 3167, 3299, 3702, 3703, 3705, 3706, 4399, 4470, 4471, 4713, 4714, 4715, 4717, 4719, 4719, 4720, 4989, 5283, 5288, 5289, 5297, 5298, 5403, 5404, 5666, 5667, 5668, 5669, 5699, 5867,

6104, 6177, 6182, 6203, 6204, 6218, 6219, 6220, 6262, 6403, 6421, 6843, 6850, 7056, 7540, 7541, 7542, 7879, 7880, 7881, 7902, 7903, 7938, 8051, 8559, 8560, 8561, 8562, 8563, 8564, 8565, 8627, 8632, 8728, 8729, 8730, 8925, 8984, 8985, 9026, 9027, 9082, 9083, 9084, 9085, 9176, 9188, 9215, 9251, 9274, 9341, 9388, 9389, 9390, 9393, 9395, 9396, 9501, 9502, 9503, 9504, 9505, 9506, 9514, 9518, 9533, 9547, 9549, 9587, 9588, 10080, 10081, 10082, 10083, 10084, 11275, 11364, 11527, 11528, 11529, 11530, 11532, 11533, 11548, 11549, 11882, 12076, 12077, 12078, 12102, 12539, 12563, 12572, 12573, 12574, 12783, 13248, 13254, 13286, 14012, 14020, 14033, 14041, 14640, 15301, 15742, 16466, 16468, 16489, 16650, 17147, 17153, 17184, 17185, 17571, 32397

BYU 2712, 4973, 4974, 5186, 5454, 5478, 5479, 5591, 12978, 18243, 18576, 20739, 21266, 35964, 37098, 37099, 37101, 37103, 37680, 37681, 37682, 37683, 37684, 37685, 38374, 39682, 41668, 43669, 46640, 49396, 49876, 49877, 49881, 49883, 53950

OMNH 27812, 27813, 27814, 27815, 27816, 27817, 27818, 27819, 27826, 28396, 35281, 35297, 35304, 35379, 35381, 38273, 39479, 42777, 42778, 46481, 46482

UTA 65363, 65364, 65365, 65366, 65367, 65368, 65369, 65370, 65377, 65378, 65379, 65380, 65381, 65382, 65396, 65403, 65404, 65432

UWYMV 1029, 1030, H-106, H-109, H-273, H-301, H-304, H-503, HCV 367

Abbreviations as in TABLE 2.1.

## CHAPTER III

RESISTANCE OF RODENT PREY TO PRAIRIE RATTLESNAKE  
(*CROTALUS VIRIDIS*) AND DESERT MASSASAUGA  
RATTLESNAKE (*SISTRURUS TERGEMINUS*  
EDWARDSII) VENOM IN AN EASTERN  
COLORADO GRASSLAND HABITAT**Abstract**

Predation has the potential to impart strong selective pressures on organisms within their environment, resulting in adaptive changes in prey that minimize risk of predation. Pressures from venomous snakes represent a unique challenge to prey, as venom represents a unique chemical arsenal tailored to incapacitate prey. In response, venom resistance has been detected in various snake prey species, and to various degrees. This study analyzes venom resistance in an eastern Colorado grassland habitat, where the Prairie Rattlesnake (*Crotalus viridis*) and Desert Massasauga Rattlesnake (*Sistrurus tergeminus edwardsii*) co-occur with a suite of grassland rodents. We test for venom resistance across rodent and snake pairings using two geographically distant field sites to determine the role of 1) predation pressure and 2) sympatry and allopatry in the presence and strength of venom resistance. Resistance is measured using median lethal dose (LD<sub>50</sub>) assays on live rodents to assess crude toxicity of venoms and using serum-based metalloproteinase inhibition assays to determine the inhibitory effect of rodent serum against snake venom metalloproteinases. Resistance appears to be present in a variety of rodent species studied, with strong resistance present in populations of the Eastern Woodrat (*Neotoma floridana*), Ord's Kangaroo Rat (*Dipodomys ordii*), and Northern Grasshopper Mouse (*Onychomys leucogaster*). Resistance appears to be poorly developed in other species, like the

House Mouse (*Mus musculus*) and Plains Pocket Mouse (*Perognathus flavescens*). Additionally, sympatry and allopatry appear to play potentially strong roles in resistance capabilities in some species, such as the Ord's Kangaroo Rat, where a sympatric pairing may generate resistance thirty times greater than an allopatric pairing. Overall, the patterns of venom resistance within a community remain complex and may be further complicated when factoring in obstacles such as strike accuracy and venom metering, further complicating the dynamic for both partners. Future study should work to characterize resistance mechanisms at the molecular level, better contextualizing the physiological means through which resistance to venoms occurs.

### **Introduction**

Predation has the ability to exert strong selective pressures on organisms that may result in behavioral (Goldenberg et al., 2014) or evolutionary change (Lee et al., 2018). Pressures associated with predation can impart enormous impacts to an ecosystem, resulting, for example, in multiple species converging on a single phenotype (Akcali and Pfennig, 2017), organisms dramatically altering activity patterns across a landscape (Fortin et al., 2005), or group dynamics changing in the face of increased predation (Thaker et al., 2010). Predators typically rely on their capabilities to overpower prey physically, but venoms to facilitate prey capture have evolved in an array of predatory species.

Venoms are a complex chemical arsenal comprised of various proteins, peptides, enzymes and other compounds that incapacitate and subdue prey items or provide protection to the organism in a defensive context (Mackessy, 2010, 2021). Venoms are widespread throughout animal phyla and are present in various invertebrates (Chun et al., 2012), fishes (Kiriake et al., 2017), reptiles (Mackessy, 2010; Mackessy and Saviola, 2016), and even several mammals (Ligabue-Braun, 2012). Venomous snakes are distinct among venomous predatory organisms for

a number of reasons, one of which being that their venoms are highly optimized for prey acquisition (Barlow et al., 2009, Mackessy and Saviola, 2016; Modahl et al., 2018) and only secondarily serve as defensive compounds (Ward-Smith et al., 2020). Ecologically, venomous snakes are also unique as predators, as they are one of only a few vertebrates specialized to consume whole prey, with very rare examples of snakes that have managed to escape this constraint (Jayne et al., 2002). Consequently, snakes are gape-limited predators, meaning that effects of their predation pressures are restricted to species small enough to be consumed whole.

As a result of strong predation pressures exerted on a community by snakes, and more specifically by their venoms, various organisms possess evolved mechanisms to counteract the function of venoms. Prey species have been under intense selective pressures associated with venoms over much of their evolutionary history, and as a result, resistance is widespread among them. Varying levels of resistance to rattlesnake venoms have evolved in tree squirrels (*Sciurus spp.*; Pomento et al., 2016), small terrestrial rodents (Perez et al., 1979; Dewit, 1982), and various lizards and amphibians (Smiley-Walters et al., 2018). This wide diversity of taxa indicates that selection strongly favors the development of venom resistance mechanisms in a variety of snake prey types. Beyond prey species, venom resistance is also present in a number of animals that prey upon venomous snakes. Given that snake predators increase their likelihood of envenomation when attempting to subdue a venomous snake, it is logical that selective pressure favoring the development of resistance exists in this context as well. Once again, resistance is widespread across predatory taxa and is present in mongoose (Herpestidae; Bdolah et al., 1997), opossums (Didelphidae; Werner and Vick, 1977) and the Honey Badger (*Mellivora capensis*; Drabeck et al., 2015). This prevalence of resistance in predators suggests that the strength of

selection associated with predation on snakes is certainly strong enough to create the evolution of intrinsic defenses.

With the occurrence of intrinsic resistance, especially in prey species, comes the potential for co-evolution, as predator venom and prey resistance continue to evolve in tandem with one another. This back-and-forth between predator and prey creates an evolutionary arms-race, aligning with the ideas put forth in the Red Queen Hypothesis (Van Valen, 1973). Evolutionary arms races, as described by Van Valen's hypothesis, are present in an array of biological systems. These arms races can play out in parasite-host dynamics, as is evident in the coevolutionary arms race between Water Fleas (*Daphnia spp.*) and their *Caullerya* parasites (Turko et al., 2018). Coevolutionary arms races are just as prevalent in a predator-prey context, with toxic newts (*Taricha granulosa*) and toxin-resistant garter snakes (*Thamnophis sirtalis*) coevolving with respect to one another representing a well-documented example (Brodie et al., 2002).

In a rodent and venomous snake pairing, a very prominent arms race dynamic is possible, one in which venom resistance and venom potency are pitted against one another. As an example, the Northern Pacific Rattlesnake (*Crotalus o. oreganus*) and California Ground Squirrel (*Otospermophilus beecheyi*) form a predator-prey pairing where venom co-evolves with venom-resistance (Coss et al., 1993). Considerable efforts have been undertaken to understand the arms-race in this particular system, and investigation has determined that in some populations, snakes appear to be "winning" the respective arms race with their co-occurring squirrel population (Holding et al., 2016). The evolutionary arms race truly is a dynamic system, thus when studying venom resistance in presently existing populations of animals, one has only a current "snapshot" of a continuously changing interaction. With additional evolutionary

innovations in resistance mechanisms through time, squirrels could overtake their rattlesnake predators in this arms race, followed by compensatory adjustments by the predator.

The rodent-snake evolutionary arms race is present in various taxa in an array of locations. This race occurs in the aforementioned California Ground Squirrel and Pacific Rattlesnake (Holding et al., 2016), the Southern Plains Woodrat (*Neotoma micropus*) and Western Diamondback Rattlesnake (*Crotalus atrox*) (Perez et al., 1978), and the Prairie Vole (*Microtus ochrogaster*) and Copperhead (*Agkistrodon contortrix*) (de Wit, 1982), to list only a few examples. The prevalence of this coevolutionary dynamic across landscapes and taxa suggests that it is a central theme involving venomous snake predation on rodents. Consequently, a central hypothesis concerning this dynamic is that resistance is present in rodent species that frequently fall prey to co-occurring venomous snakes. This trend isn't always upheld though, and surprisingly, rodents that appear to be under immense selection pressure from snake venoms may not have any evolved intrinsic resistances. Such is the case for the Cape Ground Squirrel (*Xerus inauris*), a small rodent fed upon by both sympatric Puff Adders (*Bitis arietans*) and Snouted Cobras (*Naja annulifera*). While it would be seemingly beneficial to possess venom resistance to either of these snakes, the squirrel's serum fails to inhibit the proteolytic activity of either snake's venom (Phillips et al., 2012). The lack of resistance present in this squirrel indicates that variability in resistance capabilities are present across systems. Further, the development of resistance to venoms is likely more complex than simply being under predation pressure from venomous snakes.

On the plains of Eastern Colorado, an ecosystem is present in which an array of grassland rodents interacts with two predatory rattlesnakes. The larger and more widespread Prairie Rattlesnake (*Crotalus viridis*) is present throughout much of the state, spanning the entirety of

Colorado's longitudinal breadth (Hammerson, 1999). A second species, the diminutive Desert Massasauga Rattlesnake (*Sistrurus tergeminus edwardsii*) is present in the southeastern corner of the state (Hobert et al., 2004; Wastell and Mackessy, 2016). Together, these two rattlesnakes exert pressures on their respective rodent communities as a result of their trophic interactions, but differences in feeding habits between the two species may differentially influence rodent defenses, and subsequently venom resistance. This system serves as an ideal model to study venom resistance at the community level in a multi-predator and multi-prey context.

### **House Mouse**

The House Mouse (*Mus musculus*) is a small murid rodent that traces its origins to Eurasia (Suzuki et al., 2013). The natural history and dispersal of House Mice has long been entangled with that of humans, as the House Mouse has an affinity for human-made developments. House Mice arrived in North America during the second half of the seventeenth century and rapidly dispersed throughout the continent (Tichy et al., 1994). While generally associating with human settlements, House Mice can be found naturalized throughout North America, and in particular they may contribute to rodent assemblages in modified grassland habitats. Because of their small size and superficial similarity to native North American rodents, House Mice are readily consumed by predatory animals. Predators may even incorporate introduced mice as a significant portion of their diet in areas where they are locally abundant (Teta et al., 2012), and this human-tolerant prey species may facilitate the proliferation of predators in more developed environments. Snakes frequently consume House Mice as a prey item, particularly in anthropogenic landscapes, and as such the interactions between these trophic partners warrants additional study. Additionally, the prevalence of laboratory strain House Mice as a study organism allows for comparison of wild House Mice, which may have undergone

evolutionary diversification (and possible venom resistance) to an inbred, evolutionarily naïve rodent of the same species.

### **Deer Mouse**

As one of the most abundant small rodents present at the field sites, Deer Mice (*Peromyscus maniculatus*) are likely an exceptionally important prey source for both the Prairie Rattlesnake and Desert Massasauga in regions where they are present. This has been reflected in studies analyzing the diets of both snake species (Holycross and Mackessy, 2002; Balchan et al., unpub. data; see Chapter 2), and as a result, Deer Mice may be under selective pressures from their snake predators. Deer Mice may also be a large dietary component of other predators, including various carnivorous mammals (Orrock and Fletcher, 2014) and birds (Willey, 2013; Zimmerman et al., 1996). Consequently, Deer Mice likely incur strong selective pressure favoring the evolution and maintenance of resistance to their venomous snake predators, in addition to pressures exerted by an array of other non-venomous predators throughout their environment. This cumulative pressure exerted upon these mice is clearly strong enough to facilitate adaptation, with behavioral adaptation to minimize predation risk being well characterized (Clarke et al., 1996; Connolly and Orrock, 2018).

In response to predators that subdue prey via physical means (i.e. avian and mammalian predators), Deer Mice have the potential to respond in a variety of ways. As an anti-predator response to owls, mice suppress movements during full moon-lit nights (Clarke, 1983). As a response to both mammalian and avian predation, mice may alter their activity timing to reduce predation risks dependent on the structure of their environment (Connolly and Orrock, 2018). Given the direct consequences to individual fitness associated with predation, some behavioral adaptations may be particularly long-lasting in a population, remaining present even after the

extirpation of the predator that caused the adaptive change (Orrock, 2010). With this foundational understanding of Deer Mice, and their various responses to predation, studying responses to venomous snake predation may provide unique insight into an otherwise unexplored aspect of the mouse's ecology.

### **Northern Grasshopper Mouse**

The Northern Grasshopper Mouse (*Onychomys leucogaster*) is a small cricetid rodent broadly distributed across the Great Plain and Great Basin regions. Grasshopper mice are unique among rodents in that they are obligate carnivores (Rowe and Rowe, 2015), and they frequently prey upon invertebrates and small vertebrates. Grasshopper mice even have the capacity to prey on relatively large and well-defended vertebrates such as horned lizards, further underscoring their capabilities as predatory animals (Sherbrooke, 1991). Consequently, grasshopper mice possess a far stronger bite force than similarly sized rodents (Williams et al., 2009) and exhibit behavioral adaptations that reflect a predatory lifestyle (Langley, 1994), which makes them both formidable predators and challenging prey items to subdue. Considering this, grasshopper mice do fall prey to an array of organisms in their ecosystem, including mammals, birds and reptiles, including both species of rattlesnake within this study (Holycross and Mackessy, 2002; Balchan et al., unpub. data). While Grasshopper Mice do serve as prey items within their respective food webs, they can certainly be considered well-defended when compared to other rodents, both in terms of their morphological traits and in their potential to resist venoms.

### **Ord's Kangaroo Rat**

Another abundant species across the Great Plains, the Ord's Kangaroo Rat (*Dipodomys ordii*) is a heteromyid rodent that exists in abundance throughout grassland ecosystems of eastern Colorado. As with many rodents, the kangaroo rat is nocturnally active (White and Geluso,

2007) and across its range is preyed upon by an array of co-occurring rattlesnakes (Whitford et al., 2017). Rat-rattlesnake interactions have been the subject of intense study (Freymler et al., 2019), and it is presumed that rattlesnake predation exerts great selective pressure on kangaroo rats. Kangaroo rats exhibit several behavioral and physiological responses to predatory snakes, such as potentially modifying body temperatures to confuse snake thermoreception (Schraft and Clark, 2017), performing antipredator displays to intimidate snakes (Whitford et al., 2019), and maintaining alert behavioral states following recent snake predation attempts (Freymler et al., 2017). Consequently, rattlesnakes must be rapid in their attempts to incapacitate kangaroo rats, and they may frequently fail due to lack of accuracy associated with rapid strikes or as a result of kangaroo rat escape maneuvers (Higham et al., 2017).

Of the two eastern Colorado rattlesnakes, Prairie Rattlesnakes frequently incorporate Ord's Kangaroo Rats in their diet (Balchan et al., unpub. data; Chapter 2). However, the smaller Desert Massasauga can not prey on adult kangaroo rats, as these rodents are far too large to be consumed given the gape limitations of rattlesnakes (Holycross and Mackessy, 2002). Because of this extreme difference in predation frequency between the two eastern Colorado rattlesnakes, strong predation pressure is likely exerted upon Ord's Kangaroo Rats by Prairie Rattlesnakes, while predation pressure is essentially nonexistent from Desert Massasaugas. Consequently, we expect the Ord's Kangaroo Rat to exhibit defenses toward the Prairie Rattlesnake but lack defenses toward the Desert Massasauga Rattlesnake.

### **Other Rodents Present in Colorado Grassland Communities**

The Eastern Woodrat (*Neotoma floridana*) is a large cricetid rodent broadly distributed throughout grasslands, woodlands, and marshes in eastern and central North America. Across this range, the woodrat is consumed by co-occurring rattlesnakes (Clark, 2002), and its large size

may make it particularly valuable as a prey item. As a result, woodrats may be under predation selection pressures from large rattlesnakes, with some species or populations being preyed upon heavily (Dugan and Hayes, 2012). Consequently, selective pressures may exist for woodrats to evolve resistance mechanisms to rattlesnake venoms (i.e., Perez et al., 1978; de Wit, 1982).

The Plains Pocket Mouse (*Perognathus flavescens*) is a minute heteromyid rodent distributed across the North American Great Plains. With adult body mass ranging from 6.9 to 11.5 grams (Hibbard and Beer, 1960), the pocket mouse represents one of the smallest rodents present in Colorado. This small adult body size makes it the ideal prey item for snakes whose gape limitations prohibit them from consuming larger species. Consequently, the Plains Pocket Mouse represents an ideal prey item for the Desert Massasauga Rattlesnake, and this is verified by the prevalence of this mouse in dietary analyses (Holycross and Mackessy, 2002). Conversely, this small adult body mass means that the Plains Pocket Mouse is not a particularly substantial meal for larger rattlesnakes. Thus, we do not expect this mouse to comprise a significant dietary component for adult Prairie Rattlesnakes, though it may be more frequently preyed upon by juvenile Prairie Rattlesnakes. The varied ecologies and trophic interactions associated with the rodents present throughout eastern Colorado allow for a community level view of resistance patterns present in the ecosystem.

This study analyzes patterns of venom resistance in two grassland communities in eastern Colorado using the Prairie Rattlesnake (*Crotalus viridis*), Desert Massasauga Rattlesnake (*Sistrurus tergeminus edwardsii*) and a suite of co-occurring rodents. We will explore the roles of predation pressures and geography on the strength of resistance.

H1     Rodent venom resistance to a particular species of rattlesnake should correspond with presumed predation pressure exerted upon it by that species.

P1     All rodents will display resistance to venom of the Prairie Rattlesnake.

- P2 Only smaller species of rodents will display strong resistance to venom of the Desert Massasauga Rattlesnake.
- H2 Strength of resistance should be impacted by geography, specifically sympatry and/or allopatry of populations
  - P1 Allopatric rodent/snake pairings should generate reduced venom resistance potential.
  - P2 Sympatric rodent/snake pairings should generate greater increased venom resistance potential.

## **Methods**

### **Study Design and Sample Collection**

Sample collection occurred at two field sites on privately owned land in Colorado: a site in Weld Co. (northern field site) and a site in Lincoln Co. (southern field site, approx. 100 miles due south; Figure 3.1). Both field sites are characterized as being native shortgrass and mixed grass prairie habitat, with variable (although generally minimal) levels of cattle grazing pressure. A study design incorporating two field sites was used to explore the effect of allopatry on the presence and strength of resistance among interacting species.

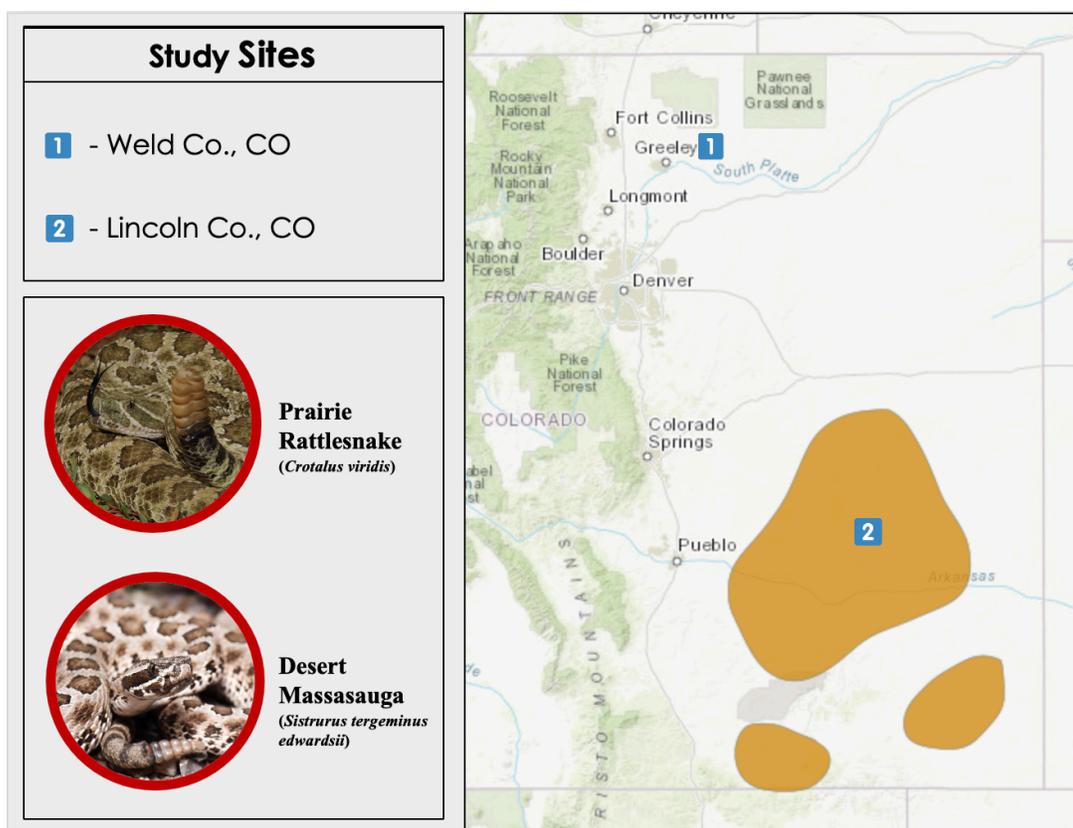


Figure 3.1. Map of study sites and select present species, indicating 1) Weld Co. and 2) Lincoln Co. field sites. Both *Crotalus viridis* (photo: David Nixon) and *Sistrurus tergeminus edwardsii* (photo: Tyler Carlson) are present at the Lincoln Co. field site, but only *Crotalus viridis* is present at the Weld Co. field site. Orange overlay represents the geographic distribution of *Sistrurus tergeminus edwardsii* in Colorado (based on Hammerson, 1999).

Two species of venomous snakes are present at the field sites. The northern field site is inhabited only by the larger Prairie Rattlesnake, while the southern field site is inhabited by both the Prairie Rattlesnake and the more diminutive Desert Massasauga Rattlesnake. Venoms were collected from both of these species at the respective sites via collection of animals at den sites, opportunistic collection of day-active snakes, and driving roads during evening and night for active snakes. Venom was manually extracted from snakes, centrifuged at 9.5k x g to pellet

cellular debris and frozen at -80 °C. Following freezing, samples were lyophilized and stored at -20 °C for later use.

The following species of rodents were trapped at field sites: Deer Mouse (*Peromyscus maniculatus*), House Mouse (*Mus musculus*), Northern Grasshopper Mouse (*Onychomys leucogaster*), Ord's Kangaroo Rat (*Dipodomys ordii*), Eastern Woodrat (*Neotoma floridana*), Plains Pocket Mouse (*Perognathus flavescens*), and Meadow Vole (*Microtus pennsylvanicus*) under permits from Colorado Parks and Wildlife (#19TR3327, issued to SPM). Rodents were trapped using Sherman live animal traps (H. B. Sherman Traps, Inc., Tallahassee, USA) baited with birdseed. Traps were set in the field in the evening and retrieved the following morning. Live rodents were transported back to the laboratory for use in assays (IACUC protocol 1905D-SM-SBirdsLM-22, to SPM). Rodents were housed in lab caging on Carefresh bedding, and were supplied with lab chow diet, bird seed and fresh water *ad libitum*.

### **Median Lethal Dose (LD<sub>50</sub>) Assays**

Median lethal dose (LD<sub>50</sub>) assays were used to assess toxicity of a venom to a population of rodents. For these assays, lyophilized venoms from three individuals were reconstituted at a concentration of 10 µg/µl into MilliQ ultra-pure water. Venom samples were selected and pooled from three individual snakes per population to account for minor individual variation in venom composition. This pooled venom solution at a concentration of 10 µg/µl was further diluted into 0.9% saline to reach desired injection doses. Rodents were initially injected at low and high doses (~1.0 µg/g and ~5.0 µg/g) of venom to establish general resistance potential. Following this initial approximation, doses were chosen at a range of concentrations, and the median lethal dose was extrapolated from the generated mortality curve. Each dosage group consisted of three individual rodents of mixed sexes, to account for any differences in toxicity between sexes.

Rodents were injected intraperitoneally with a standardized bolus dependent on species in their lower right quadrant, replaced in their caging, and mortality was recorded at 24 hours post injection. Saline controls were used for all assays. All rodent experiments were approved by the UNC-IACUC (protocol 1905D-SM-SBirdsLM-22, to SPM).

### **Metalloproteinase Inhibition Assays**

Metalloproteinase assays were used to determine the inhibitory potential of a rodent serum against rattlesnake venom metalloproteinases. Rodents were humanely euthanized by cervical dislocation and exsanguinated immediately after via bleeding from their ventricles, orbital sinus or jugular vein. Blood was collected in 1.5 ml Eppendorf tubes held on ice during collection. Following collection, whole blood was spun at 8.0k x g for 10 minutes at 4 °C in an Eppendorf refrigerated centrifuge for serum separation. Serum was then separated from whole blood using a micropipette and frozen at -80 °C.

Lyophilized snake venoms of each study population (three individuals per pooled venom) were solubilized in MilliQ ultra-pure water at a concentration of 4.0 µg/µl. Serum was collected from rodents of each available species, with tests being done on sera of three different individuals when available. Metalloproteinase assays were conducted following Aird and da Silva (1991), with additional assay controls to account for the addition of serum at 5 µl and 10 µl per assay. Briefly, assays were conducted in disposable glass culture tubes. A combination of 245 µl buffer (50 mM HEPES, 100 mM NaCl, pH 8.0) and varying amounts of venom and serum depending on trial were incubated together at room temperature (approximately 20°C) for 30 minutes. Tubes were then placed in an ice bath for 5 minutes, after which 250 µl of substrate solution (azocasein; Sigma, in buffer, 2.0 mg/ml buffer) was added to each. Tubes were incubated for a further 30 minutes at 37 °C. Following this incubation, tubes were centrifuged at

2,000 rpm, and 125  $\mu$ l of supernatant was drawn up from each. Supernatant was transferred to a well plate, triturated with 125  $\mu$ L of 0.5 M NaOH, and allowed to sit at room temperature for approximately five minutes. Absorbance readings were taken in a plate reader at 450 nm.

### **Affinity Chromatography**

Isolation of resistance-conferring serum proteins was attempted using affinity chromatography (i.e., Gutiérrez et al., 2009). A column containing Sepharose 4B CNBr-activated matrix was saturated with crude rattlesnake venom from one of the prior described populations. Once bound to the matrix and excess venom was eluted from the column, rodent serum was introduced to the column and allowed to bind to the venom matrix. Unbound serum components were then eluted from the column using wash buffer, after which the matrix was purged with an elution buffer to free bound serum proteins. The resulting eluted material, presumably containing proteins with affinity for venom molecules, was run on an SDS-PAGE gel for detection of bands that may correspond to venom resistance proteins.

## **Results**

### **Metalloproteinase Assays**

The greatest protein degradation potential is seen in the venom of the Lincoln County Desert Massasauga population (0.872  $\Delta A_{342\text{nm}}$ /minute/mg; Table 3.1), somewhat lower SVMP activity in the Lincoln County Prairie Rattlesnake venom (0.752  $\Delta A_{342\text{nm}}$ /minute/mg; Table 3.1), and greatly reduced activity in the Weld County Prairie Rattlesnake venom (0.495  $\Delta A_{342\text{nm}}$ /minute/mg; Table 3.1). All venoms tested exhibit considerable metalloproteinase activity, and thus metalloproteinases are an important component in these venoms to facilitate prey incapacitation.

Table 3.1. Snake venom metalloproteinase activities of three rattlesnake populations. Each population is represented by a pooled venom sample from three adult individuals from the same field site.

<b>Rattlesnake Population</b>	<b>Metalloproteinase Activity (<math>\Delta A_{342\text{nm}}</math>/minute/mg venom protein)</b>
<i>Crotalus viridis</i> (Weld Co.)	0.495
<i>Crotalus viridis</i> (Lincoln Co.)	0.752
<i>Sistrurus tergeminus edwardsii</i> (Lincoln Co.)	0.872

Table 3.2. Contingency table of inhibitory effect (percent inhibition) of rodent serum (mean  $\pm$  standard deviation) against snake venom metalloproteinase activity of three rattlesnake venoms.

	Lincoln County <i>S. t. edwardsii</i>	Lincoln County <i>C. viridis</i>	Weld County <i>C. viridis</i>
Weld County <i>P. maniculatus</i>	2.4% $\pm$ 2.04 n = 3	5.2% $\pm$ 2.22 n = 3	12.1% $\pm$ 8.22 n = 3
Lincoln County <i>P. maniculatus</i>	25.0% $\pm$ 16.01 n = 3	10.3% $\pm$ 3.95 n = 3	12.5% $\pm$ 5.39 n = 3
Weld County <i>O. leucogaster</i>	41.8% $\pm$ 24.45 n = 3	76.2% $\pm$ 1.44 n = 3	46.1% $\pm$ 9.96 n = 3
Lincoln County <i>O. leucogaster</i>	39.4% $\pm$ 15.21 n = 3	25.8% $\pm$ 18.96 n = 3	43.8% $\pm$ 16.01 n = 3
Weld County <i>D. ordii</i>	8.7% $\pm$ 6.46 n = 3	29.2% $\pm$ 4.25 n = 3	15.3% $\pm$ 0.31 n = 3
Lincoln County <i>D. ordii</i>	5.6% $\pm$ 5.14 n = 3	27.9% $\pm$ 8.61 n = 3	22.6% $\pm$ 8.11 n = 3
Lincoln County <i>Neotoma floridana</i>	8.4% n = 1	56.6% n = 1	25.2% n = 1
Weld County <i>Perognathus flavescens</i>	16.3% n = 1	Undetermined	Undetermined
Lincoln County <i>Perognathus flavescens</i>	14.7% n = 1	Undetermined	Undetermined

Inhibition of snake venom metalloproteinases (SVMPs) is highly variable across rodent species and populations, and some amount of variability is present even within populations of rodents. In general, Deer Mice (*Peromyscus maniculatus*) display weak serum inhibition of SVMPs when compared to other rodents tested. Inhibition of Prairie Rattlesnake SVMPs appears relatively consistent across Deer Mouse and snake population pairings, except when Weld County mice are challenged with the venom of Lincoln County rattlesnakes. Deer Mice

apparently exhibit strong local adaptation to the presence of Desert Massasauga, as co-occurring mice are over ten times as resistant to this venom as allopatric mice.

Northern Grasshopper Mice (*Onychomys leucogaster*) exhibit very strong inhibition of SVMPs across species and populations. Most notably, mice from Weld County were nearly twice as resistance to the venom of the Lincoln County Prairie Rattlesnake than they were to the venom of this snake from Weld County. Interestingly, reduced inhibition was seen with co-occurring snake-mouse pairings. It does not appear that differential resistance is present between populations to Desert Massasauga venom.

*Dipodomys ordii* also display considerable resistance to Prairie Rattlesnake SVMPs, with the pairing of Weld County rats to Weld County rattlesnakes showing reduced resistance. Neither kangaroo rat population exhibits particularly strong inhibitory effect against Desert Massasauga SVMP activity.

*Neotoma floridana* display the greatest metalloproteinase inhibition to their co-occurring Prairie Rattlesnake, aligning with hypothesized increased predation pressure being exerted by this rattlesnake. Rats are weakly inhibitory to Desert Massasauga venom, a species which presumably exerts minimal pressure on them. Finally, Plains Pocket Mice (*Perognathus flavescens*) from the two field sites do not appear to display differential resistance to Desert Massasauga SVMPs, indicating that they may not be locally adapted to predation from massasaugas.

### **Median Lethal Dose (LD<sub>50</sub>) Assays**

Dose-response curves generated for NSA strain laboratory mice using Weld County Prairie Rattlesnake and Lincoln County Prairie Rattlesnake venoms revealed a large difference in

toxicity of these venoms. Median lethal dose values were 1.3 mg/kg against Weld County venom (n = 15) and 2.4 mg/kg against Lincoln County venom (n = 15).

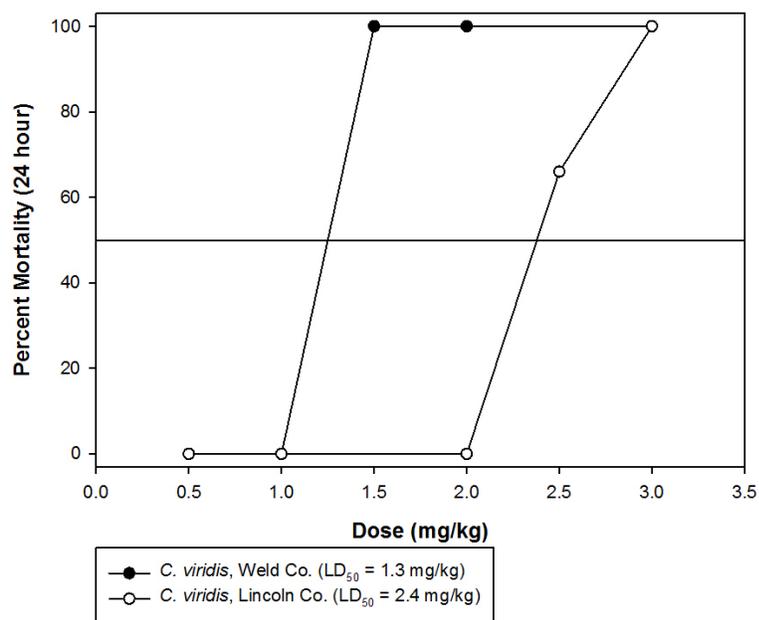


Figure 3.2. Dose-response curves for venoms from two populations of Prairie Rattlesnake venoms against NSA *Mus musculus*.

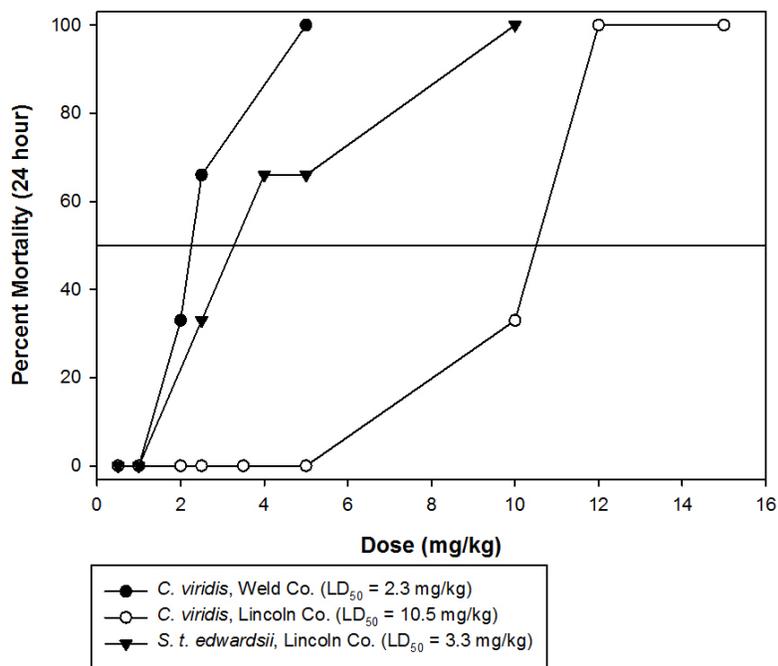


Figure 3.3. Dose-response curves for venoms from three populations of rattlesnakes against Weld County *Peromyscus maniculatus*.

Dose-response curves generated for Weld County Deer Mice using Weld County Prairie Rattlesnake, Lincoln County Prairie Rattlesnake, and Lincoln County Desert Massasauga Rattlesnake venoms. Median lethal dose values were 2.3 mg/kg against Weld County Prairie Rattlesnake venom (n = 15), 10.5 mg/kg against Lincoln County Prairie Rattlesnake venom (n = 27) and 2.4 mg/kg against Lincoln County Desert Massasauga Rattlesnake venom (n = 18).

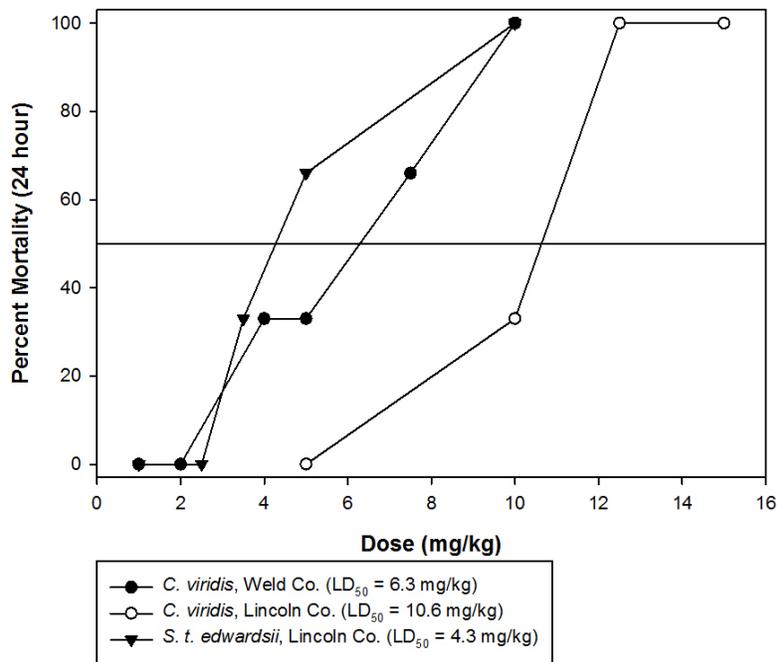


Figure 3.4. Dose-response curves for venoms from three populations of rattlesnakes against Lincoln County *Peromyscus maniculatus*.

Dose-response curves were generated for Lincoln County Deer Mice using Weld County Prairie Rattlesnake, Lincoln County Prairie Rattlesnake, and Lincoln County Desert Massasauga Rattlesnake venoms. Median lethal dose values were 6.3 mg/kg against Weld County Prairie Rattlesnake venom (n = 18) , 10.6 mg/kg against Lincoln County Prairie Rattlesnake venom (n = 12) and 4.3 mg/kg against Lincoln County Desert Massasauga Rattlesnake venom (n = 18).

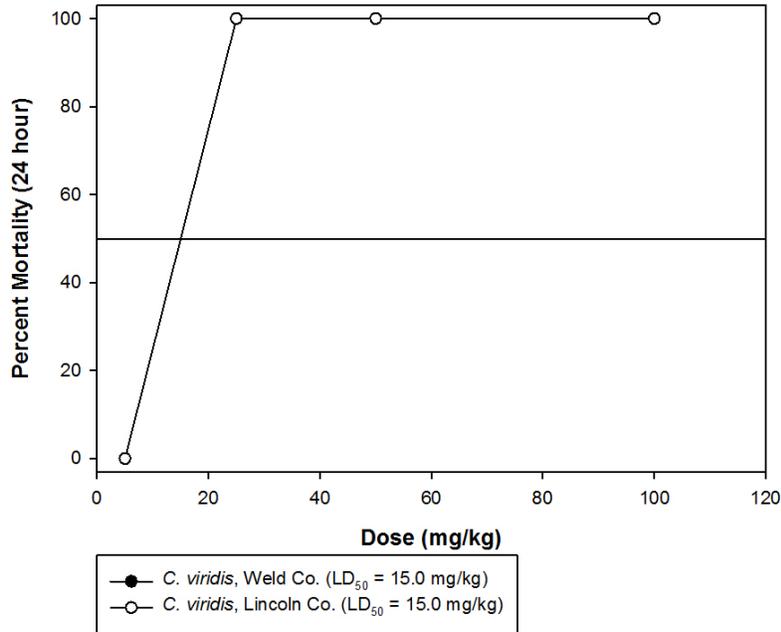


Figure 3.5. Dose-response curves for Weld County *Dipodomys ordii* against two populations of rattlesnake venoms. Both curves are depicted here but overlay each other completely.

Dose-response curves were generated for Weld County Ord's Kangaroo Rats using Weld County Prairie Rattlesnake and Lincoln County Prairie Rattlesnake venoms. Median lethal dose values were 15.0 mg/kg against Weld County Prairie Rattlesnake venom ( $n = 12$ ) and 15.0 mg/kg against Lincoln County Prairie Rattlesnake venom ( $n = 12$ ).

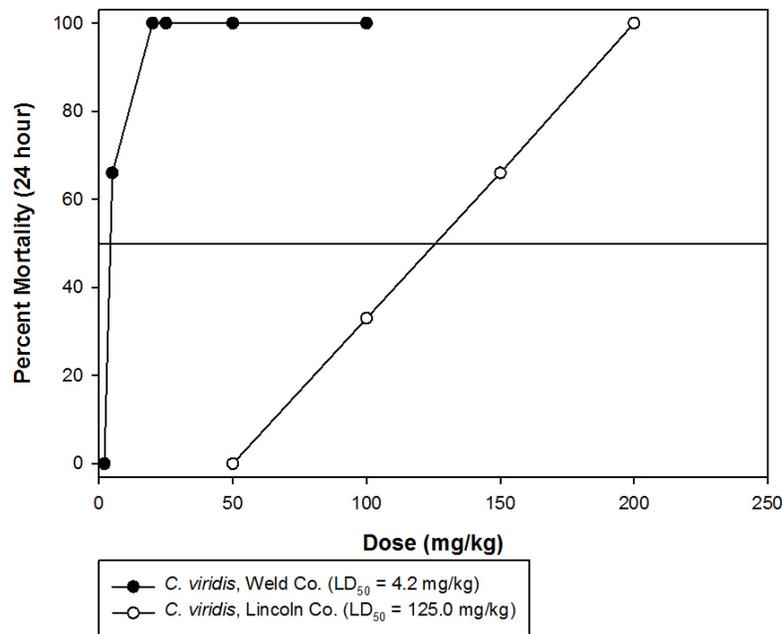


Figure 3.6. Dose-response curves for venoms from two populations of rattlesnakes against Lincoln County *Dipodomys ordii*.

Dose-response curves were generated for Lincoln County Ord's Kangaroo Rats using Weld County Prairie Rattlesnake and Lincoln County Prairie Rattlesnake venoms. Median lethal dose values were 4.2 mg/kg against Weld County Prairie Rattlesnake venom (n = 18) and 125.0 mg/kg against Lincoln County Prairie Rattlesnake venom (n = 12).

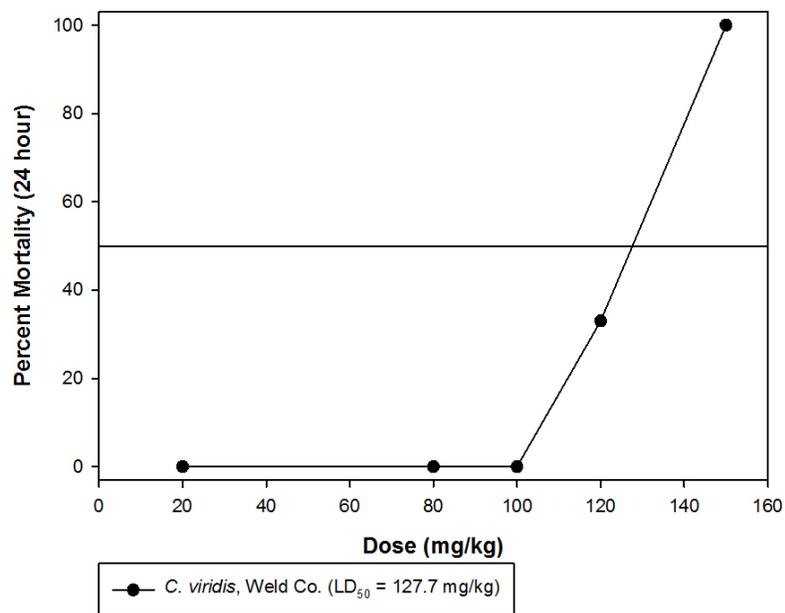


Figure 3.7. Dose-response curve for Weld Co. Prairie Rattlesnake venom against Weld County *Onychomys leucogaster*.

Dose-response curves were generated for Weld County Northern Grasshopper Mice using Weld County Prairie Rattlesnake venom. Median lethal dose value was 127.7 mg/kg against Weld County Prairie Rattlesnake venom (n = 15).

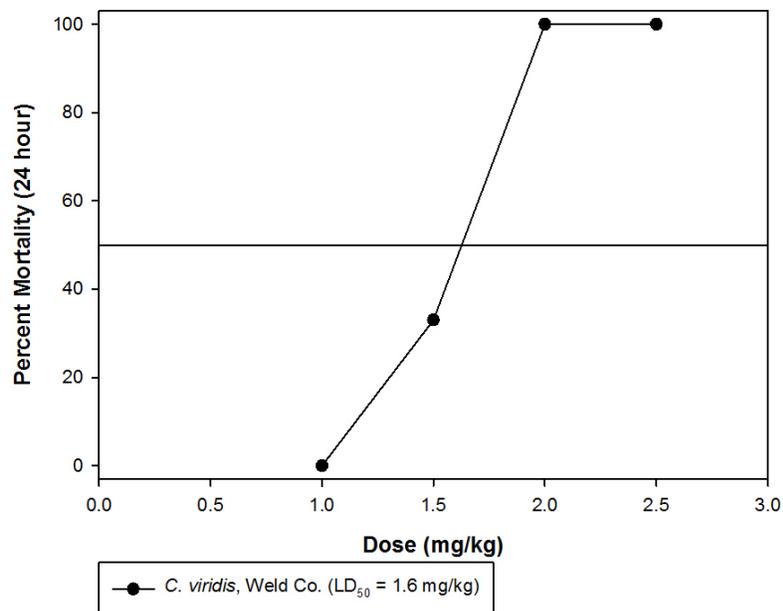


Figure 3.8. Dose-response curve for Weld Co. Prairie Rattlesnake venom against Weld County *Mus musculus*.

Dose-response curves were generated for Weld County House Mice using Weld County Prairie Rattlesnake venom. Median lethal dose value was 1.6 mg/kg against Weld County Prairie Rattlesnake venom (n = 12).

Table 3.3. Contingency table of intraperitoneal median lethal dose (IP LD<sub>50</sub>) of rattlesnake venoms against tested rodent populations.

	Lincoln County <i>S. t. edwardsii</i>	Lincoln County <i>C. viridis</i>	Weld County <i>C. viridis</i>
Weld County <i>P. maniculatus</i>	3.3 mg/kg n = 18	10.5 mg/kg n = 27	2.3 mg/kg n = 15
Lincoln County <i>P. maniculatus</i>	4.3 mg/kg n = 12	10.6 mg/kg n = 12	6.3 mg/kg n = 18
Lincoln County <i>O. leucogaster</i>	Undetermined	Undetermined	127.7 mg/kg n = 15
Weld County <i>D. ordii</i>	Undetermined	15.0 mg/kg n = 12	15.0 mg/kg n = 12
Lincoln County <i>D. ordii</i>	Undetermined	125.0 mg/kg n = 12	4.2 mg/kg n = 18
Weld County <i>M. musculus</i>	Undetermined	Undetermined	1.6 mg/kg n = 12
NSA Strain (inbred) <i>M. musculus</i>	0.60 mg/kg*	2.4 mg/kg n = 15	1.3 mg/kg n = 15

\* From Gibbs and Mackessy 2009.

### Affinity Chromatography

SDS-PAGE gel electrophoresis analysis of serum fractions collected from the affinity chromatography apparatus indicates protein bands in *Onychomys leucogaster*, *Dipodomys ordii*, *Neotoma floridana*, and *Peromyscus maniculatus* (Figure 3.9) that may represent proteins that bound to the venom saturated gel matrix. These matrix-bound proteins may represent proteins that confer resistance to the organism by binding circulating venom molecules in the animal or could be serum proteins that otherwise bind venom molecules without conferring great resistance in the organism (ex. serum albumin, McCabe et al.; unpublished data). Additional mass

spectroscopy characterization of gel bands is necessary to identify proteins isolated through affinity chromatography to confirm the presence of these as resistance proteins.

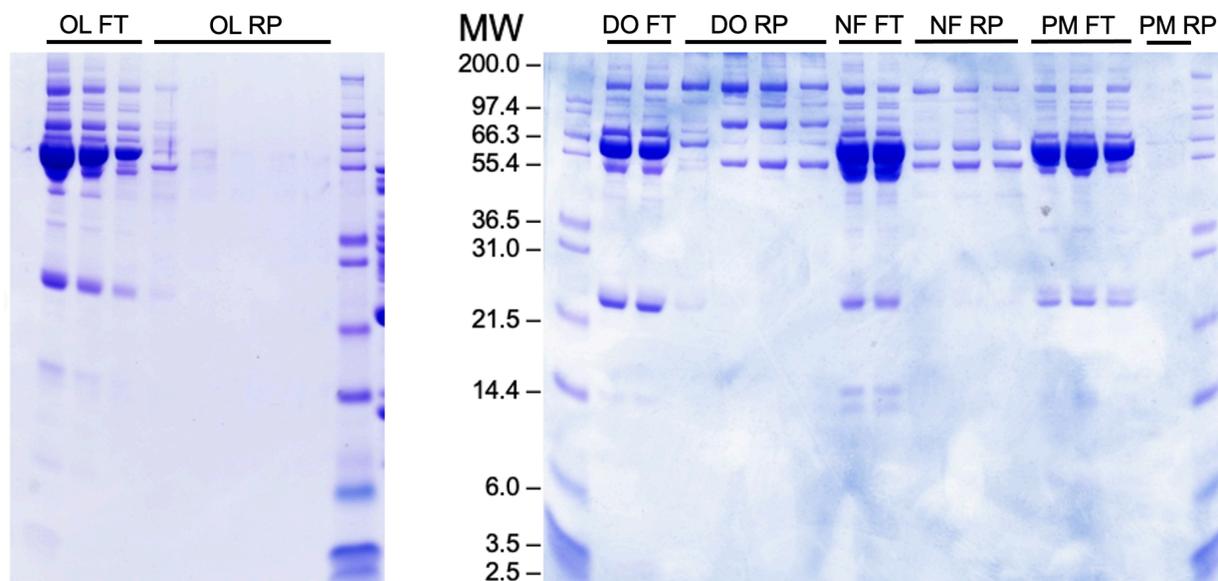


Figure 3.9. SDS-PAGE gels (A) with *Onychomys leucogaster* (OL) serum fractions and (B) *Dipodomys ordii* (DO), *Neotoma floridana* (NF), and *Peromyscus maniculatus* (PM) serum fractions. MW, molecular weight standards. FT, flow-through serum at beginning of elution containing material unbound to matrix. RP, retained serum proteins eluted from affinity chromatography matrix.

## Discussion

### Rattlesnake Venoms

Snake venoms have the potential to vary immensely among taxa (Modahl et al., 2020), across the range of a single species (Strickland et al., 2018), or even within a single individual throughout its lifetime (Mackessy, 1988; Saviola et al., 2015). The three populations of rattlesnake venoms used in this study (Weld Co. Prairie Rattlesnake, Lincoln Co. Prairie Rattlesnake, and Lincoln Co. Desert Massasauga) vary considerably from each other in composition and in toxicity. Snake venom metalloproteases are degradative enzymatic

compound found in rattlesnake venoms that functions to break down proteins, potentially facilitating digestion following consumption of prey (Mackessy, 1988, 2010). A venom can be classified as more or less degradative based on the activity of its SVMPs on a protein substrate. The greatest protein degradation potential was seen in the venom of the Lincoln County Desert Massasauga, followed by the Lincoln County Prairie Rattlesnake, and finally the Weld County Prairie Rattlesnake, with considerably lower degradation than either Lincoln Co. snakes (Table 3.1). These results indicate that both the Lincoln County Desert Massasauga and Prairie Rattlesnake possess highly degradative venoms compared to the Weld County Prairie Rattlesnake, which likely result in increased tissue destruction and hemorrhage in envenomed prey.

Conversely, crude toxicity of a venom may be a more relevant metric when evaluating venom resistance in a predator-prey context, as a venom that can effectively immobilize or incapacitate a prey item should be favored when prey is released following envenomation, a feeding strategy typical of many vipers (Saviola et al., 2013). The median lethal dose in laboratory mice for venom from the Lincoln County Desert Massasauga population was previously determined to be 0.60 mg/kg (Gibbs and Mackessy, 2009). The current study conducted median lethal dose assays for both Prairie Rattlesnake populations and found median lethal dose values of 1.3 mg/kg for Weld County snakes, and 2.4 mg/kg for Lincoln County snakes (Figure 3.2). From a crude venom lethality standpoint, the Lincoln County Desert Massasauga clearly possesses the most toxic venom against this inbred murine model, followed by the Weld County Prairie Rattlesnake and then Lincoln County Prairie Rattlesnake. As these three LD<sub>50</sub> values were generated with naïve laboratory rodents (rodents not exposed to snake predation pressures in recent evolutionary time), they can be used in a comparative context

across snake populations to assess basic differences in venom toxicity. Differences in crude toxicity result in compositional differences across the entire venom proteome, and various toxins may be optimized to incapacitate prey in different ways. However, lab mouse models provide an approximation only, and native species can show greater or lesser sensitivity to specific venoms, in part due to coevolutionary dynamics over evolutionary time (e.g., Mackessy, 1988; Smiley-Walters et al., 2018).

### **House Mouse**

House Mice are comparable in size to many native Colorado rodents, and are likely to be consumed by both species of rattlesnakes at the study sites. While House Mice are present at both the Weld and Lincoln County sites, they were only trapped in Weld County during this study. An LD<sub>50</sub> assay was conducted with these Weld County mice, using Weld County Prairie Rattlesnake venom, and the median lethal dose was determined to be 1.6 mg/kg (Figure 3.8). This value is quite similar to the median lethal dose for evolutionarily naïve laboratory mice when challenged with this same venom (1.3 mg/kg; Figure 3.2), suggesting that feral House Mice may not possess any resistance to rattlesnake venoms. The reasons behind this remain unclear, but lack of resistance could simply reflect a lack of evolutionary time for this adaptation to evolve. In addition, House Mice incur an array of predation pressures from a broad range of predators, all of which may hinder the development of venom resistance due to diffused sources of pressure. Finally, it is possible that House Mice are under constraints that prevent them from readily developing resistance mechanisms, as other rodents are present that have apparently failed to develop resistance to snake venoms (Phillips et al., 2012). Median lethal doses determined here serves only as a starting point for understanding the biochemical ecology of

rattlesnakes and House Mice in this study system, especially considering that the interactions between these partners are still evolutionarily young.

House Mice serve as important dietary components for snakes, particularly those species that can be found on anthropogenic landscapes (Slip and Shine, 1988). In fact, the majority of the diet of some snakes may consist of introduced House Mice (Wolfe et al., 2018), and high predation pressures are expected to be exerted upon these mice. House Mice are relatively new arrivals to much of their current range, having had less than five centuries to co-evolve with North American fauna. Within this timespan, it is questionable if anti-predator adaptations could have evolved, or if this comprises an evolutionary relevant timespan for these innovations. General predator avoidance and wariness appears to be present in both wild and lab strains of *Mus musculus*, suggesting that some level of anti-predator behavior may be conserved in mice regardless of environment or evolutionary timespans (Troxell-Smith et al., 2016).

### **Deer Mouse**

Predation exerted by venomous snakes imparts additional pressures beyond simply being able to avoid one's predator or alter behavioral regimes to reduce predatory risk. While resistance to venoms has been studied and identified in a variety of rodents, investigators have not previously tested for venom resistance in the Deer Mouse. The data presented confirm the presence of venom resistance in some populations of Deer Mice through comparative lethal toxicity between populations and the inhibition of snake venom metalloproteinase activity by Deer Mouse serum.

Local adaptation on the part of Weld County Prairie Rattlesnakes appears to be present in this system. Sympatric Deer Mice (Weld County) exhibit a comparatively low LD<sub>50</sub> to Weld County Prairie Rattlesnake venom (2.3 mg/kg; Figure 3.3). Conversely, toxicity toward

allopatric Deer Mice (Lincoln County) when challenged with this same venom is nearly three times lower (6.3 mg/kg; Figure 3.4). These data indicate that venom from the northern rattlesnake population exhibits a far greater toxicity to sympatric Deer Mice than to allopatric mice. The venom proteome of Weld County Prairie Rattlesnakes contains very high expression levels of myotoxin a, a non-enzymatic peptide that results in rapid incapacitation and tetanic paralysis of prey (Saviola et al., 2015). Additionally, reduced expression of snake venom metalloproteinase, an enzymatic compound resulting in the degradation of tissues, is seen in this population (Saviola et al., 2015). The relative abundance of these two toxins in Weld County Prairie Rattlesnake venom suggest that this venom is optimized to subdue prey quickly rather than significantly degrade prey tissues, as outlined previously (Mackessy, 2010).

Given the results obtained for the Weld County venom, it is somewhat surprising that Lincoln County Deer Mice are almost three times as resistant as their Weld County counterparts to Weld County Prairie Rattlesnake venom. A similar trend was found by Holding et al. (2016), where phenotype matching consistently resulted in local adaptation of the rattlesnake's venom to be more toxic to local squirrels. This same trend of snake-favored local adaptation was reiterated in another snake-prey system, but only time-to-death was locally adapted rather than overall toxicity (Smiley-Walters et al., 2017). There may be additional venom components playing into heightened toxicity of matched venom-prey pairings in the prior example, and these adapted venom components may specifically result in greater toxicity to only the co-occurring population of Deer Mice. Conversely, mice from the two field sites may have physiological differences that result in toxins interacting in different ways once envenomation has occurred. Regardless of what mechanism(s) make venom more toxic to co-occurring Deer Mice, these results indicate

that crude Weld County Prairie Rattlesnake venom may be locally adapted to its co-occurring Deer Mouse.

Snake venom metalloproteinases (SVMPs) are important components of snake venoms and may comprise large percentages of the venom proteome and assist with tissue degradation in envenomed prey, potentially facilitating later digestion (Mackessy, 2010). Metalloproteinases may also be particularly easy for prey organisms to evolve resistances to, via serum proteins that inhibit enzymatic activity (Holding et al., 2016). A trend similar to crude venom toxicities is observed when analyzing resistance of rodents to Weld County Prairie Rattlesnake venom metalloproteases alone. Weld County Deer Mice serum exhibits similar inhibition (12.1%; Table 3.2) of Weld County Prairie Rattlesnake metalloproteases than do Lincoln County Deer Mice (12.5%; Table 3.2).

When challenged with Lincoln County Prairie Rattlesnake crude venom, Deer Mice from both populations were approximately equally resistant. Consistent median lethal doses for Weld County Deer Mice (10.5 mg/kg; Figure 3.3) and Lincoln County Deer Mice (10.6 mg/kg; Figure 3.4) indicate that crude venom is equally toxic to the sympatric and allopatric mice tested, and Lincoln Co. Prairie Rattlesnake venom is considerably less toxic than Weld Co. venoms, as reflected by toxicity assays in NSA mice. This venom differs considerably from that of the Weld County Prairie Rattlesnake in that it lacks abundant myotoxin levels and instead shows high expression of SVMPs (Smith et al., unpub. data). With this toxin profile, Lincoln County Prairie Rattlesnake venom may be optimized for degradation of prey tissues rather than rapid incapacitation of prey items (cf. Mackessy, 2010). Consequently, this venom may display a reduced overall toxicity in favor of facilitating digestion of a consumed prey, with tissues beginning to degrade following envenomation.

When testing for serum inhibition of SVMP activity, we see little evidence of local adaptation for inhibition of Weld County Prairie Rattlesnake venom. Weld County Deer Mouse serum (12.1% inhibition; Table 3.2) and Lincoln County Deer Mouse serum (12.5% inhibition; Table 3.2) are approximately equal in their ability to inhibit SVMP activity. As the Lincoln County Prairie Rattlesnake venom is comprised of a much higher proportion of SVMP overall, SVMP may be a component within this venom exerting stronger resistance pressures on rodents. The greater inhibitory effect on SVMP activity in Lincoln County mouse serum is confirmed by results presented here, and Lincoln County Deer Mice consistently produce a higher percent inhibition of SVMP in comparison to their Weld County counterparts. Mice in Weld County may be under greater selective pressure from myotoxin a, resulting in reduced “resistance allocation” to SVMP, hence their overall reduced inhibitory capability when challenged with Prairie Rattlesnake venom from either site.

The Desert Massasauga is a substantially smaller rattlesnake when compared to the Prairie Rattlesnake, and as a result of its small size, small rodents and lizards make up a majority of its diet (Holycross and Mackessy, 2002). Deer Mice in particular have been identified as a prey species but appear to comprise only a very small proportion of the massasauga’s diet (Holycross and Mackessy 2002). In accordance with this presumably weak selection pressure, we see little evidence for local adaptation to Desert Massasauga venoms in Deer Mice, consistent with what would be expected.

Median lethal dose data indicate similar toxicities for Weld County mice (3.3 mg/kg; Figure 3.3) and Lincoln County mice (4.3 mg/kg; Figure 3.4) when challenged with Lincoln County Desert Massasauga venom. While these median lethal dose values are not dramatically divergent, it is apparent that the crude venom is somewhat more toxic to Weld County mice,

indicating that the Lincoln County mouse may be evolutionarily slightly ahead in the arms race dynamic with this predator-prey pairing, though this may at least in part reflect changing distributions of mice across the landscape in recent times. This contradicts systems where the snake consistently appears to be the locally adapted partner (Holding et al., 2016) and may indicate that the arms race dynamic between mouse and massasauga is being viewed at an evolutionary time point where the snake is not the locally adapted partner. When solely considering resistance to serum inhibition of snake venom metalloproteinase activity, no apparent difference in the inhibitory potential of the two mouse populations is observed (Table 3.2). Consequently, neither population of mouse is more effective at inhibiting Desert Massasauga SVMPs, but co-occurring mice exhibit a greater resistance to the venom as a whole. When considering local adaptation, LD<sub>50</sub> values may provide better biological relevance as they are better reflective of actual mortality associated with envenomation. With massasauga venom being less toxic to co-occurring Lincoln County Mice (4.3 mg/kg; Table 3.3) than Weld County mice (3.3mg/kg; Table 3.3), it is apparent that the mouse is locally adapted when considering predator-prey dynamics between these two species in Lincoln County.

### **Northern Grasshopper Mouse**

Overall, Northern Grasshopper Mice (Weld Co.) exhibit very high resistance to all rattlesnake venoms tested in this study, showing a median lethal dose of 127.7 mg/kg when challenged against sympatric Prairie Rattlesnake venom (Figure 3.7). Remarkably, this rattlesnake venom is characterized by elevated levels of myotoxin a, a highly toxic non-enzymatic compound that effectively subdues prey rapidly (Saviola et al., 2015). Resistance to Weld County Prairie Rattlesnake venom may be the result of myotoxin a inhibition, a potentially novel innovation lacking in other rodent species. Overall, we see high levels of resistance to

SVMP activity for mice of both populations against all venoms. This may be due to strong predation pressures exerted on *Onychomys leucogaster* by viperid snakes in general, as SVMPs are a conserved superfamily of venom components common to most North American pitvipers (Fox and Serrano, 2005). Further work is needed to understand mechanisms of venom resistance in this rodent, and how particular venom components are being inhibited.

Grasshopper Mice also form part of a unique venom resistance interaction with the scorpions upon which they prey. Unlike many predator-prey pairings involving venom, here venom is possessed by the prey species and is used in a defensive rather than offensive context. Scorpions form a considerable proportion of Grasshopper Mouse diets in some regions, and resistance to scorpion venoms has evolved among these mice (Rowe and Rowe, 2008). Scorpions rely on the pain inflicted by their venoms to deter predation attempts (Niermann et al., 2020), and these venoms are not optimized to incapacitate or wound mice permanently, as venoms of snake predators may be. Mutations in the sodium channels of Grasshopper Mice pain-sensing neurons contain channel variants that effectively bind venom and induce analgesia rather than pain, essentially eliminating the sting as an antipredator defense against them (Rowe et al., 2013). This predator-prey dynamic plays out across populations and landscapes to form an evolutionary-arms race dynamic (Rowe and Rowe, 2015), not unlike that apparent among some rodents and snakes (Holding et al., 2016; this chapter). Consequently, Grasshopper Mice may have a predisposition to developing resistance mechanisms to venoms generally.

Because Northern Grasshopper Mice are consumed by rattlesnakes, we expect them to be under selective pressures associated with envenomation. Additionally, Grasshopper Mice may prey on young rattlesnakes, as predation on other relatively large squamates has previously been documented (Sherbrooke, 1991). Thus, mice may encounter rattlesnake venom both during

offensive bites, and potentially also during defensive bites. In Colorado, Northern Grasshopper Mice have been found in the diet of Prairie Rattlesnakes (Balchan et al., unpub. data; see Chapter 2) and (when neonates) as a minor component of Desert Massasauga diets (Holycross and Mackessy, 2002). While Desert Massasauga predation on Grasshopper Mice has been observed, only a single individual was found to have been preyed on in the 2002 study, perhaps representing a nestling or otherwise more vulnerable individual, as adult Grasshopper Mice are of an unsuitable size for Desert Massasaugas to consume. Conversely, the Prairie Rattlesnake, given its much larger size, would be able to feed on adult Grasshopper Mice without difficulty. Therefore, we expect strong selection pressure to be exerted upon Grasshopper Mice by Prairie Rattlesnakes, and weaker pressure to be exerted on mice by Desert Massasauga Rattlesnakes, and this prediction is supported by our data (Table 3.2; Table 3.3).

### **Ord's Kangaroo Rat**

Weld County kangaroo rats appear to exhibit moderate levels of resistance to both Prairie Rattlesnake venoms. Weld County rats exhibit divergent inhibitory potentials to the SVMPs of both Prairie Rattlesnake populations, inhibiting 15.3% of Weld Co. Prairie Rattlesnake SVMP activity and 29.2% of Lincoln County Prairie Rattlesnake SVMP activity (Table 3.2). These serum results strongly suggest local adaptation, where rats display resistance to their co-occurring snake's SVMPs. Conversely, resistance to SVMPs may be limited to only one of the three subclasses of SVMPs, which are likely differentially distributed in the northern and southern *C. viridis* populations. Surprisingly, rats appear to be about equally resistant to lethal toxicity of crude venoms, with an LD<sub>50</sub> value of 15.0 mg/kg to both Prairie Rattlesnake venoms (Figure 3.5), illustrating the difference between studying only a single venom component versus crude venom as a whole. While kangaroo rats probably only rarely fall prey to the Desert

Massasauga Rattlesnake, Weld County rats still exhibited some inhibition of massasauga SVMPs (8.7% inhibition; Table 3.2)

Lincoln County kangaroo rats display a considerably different pattern of resistance in comparison to their Weld County counterparts. Serum inhibition of Prairie Rattlesnake SVMP does differ between populations, and Lincoln County rat sera inhibit 22.6% of Weld County Prairie Rattlesnake SVMP activity while inhibiting 27.9% of co-occurring Lincoln County Prairie Rattlesnake SVMP (Table 3.2). These results indicate that Lincoln County rats may be the locally adapted partner in their predator-prey interaction and may be better suited against their co-occurring rattlesnake than allopatric rats are, though it does not appear particularly powerful at the serum metalloprotease inhibition level. This local adaptation is reaffirmed when crude venom lethality is considered, as median lethal dose values diverge dramatically between venom types. When challenged with Weld County Prairie Rattlesnake venom, Lincoln County rats display a median lethal dose of 4.2 mg/kg (Figure 3.6). Conversely, when challenged with Lincoln County Prairie Rattlesnake venom, rats show a median lethal dose value of 125.0 mg/kg (Figure 3.6). This dramatic divergence in LD<sub>50</sub> values demonstrates extreme differences in resistance capabilities to Prairie Rattlesnake venoms and illustrates that Lincoln County rats are strongly locally adapted against their co-occurring snake. This may be the result of intense predation pressures exerted by the snakes, as kangaroo rat-snake interaction have been the focus of previously studies. Unsurprisingly, Lincoln County rat serum fails to inhibit significant SVMP activity from Lincoln County massasauga venom (Table 3.2). This likely reflects the lack of predation pressure exerted by massasaugas, and it suggests that specific homologs are recognized (or not) by serum factors.

## Other Rodents

Venom resistance has been well characterized in woodrats, and overall, resistance to rattlesnake venoms is well developed throughout the genus. In one example, the Southern Plains Woodrat (*Neotoma micropus*) exhibits strong resistance to the venom of the Western Diamondback Rattlesnake (*Crotalus atrox*) (Perez et al., 1978). In another, the Eastern Woodrat is effective at inhibiting the venom of the Copperhead (*Agkistrodon contortrix*) (de Wit, 1982). The occurrence of resistance in several woodrat species indicates that venom resistance occurs broadly and may be present throughout the genus. Further, venom resistance in the woodrats may be a relatively conserved character and remain well maintained across species, in part due to the fact that many components are common to all rattlesnake venoms. As a result, one might expect a woodrat of any species to possess resistance to the venom of its co-occurring viperid snake.

The distribution of the Eastern Woodrat extends into southeastern Colorado, where it co-occurs with both the Prairie Rattlesnake and the Desert Massasauga Rattlesnake. While the massasauga is far too small to prey on the woodrat, it is possible that pups may be consumed opportunistically on rare occasions. Conversely, the Prairie Rattlesnake likely consumes adult woodrats frequently, as these would represent a suitable and large meal that could sustain a snake for a considerable amount of time. Therefore, it is expected that strong predation pressures are exerted on woodrats by Prairie Rattlesnakes, and considerably weaker pressures by Desert Massasauga Rattlesnakes. As a result, one expects that rats will be highly resistant to Prairie Rattlesnake venom, and considerably less resistant to the venom of the Desert Massasauga Rattlesnake.

This predication does appear to be upheld by our results, where one tested Eastern Woodrat showed remarkably high SVMP inhibition of the venom of its co-occurring Lincoln Co.

Prairie Rattlesnakes (56.6% inhibition; Table 3.2), and high, though considerably less so, inhibition of the SVMPs of the Weld Co. Prairie Rattlesnakes (25.2% inhibition,  $n = 1$ ; Table 3.2). These results suggest, with respect to Prairie Rattlesnakes, that the woodrat is the locally adapted partner and is best at negating venom function of its co-occurring Prairie Rattlesnake. Unsurprisingly, these rats show low serum inhibition of co-occurring Desert Massasauga SVMPs (8.4% inhibition; Table 3.2). While reduced resistance due to corresponding reduction in predation pressure is expected, one might expect some level of inhibition to be present, given that SVMPs are conserved across viper venoms. However, Desert Massasauga SVMPs may be sufficiently divergent from those of Prairie Rattlesnake venoms that resistance mechanisms to the latter do not effectively work for massasauga venom.

While Plains Pocket Mice are present at both field sites, the Desert Massasauga is absent from the Weld County site. This pairing of a mouse population with snake predator (Lincoln County) and mouse population without snake predator (Weld County) allowed for exploration into the presence of local adaptation. An SVMP inhibition assay was conducted, challenging serum from Weld County and Lincoln County pocket mice against the venom of Lincoln County Desert Massasauga. Both sera performed similarly, with Weld County pocket mice inhibiting 16.3% of SVMP activity (Table 3.2) and Lincoln County pocket mice inhibiting 14.7% SVMP activity (Table 3.2). To support this trend of a lack of local adaptation, several  $LD_{50}$  approximations were conducted for the Plains Pocket Mouse but could not be completed due to low numbers of captured mice. Based on dosages that could be tested, mortality has been observed from two individuals (Lincoln Co.) at a dosage of 5.0 mg/kg and another individual at 1.0 mg/kg of Desert Massasauga venom. While not conclusive, these data suggest that the  $LD_{50}$  for this pairing may be below 1.0 mg/kg. These data indicate that pocket mice are not locally

adapted where they co-occur with Desert Massasaugas, even though they are presumably under strong selective pressures exerted by massasauga predation.

### **The Evolutionary Arms Race**

Though complex, patterns of resistance in a biological community allow exploration of the means through which co-evolution can operate among predator and prey in the context of biochemical ecology. Arms race dynamics can be important as a potential starting point for evolutionary trajectories, and selective pressures as a result of arms races may be considerably stronger than many other concurrent pressures an organism faces. In eastern Colorado, it appears that several arms races may be present between interacting snakes and rodents, with different winners and losers at present, and these arms races may be meaningful in an ecological and evolutionary context beyond what we can observe externally. The complexity of venom as a means of subduing prey cannot be understated. While venoms may have varying degrees of potency to different prey species, potentially creating a patchwork dynamic among partners and communities (Table 3.4), there may be potential for this to be overcome by dosage, further complicating our understanding of venom mediated predatory interactions (Hayes, 1995).

Table 3.4. Hypothetical patchwork dynamic across species and sites where interacting pairings may exhibit differing adaptation outcomes. Red rectangles suggest the snake as the locally adapted partner in the interaction and green rectangles suggest the rodent as the locally adapted partner in the interaction. Black rectangles indicate pairings where interactions have not been determined. These dynamics represent a snapshot in evolutionary time and may change considerably as arms-race dynamics occur.

	Lincoln County Desert Massasauga	Lincoln County Prairie Rattlesnake	Weld County Prairie Rattlesnake
Weld County Deer Mouse	Red	Red	Red
Lincoln County Deer Mouse	Green	Green	Red
Weld County Grasshopper Mouse	Green	Green	Green
Lincoln County Grasshopper Mouse	Green	Green	Green
Weld County Kangaroo Rat	Red	Red	Red
Lincoln County Kangaroo Rat	Red	Green	Red
Lincoln County Eastern Woodrat	Green	Green	Green
Weld County Plains Pocket Mouse	Red	?	?
Lincoln County Plains Pocket Mouse	Red	?	?

Notably, it appears that Plains Pocket Mice lack local adaptation entirely to Desert Massasauga venom. This mouse represents in many ways the ideal prey item for the massasauga, and this is reflected in diet studies. It would seem maladaptive to lack defenses to a venomous predator, but several conflicting pressures may inhibit the development of resistance to an animal that is also preyed upon heavily by other species. Additionally, landscape dynamics impacting the distributions of rodents and snakes may impact the development and maintenance of resistance, effectively “resetting” the dynamic when lineages and populations become vicariant across a landscape. As such, the current snapshot of the massasauga-pocket mouse dynamic may only represent a very early start to a temporally varying predator-prey interaction. Sampling at a much later timepoint could reveal the development of resistance, if the mouse and snake do indeed interact with great frequency in a trophic context. At present, it appears that the Desert Massasauga is evolutionarily “ahead” of the Plains Pocket Mouse.

An arms race dynamic also appears to be occurring between the Prairie Rattlesnake and both the Ord’s Kangaroo Rat and Northern Grasshopper Mouse. In both cases, it appears that these rodents are evolutionarily “ahead” in at least some locations. With strong pressure exerted by rattlesnakes, it would be an important evolutionary development to develop and maintain resistance. But with the advent of resistance, a rattlesnake predator should incur pressure to surpass its prey item in this interaction, thus setting the stage for an oscillatory pattern where predator and prey have the potential to evolve reciprocally. Thus, a race is created where at any given time in evolutionary history one partner may exceed the other in level of local adaptation. These small evolutionary advantages may require varying amounts of time to confer and may depend on the modularity of a system (e.g. serum proteins vs. venom composition). In any event, it is crucial to remember that local adaptation is only informative for present dynamics, and

sampling in the past or future may show trends that do not at all reflect those displayed at present.

In conclusion, venom resistance is a complex interaction involving chemically-mediated predation and prey physiological responses, factoring in an array of selective pressures exerted across a faunal community. The presence and maintenance of resistance can be attributed to far more than just single predator-single prey interactions, and dynamics may shift significantly over relatively short evolutionary times. Venom resistance represents only a single way in ecological systems where prey may shape predator phenotype, and vice versa. As a result, studies of resistance should consider ecology and physiology at multiple levels, and combine data derived from whole organism assays as well as those derived from *in vitro* assays will prove most informative. Venom resistance can reveal much about species in a free-ranging setting, and further work should continue to characterize this dynamic in natural systems, incorporating aspects beyond those that can be gleaned solely in the laboratory.

### References

- Aird S. D., and N. J. da Silva, Jr. 1991. Comparative enzymatic composition of Brazilian coral snake (*Micrurus*) venoms. *Comparative Biochemistry and Physiology B* 99:287-294.
- Akali, C. K. and D. W. Pfennig. 2017. Geographic variation in mimetic precision among different species of coral snake mimics. *Journal of Evolutionary Biology* 30:1420-1428.
- Barlow, A., Pook, C. E., Harrison, R. A., and W. Wüster. 2009. Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. *Proceedings of the Royal Society B* 276:2443-2449.
- Bdolah, A., Kochva, E., Ovadia, M., Kinamon, S., and Z. Wollberg. 1997. Resistance of the Egyptian Mongoose to sarafotoxins. *Toxicon* 35:1251-1261.

- Brodie, E. D., Ridenhour, B. J., and E. D. Brodie. 2002. The evolutionary response of predators to dangerous prey: Hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. *Evolution* 56:2067-2082.
- Chun, J. B. S., Baker, M. R., Kim, D. J., LeRoy, M., Toribo, P., and J. -P. Bingham. 2012. Cone snail milked venom dynamics – a quantitative study of *Conus purpurascens*. *Toxicon* 60:83-94.
- Clarke, J. A. 1983. Moonlight's influence on predator/prey interactions between Short-Eared Owls (*Asio flammeus*) and Deermice (*Peromyscus maniculatus*). *Behavioral Ecology and Sociobiology* 13:205-209.
- Clarke, J.A., J.T. Chopko and S.P. Mackessy. 1996. The effect of moonlight on activity patterns of adult and juvenile prairie rattlesnakes (*Crotalus viridis viridis*). *Journal of Herpetology* 30(2):192-197.
- Clark, R. W. 2002. Diet of the Timber Rattlesnake, *Crotalus horridus*. *Journal of Herpetology* 36: 494-499.
- Connolly, B. M., and J. L. Orrock. 2018. Habitat-specific capture timing of Deer Mice (*Peromyscus maniculatus*) suggests that predators structure temporal activity of prey. *Ethology* 124:105-112.
- Coss, R. G., Guse, K. L., Poran, N. S., and D. G. Smith. 1993. Development of antsnake defenses in California Ground Squirrels (*Spermophilus beecheyi*): II. Microevolutionary effects of relaxed selection from rattlesnakes. *Behavior* 124:137-164.
- de Wit, C. A. 1982. Resistance of the Prairie Vole (*Microtus ochrogaster*) and the Woodrat (*Neotoma floridana*), in Kansas, to venom of the Osage Copperhead (*Agkistrodon contortrix phaeogaster*). *Toxicon* 20:709-714.

- Drabeck, D. J., Dean, A. M., and S. A. Jansa. 2015. Why the honey badger don't care: Convergent evolution of venom-targeted nicotinic acetylcholine receptors in mammals that survive venomous snake bites. *Toxicon* 99:68-72.
- Dugan, E., and W. Hayes. 2012. Diet and feeding ecology of the Red Diamond Rattlesnake, *Crotalus ruber* (Serpentes: Viperidae). *Herpetologica* 68:203-217.
- Fortin, D., Beyer, H., Boyce, M., Smith, D., Duchesne, T., and J. Mao. 2005. Wolves influence elk movements: behavior shapes a trophic cascade in Yellowstone National Park. *Ecology* 86:1320-1330.
- Fox, J. W., and S. M. T. Serrano. 2005. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprotolysin family of metalloproteinases. *Toxicon* 45:969–985.
- Freymler, G. A., Whitford, M. D., Higham, T. E., and R. W. Clark. 2017. Recent interactions with snakes enhance escape performance of Desert Kangaroo Rats (Rodentia: Heteromyidae) during simulated attacks. *Biological Journal of the Linnean Society* 122:651–660.
- Freymler, G. A., Whitford, M. D., Higham, T. E., and R. W. Clark. 2019. Escape dynamics of free-ranging Desert Kangaroo Rats (Rodentia: Heteromyidae) evading rattlesnake strikes. *Biological Journal of the Linnean Society* 127:164–172o
- Gibbs, H. L., and S. P. Mackessy. 2009. Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. *Toxicon* 53:672-679.
- Goldenberg, S. U., Borcharding, J. and M. Heynen. 2014. Balancing the response to predation—the effects of shoal size, predation risk and habituation on behaviour of juvenile perch. *Behavioral Ecology and Sociobiology* 68:989-998.

- Gutiérrez J. M., Lomonte B., León, G., Alape-Girón, A., Flores-Díaz, M., Sanz, L., Angulo, Y., and J. J. Calvete. 2009. Snake venomomics and antivenomics: proteomic tools in the design and control of antivenoms for the treatment of snakebite envenoming. *Journal of Proteomics* 72:165-182.
- Hayes, W. K. 1995. Venom metering by juvenile prairie rattlesnakes, *Crotalus v. viridis*: effects of prey size and experience. *Animal Behaviour* 50:33-40.
- Hammerson, G. A. 1999. Amphibians and Reptiles in Colorado. Second edition. University Press of Colorado, Niwot, Colorado.
- Hibbard, E. A., and J. R. Beer. 1960. The Plains Pocket Mouse in Minnesota. *Flicker*, 32:89-94.
- Higham, T. E., Clark, R. W., Collins, C. E., Whitford, M. D., G.A. Freymiller. 2017. Rattlesnakes are extremely fast and variable when striking at kangaroo rats in nature: three-dimensional high-speed kinematics at night. *Scientific Reports* 7:40412
- Hobert, J. P., Montgomery, C. E., and S. P. Mackessy. 2004. Natural history of the Massasauga, *Sistrurus catenatus edwardsii*, in southeastern Colorado. *Southwestern Naturalist* 49:321-326.
- Holding, M. L., Biardi, J. E., and H. L. Gibbs. 2016. Coevolution of venom function and venom resistance in a rattlesnake predator and its squirrel prey. *Proceedings of the Royal Society B*. 283:20152841.
- Holding, M. L., Drabeck, D. H., Jansa, S. A. and H. L. Gibbs. 2016. Venom resistance as a model for understanding the molecular basis of complex coevolutionary adaptations. *Integrative and Comparative Biology* 56:1032-1043.

- Holycross, A. T., and S. P. Mackessy. 2002. Variation in the diet of *Sistrurus catenatus* (Massasauga), with emphasis on *Sistrurus catenatus edwardsii* (Desert Massasauga). *Journal of Herpetology* 36:454-464.
- Jayne, B. C., Voris, H. K., and P. K. L. Ng. 2002. Snake circumvents constraints on prey size. *Nature* 418:143.
- Kiriake, A., Ishizaki, S., Nagashima, Y., and K. Shiomi. 2017. Occurrence of a Stonefish toxin-like toxin in the venom of the Rabbitfish *Siganus fuscescens*. *Toxicon* 140: 139-146.
- Langley, W. M. 1994. Comparison of predatory behaviors of Deer Mice (*Peromyscus maniculatus*) and Grasshopper Mice (*Onychomys leucogaster*). *Journal of Comparative Psychology* 108:394-400.
- Lee, C.-Y., Yo, S.-P., Clark, R. W., Hsu, J. -Y., Liao, C.-P., Tseng, H.-Y., and W.-S. Huang. 2018. The role of different visual characters of weevils signalling aposematism to sympatric lizard predators. *Journal of Zoology* 306:36-47.
- Ligabue-Braun, R., Verli, H., and C. R. Carlini. 2012. Venomous mammals: a review. *Toxicon* 59: 680-695.
- Mackessy, S. P. 1988. Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *Crotalus viridis oreganus*. *Copeia* 1988:92-101.
- Mackessy, S. P. 2010. Evolutionary trends in venom composition in the Western Rattlesnakes (*Crotalus viridis* sensu lato): toxicity vs. tenderizers. *Toxicon* 55:1463-1474
- Mackessy, S. P., and A. J. Saviola. 2016. Understanding biological roles of venoms among the Caenophidia: the importance of rear-fanged snakes. *Integrative and Comparative Biology* 56:1004-1021.

- Modahl, C. M., Roointan, A., Rogers, J., Currier, K., and S. P. Mackessy. 2020 Interspecific and intraspecific venom enzymatic variation among Cobras (*Naja* sp. and *Ophiophagus hannah*). *Comparative Biochemistry and Physiology, Part C* 232:108743.
- Niermann, C. N., Tate, T. G. Suto, A. L., Barajas, R., White, H. A., Guswiler, O. D., Secor, S. M., Rowe, A. H., and M. P. Rowe. 2020. Defensive venoms: is pain sufficient for predator deterrence? *Toxins* 12:260.
- Orrock, J. L. 2010. When the ghost of predation has passed: do rodents from islands with and without fox predators exhibit aversion to fox cues? *Ethology* 116: 338-345.
- Orrock, J. L., and R. J. Fletcher, Jr. 2014. An island-wide predator manipulation reveals immediate and long-lasting matching of risk by prey. *Proceedings of the Royal Society B* 281:20140391.
- Pawlak, J., Mackessy, S. P., Fry, B. G., Bhatia, M., Mourier, G., Fruchart-Gaillard, C., Servent, D., Ménez, R., Stura, E., Ménez A., and R. M. Kini. 2006. Denmotoxin: a three-finger toxin from colubrid snake *Boiga dendrophila* (Mangrove Catsnake) with bird-specific activity. *Journal of Biological Chemistry* 281:29030-29041.
- Pawlak, J., Mackessy, S. P., Sixberry, N. M., Stura, E. A., Le Du, M. H., Menez, R., Foo, C. S., Menez, A., Nirthanan S., and R. M. Kini. 2009. Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. *FASEB Journal* 23:534-545.
- Perez, J. C., Haws, W. C., and C. H. Hatch. 1978. Resistance of woodrats (*Neotoma micropus*) to *Crotalus atrox* venom. *Toxicon* 16:198-200.

- Perez, J. C., Pichyangkul, S., and V. E. Garcia. 1979. The resistance of three species of warm-blooded animals to Western Diamondback Rattlesnake (*Crotalus atrox*) venom. *Toxicon* 17:601-607.
- Phillips, M. A., Waterman, J. M., Du Plessis, P., Smit, M., and N. C. Bennett. 2012. No evidence for proteolytic venom resistance in southern African ground squirrels. *Toxicon* 60:760-763
- Pomanto, A. M., Perry, B. W., Denton, R. D., Gibbs, H. L., and M. L. Holding. 2016. No safety in the trees: local and species-level adaptation of an arboreal squirrel to the venom of sympatric rattlesnakes. *Toxicon* 118:149-155.
- Poran, N. S., and H. Heatwole. 1995. Resistances of sympatric and allopatric eels to sea snake venoms. *Copeia* 1995(1):136-147.
- Poran, N. S., Coss, R. G., and E. Benjamini. 1987. Resistance of California Ground Squirrels (*Spermophilus beecheyi*) to the venom of the Northern Pacific Rattlesnake (*Crotalus viridis oregonus*): a study of adaptive variation. *Toxicon* 25:767-777.
- Rowe, A. H., and M. P. Rowe. 2008. Physiological resistance of grasshopper mice (*Onychomys spp.*) to Arizona Bark Scorpion (*Centruroides exilicauda*) venom. *Toxicon* 52:597-605.
- Rowe, A. H., and M. P. Rowe. 2015. Predatory grasshopper mice. *Current Biology* 25:R1023-R1026.
- Rowe, A., Xiao, Y., Rowe, M., Cummins, T., and H. Zakon. 2013. Voltage-gated sodium channel in Grasshopper Mice defends against Bark Scorpion toxin. *Science* 342:441-446.
- Saviola, A. J., Pla, D., Sanz, L., Castoe, T. A., Calvete, J. J., and S. P. Mackessy. 2015. Comparative venomomics of the Prairie Rattlesnake (*Crotalus viridis viridis*) from Colorado: identification of a novel pattern of ontogenetic changes in venom composition

- and assessment of the immunoreactivity of the commercial antivenom CroFab®. *Journal of Proteomics* 121:28-43.
- Saviola, A. J., Chiszar, D., Busch, C., and S. P. Mackessy. 2013. Molecular basis for prey relocation in viperid snakes. *BMC Biology* 11:20.
- Schraft, H. A., and R. W. Clark. 2017. Kangaroo rats change temperature when investigating rattlesnake predators. *Physiology & Behavior* 173:174-178.
- Sherbrooke, W. 1991. Behavioral (predator-prey) interactions of captive Grasshopper Mice (*Onychomys torridus*) and horned lizards (*Phrynosoma cornutum* and *P. modestum*). *The American Midland Naturalist* 126:187-195.
- Slip, D., and R. Shine. 1988. Feeding habits of the Diamond Python, *Morelia s. spilota*: ambush predation by a boid snake. *Journal of Herpetology* 22:323-330.
- Smiley-Walters, S. A., Farrell, T. M., and H. L. Gibbs. 2018. The importance of species: Pygmy Rattlesnake venom toxicity differs between native prey and related non-native species. *Toxicon* 144:42-47.
- Smiley-Walters, S., Farrell, T. M., and H. L. Gibbs. 2017. Evaluating local adaptation of a complex phenotype: reciprocal tests of Pigmy Rattlesnake venoms on treefrog prey. *Oecologia* 184:739-748.
- Strickland, J. L., Smith, C. F., Mason, A. J., Schield, D. R., Borja, M., Castañeda-Gaytán, G., Spencer, C. L., Smith, L. L., Trápaga, A., Bouzid, N. M., Campillo-García, G., Flores-Villela, O. A., Antonia-Rangel, D., Mackessy, S. P., Castoe, T. A., Rokyta, D. R., and C. L. Parkinson. 2018. Evidence for divergent patterns of local selection driving venom variation in Mojave Rattlesnakes (*Crotalus scutulatus*). *Scientific Reports* 8:1-15.

- Suzuki, H., Nunome, M., Kinoshita, G., Aplin, K.P., Vogel, P., Kryukov, A.P., Jin, M.L., Han, S.H., Maryanto, I., Tsuchiya, K., Ikeda, H., Shiroishi, T., Yonekawa, H., and K. Moriwaki. 2013. Evolutionary and dispersal history of Eurasian House Mice *Mus musculus* clarified by more extensive geographic sampling of mitochondrial DNA. *Heredity* 111:375-390.
- Teta, P., Herculini, C., and G. Cueto. 2012. Variation in the diet of Western Barn Owls (*Tyto alba*) along an urban-rural gradient. *The Wilson Journal of Ornithology* 124:589-596.
- Thaker, M., Vanak, A. T., Owen, C. R., Ogden, M. B., and R. Slotow. 2010. Group dynamics of Zebra and Wildebeest in a woodland savanna: effects of predation risk and habitat density. *PLoS One* 5(9):e12758.
- Tichy, H., Zaleska-Rutczynska, Z., O'Huigin, C., Figueroa, F., and J. Klein. 1994. Origin of the North American House Mouse. *Folia Biologica* 40:483-496.
- Troxell-Smith, S. M., Tutka, M. J., Albergo, J. M., Balu, D., Brown, J. S., and J. P. Leonard. 2016. Foraging decisions in wild versus domestic *Mus musculus*: what does life in the lab select for? *Behavioural Processes* 122:43-50.
- Turko, P., Tellenbach, C., Keller, E., Tardent, N., Keller, B., Spaak, P., and J. Wolinska. 2018. Parasites driving host diversity: incidence of disease correlated with *Daphnia* clonal turnover. *Evolution* 72:619-629.
- Van Valen, L. 1973. A new evolutionary law. *Evolutionary Theory* 1:1-30.
- Ward-Smith, H., Arbuckle, K., Naude, A., and W. Wüster. 2020. Fangs for the memories? A survey of pain in snakebite patients does not support a strong role for defense in the evolution of snake venom composition. *Toxins* 12:201.

- Wastell, A. R., and S. P. Mackessy. 2016. Desert Massasauga Rattlesnakes (*Sistrurus catenatus edwardsii*) in southeastern Colorado: life history, reproduction, and communal hibernation. *Journal of Herpetology* 50:594-603.
- Werner, R. M., and J. A. Vick. 1977. Resistance of the Opossum (*Didelphis virginiana*) to envenomation by snakes of the family Crotalidae. *Toxicon* 15:29-32.
- White, J. A., and K. Geluso. 2007. Seasonal differences in onset of surface activity of Ord's Kangaroo Rat (*Dipodomys ordii*). *Journal of Mammalogy* 88:234-240.
- Whitford, M. D., Freymiller, G. A., and R. W. Clark. 2019. Managing predators: the influence of kangaroo rat antipredator displays on sidewinder rattlesnake hunting behavior. *Ethology* 125:450-456.
- Whitford, M., D., Freymiller, G. A., and R. W. Clark. 2017. Avoiding the serpent's tooth: predator-prey interactions between free-ranging Sidewinder Rattlesnakes and Desert Kangaroo Rats. *Animal Behaviour* 130:73-78.
- Willey, D. W. 2013. Diet of the Mexican Spotted Owls in Utah and Arizona. *The Wilson Journal of Ornithology* 125:775-781.
- Williams, S. H., Peiffer, E., and S. Ford. 2009. Gape and bite force in the rodents *Onychomys leucogaster* and *Peromyscus maniculatus*: does jaw-muscle anatomy predict performance? *Journal of Morphology* 270:1338-1347.
- Wolfe, A. K., Bateman, P. W., and P. A. Fleming. 2018. Does urbanization influence the diet of a large snake? *Current Zoology* 64:311-318.
- Zimmerman, G., Stapp, P., and B. Van Horne. 1996. Seasonal variation in the diet of Great Horned Owls (*Bubo virginianus*) on shortgrass prairie. *The American Midland Naturalist* 136:149-156.

## CHAPTER IV

### CONCLUSIONS

New developments consistently occur to bolster our understanding of how snakes interact with their environments in a trophic context. Traditional ideas of snakes being relatively benign and non-specific predators have largely been abandoned with the continuation of study on snake diets. Snake are now understood as being complex predators, exhibiting dietary specialization (Madsen and Shine, 1996), prey luring behaviors (Glaudas and Alexander, 2016), and dangerous prey avoidance (Phillips et al., 2010), plus other relatively complex behavioral components not traditionally associated with reptiles. Venoms add an additional level of complexity to snake predation dynamics, as they provide a distinct way of facilitating incapacitation of prey. Venoms have the potential to be highly variable (Strickland et al. 2018) while also exhibiting a great degree of specificity (Pawlak et al., 2006; Heyborne and Mackessy, 2013). In response to both these behavioral attributes (i.e., Bleicher et al., 2020) and these biochemical attributes (Poran et al., 1987), snakes have the potential to impact the prey species within their ecosystem radically.

In addition to understanding roles within natural systems, a thorough contextualization of snake feeding ecology may reveal the factors mediating venom variation in many snake species, with particular importance to medically significant taxa. Recent designation of snakebite as a “Neglected Tropical Disease” by the World Health Organization (2019) has underscored the severity of this malady, particularly in the tropics. Snakebite results in hundreds of thousands of cases of morbidity and mortality globally, and these astonishingly high numbers are also related to antivenom availability and accessibility. This antivenom issue is further complicated by

venom variation and lack of cross reactivity among some snake populations. For example, a Spectacled Cobra (*Naja naja*) envenomation in Sri Lanka cannot effectively be treated with antivenom manufactured for the same species in India (Sintiprungrat et al., 2016). Understanding how diet impacts venom biochemistry may provide us with predictive power and allow us to make better decisions when choosing snakes to use for a regionally specific antivenom.

## **Summary of Chapter II**

Chapter II described the diet of the Prairie Rattlesnake (*Crotalus viridis*) across much of its geographical distribution. While previous studies have explored the diet of this taxon at a population level (Hill et al. 2001), we provide the first characterization of its diet across the extensive latitudinal distribution through which it occurs. Data from other medium-large bodied rattlesnakes suggest the presence of dietary ontogeny, with an ectotherm to endotherm shift in prey items present throughout a snake's lifespan, and also indicate a general prevalence of mammalian prey in the diet of adults (ex. Dugan and Hayes, 2002; Glaudas et al., 2008).

Based on diet data collected from 449 preserved specimens of Prairie Rattlesnake, we recovered a broad sampling of prey items distributed throughout the year and across the species' range. Mammalian prey was found to comprise the majority of prey classes consumed, with the majority of prey items being small rodents. To a lesser degree, avian and lizard prey was also consumed by Prairie Rattlesnakes. Data showed weak support for an ontogenetic dietary shift, though lizard predation did appear to be biased towards snakes of smaller body lengths, indicating that lizard predation may occur more frequently in smaller snakes. Additional sampling of snakes, particularly those from southern regions of the distribution where lizards increase in abundance will be needed to better understand the presence of dietary ontogeny. We found little evidence to suggest latitudinal differences in diet, though lizard prey items were

recovered from more southerly occurring snakes, but further sampling is needed to clarify this. Additionally, we found no evidence for variability in seasonality impacting foraging duration, and snakes from more southerly areas were not found to have fed earlier or later into their active season than others.

Thorough contextualization of a species' diet is important from a number of standpoints. Firstly, diet functions as a major avenue through which an animal interacts with its environment. The assumption that snakes serve as opportunistic, generalist predators within their environments both discredits their potential to play strong mediating roles in regulating preferred prey species numbers and undermines the growing realization that snakes have complex foraging behaviors and preferences. To understand, and further, to conserve snakes, we must have a knowledge of how they fit into their ecosystems in a trophic context and how these myriad partners interact. Secondly, diet may be one of the main drivers of venom evolution and variation (Mackessy, 1988; Barlow et al., 2009), and understanding diets and foraging strategies may provide predictive power in understanding how and why venom varies across a landscape. With snakebite being a major global crisis, dietary data may be important in designing and distributing more efficacious antivenoms. Overall, a considerable amount of additional research is needed to understand better the diets of snakes, and this study represents only the beginning stages of understanding feeding ecology of the Prairie Rattlesnake. Further sampling is needed to generate a better representation of this snake's diet across its geographical distribution.

### **Summary of Chapter III**

Chapter III investigated patterns of resistance to rattlesnake venoms across a rodent community in the grasslands of eastern Colorado. Resistance to snake venoms is present and has been characterized in a number of systems (Poran et al., 1987; Poran and Heatwole 1995), yet

remains apparently absent in others (Phillips et al. 2012). The factors influencing the development and maintenance of resistance are poorly understood, and previous studies have focused on isolating resistance to single predator-prey pairings. This study analyzed resistance at the community level, incorporating two venomous snakes, and the array of co-occurring rodent prey items present. Additionally, the use of two field sites allowed for reciprocal comparisons of sympatric and allopatric predator-prey population pairings, as well as experimentation with two divergent venom phenotypes in the same rattlesnake species.

This study used LD<sub>50</sub> assays and serum metalloprotease inhibition assays to determine resistance capabilities of various rodents to the venoms of Prairie Rattlesnakes (*Crotalus viridis*) from two field sites (with divergent venom phenotypes) and Desert Massasauga Rattlesnakes (*Sistrurus tergeminus edwardsii*) from a single field site. Results indicate high levels of resistance to venoms in populations of the Eastern Woodrat (*Neotoma floridana*), Ord's Kangaroo Rat (*Dipodomys ordii*), and Northern Grasshopper Mouse (*Onychomys leucogaster*). Comparatively low levels of resistance were found in populations of Deer Mouse (*Peromyscus maniculatus*), House Mouse (*Mus musculus*), and Plains Pocket Mouse (*Perognathus flavescens*). Additional characterization of rodent serum using affinity chromatography suggests the presence of venom-binding proteins in several rodent taxa that may confer resistance to snake venom molecules.

This variability in resistance across prey species illustrates the dynamic nature of trophic interactions when conducting analyses at the community level. Venom resistance and snake diet have the potential to show a large interplay, as a highly venom-resistant rodent may be less likely to be incapacitated by a snake's envenomation, thus appearing in a snake's diet less frequently. Conversely, high levels of resistance in rodents are likely to be the result of strong predation

pressures from venomous snakes, and we might conversely expect resistant rodents to appear frequently in snake diets to align with this idea of strong predation pressure. While resistance is certainly of adaptive benefit to prey items, snakes may have ways to readily overcome these barriers, for example metering an excess amount of venom into a prey item to ensure incapacitation (Hayes, 1995). Regardless of outcome for either predator or prey, venom resistance illustrates the evolutionary arms-race dynamic, where evolution of modified venom phenotypes and concurrent resistance in prey species can occur in concert. Most notably, this dynamic is only viewable at any one point in evolutionary time in the context of most studies, and it is important to note that dynamics can be expected to change throughout evolutionary time. Further research should explore the progression of these arms-races over evolutionary time, building on previously generated datasets and re-sampling at a later time point to detect change in venoms and/or resistance.

## **Conclusions**

Based on the investigations outlined in this thesis, it is clear that snakes have the potential to exert strong pressures on their prey species. Dietary study indicates that rattlesnake predation may preferentially impact a few species within a given rodent assemblage, and venom resistance studies indicate that beyond simply suppressing numbers, predation pressures by snakes may influence the physiology of their prey species. Further studies should work to better integrate the interplay between diet and resistance to venoms, perhaps focusing in on a single study site and teasing apart both of these complex topics within. Continued research will allow us to understand better the factors that influence and preserve venom variation across a landscape, and help us better contextualize the place of snakes within their ecosystems.

## Complete Reference List

- Aird S. D., and N. J. da Silva Jr. 1991. Comparative enzymatic composition of Brazilian coral snake (*Micrurus*) venoms. *Comparative Biochemistry and Physiology B* 99:287-294.
- Akali, C. K. and D. W. Pfennig. 2017. Geographic variation in mimetic precision among different species of coral snake mimics. *Journal of Evolutionary Biology* 30:1420-1428.
- Almeida-Santos, S. M., Antoniazzi, M. M., Sant'Anna, O. A., and C. Jared. 2000. Predation by the opossum *Didelphis marsupialis* on the rattlesnake *Crotalus durissus*. *Current Herpetology* 19:1-9.
- Ang, L. J., Sanjay, S., and T. Sangtam. 2014. Ophthalmia due to spitting cobra venom in an urban setting - a report of three cases. *Middle East African Journal of Ophthalmology* 21:259-261.
- Armstrong, D., Fitzgerald, J., and C. Meaney. 2011. Mammals of Colorado, Second Edition. University Press of Colorado, Boulder, Colorado.
- Avila-Villegas, H., Martins, M., and G. Arnaud. 2007. Feeding ecology of the endemic rattleless rattlesnake, *Crotalus catalinensis*, of Santa Catalina Island, Gulf of California, Mexico. *Copeia* 1:80-84.
- Barchan, D., Kachalsky, S., Neumann, D., Vogel, Z., Ovadia, M., Kochva, E., and S. Fuchs. 1992. How the mongoose can fight the snake: the binding site of the mongoose acetylcholine receptor. *Proceedings of the National Academy of Sciences* 89:7717-7721.

- Barchan, D., Ovadia, M., Kochva, E., and S. Fuchs. 1995. The binding site of the nicotinic acetylcholine receptor in animal species resistant to alpha-bungarotoxin. *Biochemistry* 34:9172-9176.
- Barlow, A., Pook, C. E., Harrison, R. A., and W. Wüster. 2009. Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. *Proceedings of the Royal Society B: Biological Sciences* 276:2443-2449.
- Bdolah, A., Kochva, E., Ovadia, M., Kinamon, S., and Z. Wollberg. 1997. Resistance of the Egyptian Mongoose to sarafotoxins. *Toxicon* 35:1251-1261.
- Begg, C. M., Begg, K. S., Du Toit, J. T., and M. G. L. Mills. 2003. Sexual and seasonal variation in the diet and foraging behaviour of a sexually dimorphic carnivore, the Honey Badger (*Mellivora capensis*). *Journal of Zoology* 260:301-316.
- Bergman, E. J., Garrott, R. A., Creel, S., Borkowski, J. J., Jaffe, R., and F. G. R. Watson. 2006. Assessment of prey vulnerability through analysis of wolf movements and kill sites. *Ecological Applications* 16:273-284.
- Bleicher, S. S., Kotler, B. P., Downs, C. J., and J. S. Brown. 2020. Playing to their evolutionary strengths; heteromyid rodents provide opposite snake evasion strategies in the face of known and novel snakes. *Journal of Arid Environments* 173:104025.
- Blouin-Demers, G., and P. J. Weatherhead. 2001. Habitat use by Black Rat Snakes (*Elaphe obsoleta obsoleta*) in fragmented forests. *Ecology* 82:2882-2896.
- Brierley, A. S., and M. J. Cox. 2010. Shapes of krill swarms and fish schools emerge as aggregation members avoid predators and access oxygen. *Current Biology* 20:1758-1762.

- Brodie, E. D., III, and E. D. Brodie, Jr. 2015. Predictably convergent evolution of sodium channels in the arms race between predators and prey. *Brain, Behavior and Evolution* 86:48-57.
- Brodie, E. D., Ridenhour, B. J., and E. D. Brodie. 2002. The evolutionary response of predators to dangerous prey: Hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. *Evolution* 56:2067-2082.
- Brower, L. P. and W. H. Calvert. 1985. Foraging dynamics of bird predators on overwintering Monarch Butterflies in Mexico. *Evolution* 39:852-868.
- Bundy, A., Heymans, J. J., Morissette, L., and C. Savenkoff. 2009. Seals, cod and forage fish: a comparative exploration of variations in the theme of stock collapse and ecosystem change in four Northwest Atlantic ecosystems. *Progress in Oceanography* 81:188-206.
- Canova, L., and A. Gentili. 2008. Diet of the asp viper (*Vipera aspis*) in woodland habitats of the Po plain (NW Italy). *Acta Herpetologica* 3:175-178.
- Catanese, J.J., and L. F. Kress. 1993. Opossum serum alpha1-proteinase inhibitor: purification, linear sequence, and resistance to inactivation by rattlesnake venom metalloproteinase. *Biochemistry* 32:509-515.
- Ceballos, G., Pacheco, J., and R. List. 1999. Influence of Prairie Dogs (*Cynomys ludovicianus*) on habitat heterogeneity and mammalian diversity in Mexico. *Journal of Arid Environments* 41:161-172.
- Chen, Q., Deng, H., Brauth, S. E., Ding, L., and Y. Tang. 2012. Reduced performance of prey targeting in pit vipers with contralaterally occluded infrared and visual senses. *PLoS ONE* 7:e34989.

- Chun, J. B. S., Baker, M. R., Kim, D. J., LeRoy, M., Toribo, P., and J. -P. Bingham. 2012. Cone snail milked venom dynamics – a quantitative study of *Conus purpurascens*. *Toxicon* 60:83-94.
- Clark, J. A. 1983. Moonlight's influence on predator/prey interactions between Short-Eared Owls (*Asio flammeus*) and Deermice (*Peromyscus maniculatus*). *Behavioral Ecology and Sociobiology* 13:205-209.
- Clark, R. W. 2002. Diet of the Timber Rattlesnake, *Crotalus horridus*. *Journal of Herpetology* 36: 494-499.
- Clarke, J. A., Chopko, J. T., and S. P. Mackessy. 1996. The effect of moonlight on activity patterns of adult and juvenile Prairie Rattlesnakes (*Crotalus viridis viridis*). *Journal of Herpetology* 30:192-197.
- Connolly, B. M., and J. L. Orrock. 2018. Habitat-specific capture timing of Deer Mice (*Peromyscus maniculatus*) suggests that predators structure temporal activity of prey. *Ethology* 124:105-112.
- Cook, L. M., and I. J. Saccheri. 2013. The peppered moth and industrial melanism: evolution of a natural selection case study. *Heredity* 110:207-212.
- Coss, R. G., Guse, K. L., Poran, N. S., and D. G. Smith. 1993. Development of antisnake defenses in California Ground Squirrels (*Spermophilus beecheyi*): II. Microevolutionary effects of relaxed selection from rattlesnakes. *Behavior* 124:137-164.
- Daltry, J. C., Wüster, W., and R. S. Thorpe. 1996. Diet and snake venom evolution. *Nature* 379:537-540.

- Davis, J. R., and T. C. Theimer. 2003. Increased Lesser Earless Lizard (*Holbrookia maculata*) abundance on Gunnison's Prairie Dog colonies and short term responses to artificial prairie dog burrows. *American Midland Naturalist* 150:282-290
- de Wit, C. A. 1982. Resistance of the Prairie Vole (*Microtus ochrogaster*) and the Woodrat (*Neotoma floridana*), in Kansas, to venom of the Osage Copperhead (*Agkistrodon contortrix phaeogaster*). *Toxicon* 20:709-714.
- Drabeck, D. J., Dean, A. M., and S. A. Jansa. 2015. Why the honey badger don't care: Convergent evolution of venom-targeted nicotinic acetylcholine receptors in mammals that survive venomous snake bites. *Toxicon* 99:68-72.
- Dugan, E. A., and W. K. Hayes. 2002. Diet and feeding ecology of the Red Diamond Rattlesnake, *Crotalus ruber* (Serpentes: Viperidae). *Herpetologica* 68:203-217.
- Dunson, W. A. 1969. Electrolyte excretion by the salt gland of the Galápagos Marine Iguana. *American Journal of Physiology* 216:995-1002.
- Essener, R. L. Jr., Patel, R., and S. M. Reilly. 2014. Ontogeny of body shape and diet in Freshwater Drum (*Aplodinotus grunniens*). *Transactions of the Illinois State Academy of Science* 107:27-30.
- Feldman, C. R., Brodie, E. D. Jr., Brodie, E. D., III., and M. E. Pfrender. 2012. Constraint shapes convergence in tetrodotoxin resistant sodium channels of snakes. *Proceedings of the National Academy of Sciences* 109:4556-4561.
- Ferraz, C. R., Arrahman, A., Xie, C., Casewell, N. R., Lewis, R. J., Kool, J., and F. C. Cardoso. 2019. Multifunctional toxins in snake venoms and therapeutic implications: from pain to hemorrhage and necrosis. *Frontiers in Ecology and Evolution* 7:218.

- Fitch, H. S., and H. Twining. 1946. Feeding habits of the Pacific Rattlesnake. *Copeia* 1946:64-71.
- Fitzgerald, K. T., Shipley, B. K., Newquist, K. L., Vera, R., and A. A. Flood. 2013. Additional observations and notes on the natural history of the Prairie Rattlesnake (*Crotalus viridis*) in Colorado. *Topics in Companion Animal Medicine* 28:167-76.
- Ford, N. B., and P. Hampton. 2009. Ontogenetic and sexual differences in diet in an actively foraging snake, *Thamnophis proximus*. *Canadian Journal of Zoology* 87:254-261.
- Forsman, A. 1996. Body size and net energy gain in gape-limited predators: a model. *Journal of Herpetology* 30:307-319.
- Fortin, D., Beyer, H., Boyce, M., Smith, D., Duchesne, T., and J. Mao. 2005. Wolves influence elk movements: behavior shapes a trophic cascade in Yellowstone National Park. *Ecology* 86:1320-1330.
- Freymler, G. A., Whitford, M. D., Higham, T. E., and R. W. Clark. 2017. Recent interactions with snakes enhance escape performance of Desert Kangaroo Rats (Rodentia: Heteromyidae) during simulated attacks. *Biological Journal of the Linnean Society* 122:651–660.
- Freymler, G. A., Whitford, M. D., Higham, T. E., and R. W. Clark. 2019. Escape dynamics of free-ranging Desert Kangaroo Rats (Rodentia: Heteromyidae) evading rattlesnake strikes. *Biological Journal of the Linnean Society* 127:164–172o
- Gardiner, L., Somers, C., Martino, J., Parker, D., and R. Poulin. 2013. Balancing the dumbbell: summer habitats need protection in addition to winter dens for northern snake communities. *The Journal of Wildlife Management* 77:975-982.

- Gibbs, H. L., and S. P. Mackessy. 2009. Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. *Toxicon* 53:672-679.
- Glaudas, X., and G. J. Alexander. 2016 A lure at both ends: aggressive visual mimicry signals and prey-specific luring behaviour in an ambush-foraging snake. *Behavioural Ecology and Sociobiology* 71:2.
- Glaudas, X., Glennon, K. L., Martins, M., Luiselli, L., Fearn, S., Trembath, D. F., Jelic, D., and G. J. Alexander. 2018. Foraging mode, relative prey size and diet breadth: A phylogenetically explicit analysis of snake feeding ecology. *Journal of Animal Ecology* 88:757:767.
- Glaudas, X., Jezkova, T., and J. A. Rodríguez-Robles. 2008. Feeding ecology of the Great Basin Rattlesnake (*Crotalus lutosus*, Viperidae). *Canadian Journal of Zoology* 86:723-734.
- Gloyd, H. K. 1933. An unusual feeding record for the Prairie Rattlesnake. *Copeia* 1933:98.
- Goetz, S. M., Petersen, C. E., Rose, R. K., Kleopfer, J. D. and A. H. Savitzky. 2016. Diet and foraging behaviors of Timber Rattlesnakes, *Crotalus horridus*, in eastern Virginia. *Journal of Herpetology* 50:520-526.
- Goetz, S. M., Piccolomini, S., Hoffman, M., Bogan, J., Holding, M. T., Mendonça, M. T., and D. A. Steen. 2019. Serum-based inhibition of pitviper venom by eastern indigo snakes (*Drymarchon couperi*). *Biology Open* 8:bio040964.
- Goldenberg, S. U., Borcharding, J., and M Heynen. 2014. Balancing the response to predation—the effects of shoal size, predation risk and habituation on behaviour of juvenile perch. *Behavioral Ecology and Sociobiology* 68:989-998.

- Gren, E. C. K., Kelln, W., Travis, Z. D., Fox, G., Person, C., and W. K. Hayes. 2016. Diet and venom ontogeny in insular and high-altitude populations of the Southern Pacific Rattlesnake (*Crotalus oreganus helleri*). *Toxicon* 117:108.
- Gutiérrez J. M., Lomonte B., León, G., Alape-Girón, A., Flores-Díaz, M., Sanz, L., Angulo, Y., and J. J. Calvete. 2009. Snake venomomics and antivenomics: proteomic tools in the design and control of antivenoms for the treatment of snakebite envenoming. *Journal of Proteomics* 72:165-182.
- Hammerson, G. A. 1999. Amphibians and Reptiles in Colorado. Second edition. University Press of Colorado, Niwot, Colorado.
- Hayes, W. 1992. Prey-handling and envenomation strategies of Prairie Rattlesnakes (*Crotalus v. viridis*) feeding on mice and sparrows. *Journal of Herpetology* 26:496-499.
- Hayes, W. K. 1995. Venom metering by juvenile Prairie Rattlesnakes, *Crotalus v. viridis*: effects of prey size and experience. *Animal Behaviour* 50:33-40.
- Heatwole, H., Poran, N., and P. King. 1999. Ontogenetic changes in the resistance of Bullfrogs (*Rana catesbeiana*) to the venom of Copperheads (*Agkistrodon contortrix contortrix*) and Cottonmouths (*Agkistrodon piscivorus piscivorus*). *Copeia* 1999:808-814.
- Heyborne, W. H., and S. P. Mackessy. 2013. Isolation and characterization of a taxon-specific three-finger toxin from the venom of the Green Vinesnake (*Oxybelis fulgidus*; family Colubridae). *Biochimie* 95:1923-1932
- Hibbard, E. A., and J. R. Beer. 1960. The Plains Pocket Mouse in Minnesota. *Flicker*, 32:89-94.
- Higham, T. E., Clark, R. W., Collins, C. E., Whitford, M. D., and G. A. Freymiller. 2017. Rattlesnakes are extremely fast and variable when striking at kangaroo rats in nature: three-dimensional high-speed kinematics at night. *Scientific Reports* 7:40412

- Hill, M. M. A., Powell, G. L., and A. P. Russell. 2001. Diet of the Prairie Rattlesnake, *Crotalus viridis viridis*, in southeastern Alberta. *Canadian Field-Naturalist* 115:241-246.
- Hobert, J. P., Montgomery, C. E., and S. P. Mackessy. 2004. Natural history of the Massasauga, *Sistrurus catenatus edwardsii*, in southeastern Colorado. *Southwestern Naturalist* 49:321-326.
- Holding, M. L., Biardi, J. E., and H. L. Gibbs. 2016. Coevolution of venom function and venom resistance in a rattlesnake predator and its squirrel prey. *Proceedings of the Royal Society B*. 283:20152841.
- Holding, M. L., Drabek, D. H., Jansa, S. A., and H. L. Gibbs. 2016. Venom resistance as a model for understanding the molecular basis of complex coevolutionary adaptations. *Integrative and Comparative Biology* 56:1032-1043.
- Holycross, A. and S. P. Mackessy. 2002. Variation in the diet of *Sistrurus catenatus* (Massasauga), with emphasis on *S. c. edwardsii* (Desert Massasauga). *Journal of Herpetology* 36:454-464.
- Jansa, S. A., and R. S. Voss. 2011. Adaptive evolution of the venom-targeted vWF protein in opossums that eat pitvipers. *PLoS ONE* 6:e20997.
- Jayne, B. C., Voris, H. K., and P. K. L. Ng. 2002. Snake circumvents constraints on prey size. *Nature* 418:143.
- Kiriake, A., Ishizaki, S., Nagashima, Y., and K. Shiomi. 2017. Occurrence of a Stonefish toxin-like toxin in the venom of the Rabbitfish *Siganus fuscescens*. *Toxicon* 140: 139-146.
- Kittle, A. M., Fryxell, J. M., Desy, G. E., and J. Hamr. 2008. The scale-dependent impact of Wolf predation risk on resource selection by three sympatric ungulates. *Oecologia* 157:163-175.

- Klauber, L. M. 1956. Rattlesnakes: Their Habits, Life Histories, and Influence on Mankind (abridged edition). University of California Press, Berkeley, California.
- Kross, S. M., Barbour, R. P., and B. L. Martinico. 2016. Agricultural land use, Barn Owl diet, and vertebrate pest control implications. *Agriculture, Ecosystems & Environment* 223:167-174.
- Lambert, S., and W. H. Reid. 1981. Biogeography of the Colorado herpetofauna. *The American Midland Naturalist* 106: 145-156.
- Langley, W. M. 1994. Comparison of predatory behaviors of Deer Mice (*Peromyscus maniculatus*) and Grasshopper Mice (*Onychomys leucogaster*). *Journal of Comparative Psychology* 108:394-400.
- Lee, C.-Y., Yo, S.-P., Clark, R. W., Hsu, J. -Y., Liao, C.-P., Tseng, H.-Y., and W.-S. Huang. 2018. The role of different visual characters of weevils signalling aposematism to sympatric lizard predators. *Journal of Zoology* 306:36-47.
- Ligabue-Braun, R., Verli, H., and C. R. Carlini. 2012. Venomous mammals: a review. *Toxicon* 59: 680-695.
- Lignot, J. -H., Helmstetter, C., and S. M. Secor. 2005. Postprandial morphological response of the intestinal epithelium of the Burmese Python (*Python molurus*). *Comparative Biochemistry and Physiology, Part A* 141:280-291.
- Loughran, C. L., Nowak, E. M., Schofer, J., Sullivan, K. O., and B. K. Sullivan. 2013. Lagomorphs as prey of Western Diamond-Backed Rattlesnakes (*Crotalus atrox*) in Arizona. *The Southwestern Naturalist* 58:502-505.
- Loughry, W. J. 1987. Harassment by Black-Tailed Prairie Dogs. *Behaviour* 103:27-48.

- Luther, D. A. and E. P. Derryberry. 2012. Birdsongs keep pace with city life: changes in song over time in an urban songbird affects communication. *Animal Behaviour* 83:1059-1066.
- Macartney, J. M. 1989. Diet of the Northern Pacific Rattlesnake, *Crotalus viridis oregonus*, in British Columbia. *Herpetologica* 45:299-304.
- Mackessy, S. P. 1988. Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *Crotalus viridis oregonus*. *Copeia* 1988:92-101.
- Mackessy, S. P. 2010. Evolutionary trends in venom composition in the Western Rattlesnakes (*Crotalus viridis* sensu lato): toxicity vs. tenderizers. *Toxicon* 55:1463-1474
- Mackessy, S. P. 2010a. Handbook of Venoms and Toxins of Reptiles. CRC Press/Taylor & Francis Group, Boca Raton, Florida.
- Mackessy, S. P. 2010b. The evolution of venom composition in the Western Rattlesnakes (*Crotalus viridis* sensu lato): toxicity versus tenderizers. *Toxicon* 55:1463-1474.
- Mackessy, S. P. 2017. The Desert Massasauga (*Sistrurus tergeminus edwardsii*) in Colorado: from biome to proteome. Pp. 253–264 in Dreslik, M. J., W. K. Hayes, S. J. Beaupre, and S. P. Mackessy (eds.), The Biology of Rattlesnakes II. ECO Herpetological Publishing and Distribution, Rodeo, New Mexico.
- Mackessy, S. P. Desert Massasauga Rattlesnake (*Sistrurus catenatus edwardsii*): a technical conservation assessment. 2005. USDA Forest Service, Rocky Mountain Region. 57 p. <http://www.fs.fed.us/r2/projects/scp/assessments/massasauga.pdf>
- Mackessy, S. P., and A. J. Saviola. 2016. Understanding biological roles of venoms among the Caenophidia: the importance of rear-fanged snakes. *Integrative and Comparative Biology* 56:1004-1021.

- Mackessy, S. P., Leroy, J., Mociño-Deloya, E., Setser, K., Bryson, R. W., and A. J. Saviola. 2018. Venom ontogeny in the Mexican Lance-Headed Rattlesnake (*Crotalus polystictus*). *Toxins* 10:271.
- Mackessy, S. P., and A. J. Saviola. 2016. Understanding biological roles of venoms among the Caenophidia: the importance of rear-fanged snakes. *Integrative and Comparative Biology* 56:1004-1021.
- Mackessy, S. P., Sixberry, N. M., Heyborne, W. H. and T. Fritts. 2006 Venom of the Brown Treesnake, *Boiga irregularis*: ontogenetic shifts and taxa-specific toxicity. *Toxicon* 47:537-548.
- Mackessy, S. P., Williams, K., and K. G. Ashton. 2003. Ontogenetic variation in venom composition and diet of *Crotalus oreganus concolor*: a case of venom paedomorphosis? *Copeia* 2003:769-782.
- Mackessy, S. P., Williams, K., and K. Ashton. 2003. Characterization of the venom of the Midget Faded Rattlesnake (*Crotalus oreganus concolor*): a case of venom paedomorphosis? *Copeia* 4:769-782.
- Mackessy, S.P. 2010. The evolution of venom composition in the Western Rattlesnakes (*Crotalus viridis* sensu lato): toxicity versus tenderizers. *Toxicon* 55:1463-1474.
- Madsen, T. and R. Shine. 1996. Seasonal migration of predators and prey--a study of pythons and rats in tropical Australia. *Ecology* 77: 149-156
- Maerz, J. C., Karuzas, J. M., Madison, D. M., and B. Blossey. 2005. Introduced invertebrates are important prey for a generalist predator. *Diversity and Distributions* 11:83-90.
- Maritz, B., Alexander, G. J., and R. A. Maritz. 2019. The underappreciated extent of cannibalism and ophiophagy in African cobras. *Ecology* 100:e02522.

- Metz, M., Piliponsky, A. M., Chen, C. C., Lammel, V., Abrink, M., Pejler, G., Tsai, M., and S. J. Galli. 2006. Mast cells can enhance resistance to snake and honeybee venoms. *Science* 313:526-530.
- Modahl, C. M., Mrinalini, Frietze S. E., and S. P. Mackessy. 2018. Adaptive evolution of prey-specific three-finger toxins in the Amazon Puffing Snake, *Spilotes sulphureus*. *Proceedings of the Royal Society B – Biological Sciences* 285:20181003.
- Modahl, C. M., Roointan, A., Rogers, J., Currier, K., and S. P. Mackessy. 2020 Interspecific and intraspecific venom enzymatic variation among Cobras (*Naja* sp. and *Ophiophagus hannah*). *Comparative Biochemistry and Physiology, Part C* 232:108743.
- Nachappa, P., Margolies, D. C., Nechols, J. R., and J. F. Campbell. 2011. Variation in predator foraging behaviour changes predator-prey spatio-temporal dynamics. *Functional Ecology* 25:1309-1317.
- Naef-Danzer, B., and M. U. Grüebler. 2016. Post-fledging survival of altricial birds: ecological determinants and adaptation. *Journal of Field Ornithology* 87:227-250
- Neumann, D., Barchan, D., Horowitz, M., Kochva, E., and S. Fuchs. 1989. Snake acetylcholine receptor: cloning of the domain containing the four extracellular cysteines of the alpha sub-unit. *Proceedings of the National Academy of Sciences* 86:7255-7259.
- Niermann, C. N., Tate, T. G. Suto, A. L., Barajas, R., White, H. A., Guswiler, O. D., Secor, S. M., Rowe, A. H., and M P. Rowe. 2020. Defensive venoms: is pain sufficient for predator deterrence? *Toxins* 12:260.
- Nowak, E. M., Schuett, G. W., Theimer, T. C., Sisk, T. D., and K. Nishikawa. 2015. Does short-term provisioning of resources to prey result in behavioral shifts by rattlesnakes? *Wildlife Management* 79:357-372

- O'Donnell, R. P., Shine, R., and R. T. Mason. 2004. Seasonal anorexia in the male Red-Sided Garter Snake, *Thamnophis sirtalis parietalis*. *Behavioral Ecology and Sociobiology* 56:413-419.
- Orrock, J. L. 2010. When the ghost of predation has passed: do rodents from islands with and without fox predators exhibit aversion to fox cues? *Ethology* 116: 338-345.
- Orrock, J. L., and R. J. Fletcher, Jr. 2014. An island-wide predator manipulation reveals immediate and long-lasting matching of risk by prey. *Proceedings of the Royal Society B* 281:20140391.
- Pahari, S., S.P. Mackessy and R.M. Kini. 2007. The venom gland transcriptome of the Desert Massasauga Rattlesnake (*Sistrurus catenatus edwardsii*): towards an understanding of venom composition among advanced snakes (superfamily Colubroidea). *BMC Molecular Biology* 8:115.
- Pawlak, J., Mackessy, S. P., Fry, B. G., Bhatia, M., Mourier, G., Fruchart-Gaillard, C., Servent, D., Ménez, R., Stura, E., Ménez A., and R. M. Kini. M. 2006. Denmotoxin: a three-finger toxin from colubrid snake *Boiga dendrophila* (Mangrove Catsnake) with bird-specific activity. *Journal of Biological Chemistry* 281:29030-29041.
- Pawlak, J., Mackessy, S. P., Sixberry, N. M., Stura, E. A., Le Du, M. H., Menez, R., Foo, C. S., Menez, A., Nirthanan S., and R. M. Kini. 2009. Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. *FASEB Journal* 23:534-545.
- Pearson, D., Shine, R., and R. How. 2002. Sex-specific niche partitioning and sexual size dimorphism in Australian Pythons (*Morelia spilota imbricata*). *Biological Journal of the Linnean Society* 77:113-125.

- Perales, J., Neves-Ferreira, A. G. C., Valente, R. H., and G. B. Domont. 2005. Natural inhibitors of snake venom hemorrhagic metalloproteinases. *Toxicon* 45:1013–1020.
- Perez, J. C., and E. E. Sanchez. 1999. Natural protease inhibitors to hemorrhagins in snake venoms and their potential use in medicine. *Toxicon* 37:703–728.
- Perez, J. C., Haws, W. C., and C. H. Hatch. 1978. Resistance of woodrats (*Neotoma micropus*) to *Crotalus atrox* venom. *Toxicon* 16:198-200.
- Perez, J. C., Pichyangkul, S., and V. E. Garcia. 1979. The resistance of three species of warm-blooded animals to Western diamondback rattlesnake (*Crotalus atrox*) venom. *Toxicon* 17:601–607.
- Phillips, B. L., Greenlees, M. J., Brown, G. P., and R. Shine. 2010. Predator behaviour and morphology mediates the impact of an invasive species: Cane Toads and Death Adders in Australia. *Animal Conservation* 13: 53-59.
- Phillips, M. A., Waterman, J. M., Du Plessis, P., Smit, M., and N. C. Bennett. 2012. No evidence for proteolytic venom resistance in southern African ground squirrels. *Toxicon* 60:760-763.
- Platt, S. G., Rainwater, T. R., Leavitt, D. J., and S. M. Miller. 2009. Diet of Barn Owls (*Tyto alba*) in Northern Belize. *Southwestern Naturalist* 54:104-107.
- Pomoto, A. M., Perry, B. W., Denton, R. D., Gibbs, H. L., and M. L. Holding. 2016. No safety in the trees: local and species-level adaptation of an arboreal squirrel to the venom of sympatric rattlesnakes. *Toxicon* 118:149-155.
- Poran, N. S., and H. Heatwole. 1995. Resistances of sympatric and allopatric eels to sea snake venoms. *Copeia* 1995(1):136-147.

- Poran, N. S., Coss, R. G., and E. Benjamini. 1987. Resistance of California ground squirrels (*Spermophilus beecheyi*) to the venom of the Northern Pacific rattlesnake (*Crotalus viridis oregonus*): A study of adaptive variation. *Toxicon* 25:767-777.
- Pough, F. H. 1980. The advantages of ectothermy for tetrapods. *American Naturalist* 115:92-112.
- Prenter, J., Weldon, C. W., and P. W. Taylor. 2013. Age-related activity patterns are moderated by diet in Queensland Fruit Flies *Bactrocera tryoni*. *Physiological Entomology* 38:260-267.
- Reinert, H., MacGregor, G., Esch, M., Bushar, L., and R. Zappalorti. 2011. Foraging ecology of Timber Rattlesnakes, *Crotalus horridus*. *Copeia* 2011:430-442.
- Reiserer, R. S., Schuett, G. W., and H. W. Greene. 2018. Seed ingestion and germination in rattlesnakes: overlooked agents of rescue and secondary dispersal. *Proceedings of the Royal Society B* 285:20172755.
- Rodriguez-Robles, J. A. 2002. Feeding ecology of North American Gopher Snakes (*Pituophis catenifer*, Colubridae). *Biological Journal of the Linnean Society* 77:165-183.
- Rowe A. H., and M. P. Rowe. 2008. Physiological resistance of Grasshopper Mice (*Onychomys spp.*) to Arizona Bark Scorpion (*Centruroides exilicauda*) venom. *Toxicon* 52:597-605.
- Rowe, A. H., and M. P. Rowe. 2015. Predatory grasshopper mice. *Current Biology* 25:R1023-R1026.
- Rowe, A. H., and M. P. Rowe. 2006. Risk assessment by Grasshopper Mice (*Onychomys spp.*) feeding on neurotoxic prey (*Centruroides spp.*). *Animal Behaviour* 71:725-734.
- Rowe, A. H., and M. P. Rowe. 2008. Physiological resistance of grasshopper mice (*Onychomys spp.*) to Arizona Bark Scorpion (*Centruroides exilicauda*) venom. *Toxicon* 52:597-605.

- Rowe, A. H., Xiao, Y., Rowe, M. P., Cummins, T. R., and H. H. Zakon. 2013. Voltage-gated sodium channel in Grasshopper Mice defends against Bark Scorpion toxin. *Science* 342:441-446.
- Sanz, L., Gibbs, H. L., Mackessy, S. P., and J. J. Calvete. 2006. Venom proteomes of closely related *Sistrurus* rattlesnakes with divergent diets. *Journal of Proteome Research* 5:2098-2112.
- Savarino-Drago, A., and I. Ruvalcaba-Ortega. 2019. A new bird nest predator: Mexican Dusky Rattlesnake (*Crotalus triseriatus*) predation on Sierra Madre Sparrow (*Xenospiza baileyi*) nestlings. *Wilson Journal of Ornithology* 131:663-666.
- Saviola, A. J., Chiszar, D., Busch, C., and S. P. Mackessy. 2013. Molecular basis for prey relocation in viperid snakes. *BMC Biology* 11:20.
- Saviola, A. J., Chiszar, D., and S. P. Mackessy. 2012. Ontogenetic shift in response to prey-derived chemical cues in Prairie Rattlesnakes *Crotalus viridis viridis*. *Current Zoology* 58:549-555.
- Saviola, A. J., Pla, D., Sanz, L., Castoe, T. A., Calvete, J. J., and S. P. Mackessy. 2015. Comparative venomomics of the Prairie Rattlesnake (*Crotalus viridis viridis*) from Colorado: identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom CroFab®. *Journal of Proteomics* 121:28-43.
- Schild, D. R., Card D. C., Hales N. R., Perry, B. W., Pasquesi, G. I. M., Blackmon, H., Adams, R. H., Corbin, A. B., Smith, C. F., Ramesh, B., Demuth, J. P., Betrán, E., Tollis, M., Meik, J. M., Mackessy, S. P., and T. A. Castoe.. 2019. The origins and evolution of

- chromosomes, dosage compensation, and mechanisms underlying venom regulation in snakes. *Genome Research* 29:590-601.
- Schraft, H. A., and R. W. Clark. 2017. Kangaroo rats change temperature when investigating rattlesnake predators. *Physiology & Behavior* 173:174-178.
- Schuett, G. W, Repp, R. A., Spencer, C. L., Beaman, K. R., and C. W. Painter. 2016. Western Diamond-backed Rattlesnake, *Crotalus atrox* (Baird and Girard 1853). In Rattlesnakes of Arizona. Eds: G.W. Schuett, M.J. Feldner, R.S. Reiserer, and C.F. Smith. Pp. 333-394
- Secor, S. M. 2003. Gastric function and its contribution to the postprandial metabolic response of the Burmese Python, *Python molurus*. *Journal of Experimental Biology* 206:1621-1630.
- Secor, S. M., and J. Diamond. 1998. A vertebrate model of extreme physiological regulation. *Nature* 395:659-662.
- Servent, D., Menez, R., Stura, E., Menez, A., and R. M. Kini. 2006. Denmotoxin, a three-finger toxin from the Colubrid snake *Boiga dendrophila* (Mangrove Catsnake) with bird-specific activity. *The Journal of Biological Chemistry* 281:29030-29041.
- Sherbrooke, W. 1991. Behavioral (predator-prey) interactions of captive Grasshopper Mice (*Onychomys torridus*) and horned lizards (*Phrynosoma cornutum* and *P. modestum*). *American Midland Naturalist* 126:187-195.
- Shine, R., Reed, R. N., Shetty, S., and H. G. Cogger. 2002. Relationships between sexual dimorphism and niche partitioning within a clade of sea-snakes (Laticaudinae). *Oecologia* 133:45-53.
- Shipley, B. K. Reading, R. P., and B. J. Miller. 2008. Capture rates of reptiles and amphibians on Black-Tailed Prairie Dog (*Cynomys ludovicianus*) colonies and on uncolonized prairie in Colorado. *Western North American Naturalist* 68:245-248.

- Shiple, B. K., Chiszar, D., Fitzgerald, K. T., and A. Saviola. J. 2013. Spatial ecology of Prairie Rattlesnakes (*Crotalus viridis*) associated with Black-Tailed Prairie Dog (*Cynomys ludovicianus*) colonies in Colorado. *Herpetological Conservation and Biology* 8:240-250.
- Shiple, B. K., and R. P. Reading. 2006. A comparison of herpetofauna and small mammal diversity on Black-Tailed Prairie Dog (*Cynomys ludovicianus*) colonies and non-colonized grasslands in Colorado. *Journal of Arid Environments* 66:27-41.
- Sinclair, E. H., and T. K. Zeppelin. 2002. Seasonal and spatial differences in diet in the western stock of Steller Sea Lions. *Journal of Mammalogy* 83:973-990.
- Sintiprungrat, K., Watcharatanyatipa, K., Senevirathnec, W. D. S. T., Chaisuriyaa, P., Chokchaichamnankit, D., Srisomsap, C., and K. Ratanabanangkoona. 2016. A comparative study of venomomics of *Naja naja* from India and Sri Lanka, clinical manifestations and antivenomics of an Indian polyspecific antivenom. *Journal of Proteomics* 30:131-143.
- Sivan, J., Kam, M., Hadad, S., Degen, A. A., Rozenboim, I., and A. Rosenstrauch. 2013. Temporal activity and dietary selection in two coexisting desert snakes, the Saharan Sand Viper (*Cerastes vipera*) and the Crowned Leafnose (*Lytorhynchus diadema*). *Zoology* 116:113-117.
- Slip, D., and R. Shine. 1988. Feeding habits of the Diamond Python, *Morelia s. spilota*: ambush predation by a boid snake. *Journal of Herpetology* 22:323-330.
- Smiley-Walters, S. A., Farrell, T. M., and H. L. Gibbs. 2018. The importance of species: Pygmy Rattlesnake venom toxicity differs between native prey and related non-native species. *Toxicon* 144:42-47.

- Smiley-Walters, S., Farrell, T. M., and H. L. Gibbs. 2017. Evaluating local adaptation of a complex phenotype: reciprocal tests of Pigmy Rattlesnake venoms on treefrog prey. *Oecologia* 184:739-748.
- Smith, M. T. 2014. Induction of phenotypic plasticity in rattlesnake trophic morphology by diet manipulation. *Journal of Morphology* 275:1339-1348.
- Stiles, B. G., Sexton, F. W., and S. A. Weinstein. 1991. Antibacterial effects of different snake venoms: purification and characterization of antibacterial proteins from *Pseudechis australis* (Australian King Brown or Mulga Snake) venom. *Toxicon* 29:1129-1141.
- Strickland, J. L., Smith, C. F., Mason, A. J., Schield, D. R., Borja, M., Castañeda-Gaytán, G., Spencer, C. L., Smith, L. L., Trápaga, A., Bouzid, N. M., Campillo-García, G., Flores-Villela, O. A., Antonia-Rangel, D., Mackessy, S. P., Castoe, T. A., Rokyta, D. R., and C. L. Parkinson. 2018. Evidence for divergent patterns of local selection driving venom variation in Mojave Rattlesnakes (*Crotalus scutulatus*). *Scientific Reports* 8:1-15.
- Suzuki, H., Nunome, M., Kinoshita, G., Aplin, K.P., Vogel, P., Kryukov, A.P., Jin, M.L., Han, S.H., Maryanto, I., Tsuchiya, K., Ikeda, H., Shiroishi, T., Yonekawa, H., and K. Moriwaki. 2013. Evolutionary and dispersal history of Eurasian House Mice *Mus musculus* clarified by more extensive geographic sampling of mitochondrial DNA. *Heredity* 111:375-390.
- Takacs, Z., Wilhelmsen, K.C., and S. Sorota. 2004. Cobra (*Naja spp.*) nicotinic acetylcholine receptor exhibits resistance to Erabu Sea Snake (*Laticauda semifasciata*) short-chain a-neurotoxin. *Journal of Molecular Evolution* 58:516–526.
- Taylor, E. N. 2001. Diet of the Baja California Rattlesnake, *Crotalus enyo* (Viperidae). *Copeia* 2001:553-555.

- Teta, P., Herculini, C., and G. Cueto. 2012. Variation in the diet of Western Barn Owls (*Tyto alba*) along an urban-rural gradient. *The Wilson Journal of Ornithology* 124:589-596.
- Thaker, M., Vanak, A. T., Owen, C. R., Ogden, M. B., and R. Slotow. 2010. Group dynamics of Zebra and Wildebeest in a woodland savanna: effects of predation risk and habitat density. *PLoS One* 5(9):e12758.
- Thomas, R. G., and F. H. Pough. 1979. The effect of rattlesnake venom on digestion of prey. *Toxicon* 17:221-228.
- Thompson, I. D., Wiebe, P. A., Mallon, E., Rodgers, A. R., Fryxwell, J. M., Baker, J. A., and D. Reid. 2015. Factors influencing the seasonal diet selection by Woodland Caribou (*Rangifer tarandus tarandus*) in boreal forests in Ontario. *Canadian Journal of Zoology* 93:87-98.
- Tichy, H., Zaleska-Rutczynska, Z., O'Huigin, C., Figueroa, F., and J. Klein. 1994. Origin of the North American House Mouse. *Folia Biologica* 40:483-496.
- Troxell-Smith, S. M., Tutka, M. J., Albergo, J. M., Balu, D., Brown, J. S., and J.P. Leonard. 2016. Foraging decisions in wild versus domestic *Mus musculus*: what does life in the lab select for? *Behavioural Processes* 122:43-50.
- Turko, P., Tellenbach, C., Keller, E, Tardent, N, Keller, B., Spaak, P., and J. Wolinska. 2018. Parasites driving host diversity: incidence of disease correlated with *Daphnia* clonal turnover. *Evolution* 72:619-629.
- Van Valen, L. 1973. A new evolutionary law. *Evolutionary Theory* 1:1-30.
- Wallace, R. L., and L. V. Diller. 1990. Feeding ecology of the rattlesnake, *Crotalus viridis oregonus*, in Northern Idaho. *Journal of Herpetology* 24:246-253.

- Ward-Smith, H., Arbuckle, K., Naude, A., and W. Wüster. 2020. Fangs for the memories? A survey of pain in snakebite patients does not support a strong role for defense in the evolution of snake venom composition. *Toxins* 12:201.
- Wasko, D. K., and M. Sasa. 2009. Activity patterns of a neotropical ambush predator: spatial ecology of the Fer-De-Lance (*Bothrops asper*, Serpentes: Viperidae) in Costa Rica. *Biotropica* 41:241-249.
- Wastell, A. R., and S. P. Mackessy. 2011. Spatial ecology of the Desert Massasauga Rattlesnake (*Sistrurus catenatus edwardsii*) in southeastern Colorado: habitat and resource utilization. *Copeia* 2011:29–37.
- Wastell, A. R., and S. P. Mackessy. 2016. Desert Massasauga Rattlesnakes (*Sistrurus catenatus edwardsii*) in southeastern Colorado: life history, reproduction, and communal hibernation. *Journal of Herpetology* 50:594-603.
- Webb, J. K., and M. J. Whiting. 2005. Why don't small snakes bask? Juvenile Broad-Headed Snakes trade thermal benefits for safety. *Oikos* 110:515-522.
- Webb, J. K., and R. Shine. 1998. Using thermal ecology to predict retreat-site selection by an endangered snake species. *Biological Conservation*, 86:233-242.
- Webber, M. M., Jezkova, T., J. A. Rodriguez-Robles. 2016. Feeding ecology of Sidewinder Rattlesnakes, *Crotalus cerastes* (Viperidae). *Herpetologica* 72:324-330.
- Werner, R. M., and J. A. Vick. 1977. Resistance of the Opossum (*Didelphis virginiana*) to envenomation by snakes of the family Crotalidae. *Toxicon* 15:29-32.
- White, J. A., and K. Geluso. 2007. Seasonal differences in onset of surface activity of Ord's Kangaroo Rat (*Dipodomys ordii*). *Journal of Mammalogy* 88:234-240.

- Whitford, M. D., Freymiller, G. A., and R. W. Clark. 2019. Managing predators: the influence of kangaroo rat antipredator displays on sidewinder rattlesnake hunting behavior. *Ethology* 125:450-456.
- Whitford, M., D., Freymiller, G. A., and R. W. Clark. 2017. Avoiding the serpent's tooth: predator–prey interactions between free-ranging Sidewinder Rattlesnakes and Desert Kangaroo Rats. *Animal Behaviour* 130:73-78.
- Willey, D. W. 2013. Diet of the Mexican Spotted Owls in Utah and Arizona. *The Wilson Journal of Ornithology* 125:775-781.
- Williams, S. H., Peiffer, E., and S. Ford. 2009. Gape and bite force in the rodents *Onychomys leucogaster* and *Peromyscus maniculatus*: does jaw-muscle anatomy predict performance? *Journal of Morphology* 270:1338-1347.
- Wohl, E. 2013. Landscape-scale carbon storage associated with Beaver dams. *Geophysical Research Letters* 40:3631-3636.
- Wolfe, A. K., Bateman, P. W., and P. A. Fleming. 2018. Does urbanization influence the diet of a large snake? *Current Zoology* 64:311-318.
- World Health Organization. 2019. Snakebite Envenoming. A Strategy for Prevention and Control. Geneva, Switzerland: World Health Organization. 50 p.  
<https://www.who.int/snakebites/resources/9789241515641/en/>
- Zimmerman, G., Stapp, P., and B. Van Horne. 1996. Seasonal variation in the diet of Great Horned Owls (*Bubo virginianus*) on shortgrass prairie. *The American Midland Naturalist* 136:149-156.

APPENDIX A  
INSTITUTIONAL ANIMAL CARE AND USE  
COMMITTEE APPROVALS



UNIVERSITY OF  
**NORTHERN COLORADO**

---

**Institutional Animal Care and Use Committee**

Date: *August 19, 2020*

Principal Investigator: *Dr. Stephen Mackessy*

Committee Action: **IACUC Protocol- Amendment Approval**  
Action Date: *August 19, 2020*

Protocol Number: *1905D-SM-SBirdsLM-22*  
Protocol Title: *Toxicity of Venoms and Purified Toxins to Rodents, Lizards and Birds*

Expiration Date: *March 28, 2021*

The University of Northern Colorado Institutional Animal Care and Use Committee (IACUC) APPROVED your amendment to animal use protocol, *Toxicity of Venoms and Purified Toxins to Rodents, Lizards and Birds–1905D-SM-SBirdsLM-22*. All requested changes (addition of ketamine use for venom extraction) are incorporated into this protocol and are effective as of August 19, 2020.

The committee's review was based on the requirements of the Government Principles, Public Health Policy, USDA Animal Welfare Act and Regulations, the Guide for the Care and Use of Laboratory Animals, as well as university policies and procedures related to the care and use of animals at the UNC. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI is approved to perform the experiments or procedures as described in the amendment request as approved by the committee.

If you have any questions, please contact the UNC Animal Care and Use Program (ACUP) Director, Laura Martin, at 734-730-6631 or via e-mail at [laura.martin@unco.edu](mailto:laura.martin@unco.edu). Additional information concerning the requirements for the protection and use of animal subjects at UNC may be found at the ACUP website, <https://www.unco.edu/research/research-integrity-and-compliance/iacuc/>, at the Office of Laboratory Animal Welfare website, <https://olaw.nih.gov/>, or at the USDA Animal Plant and Health Inspection Services.

Sincerely,

Laura W. Martin  
Director of Compliance and Operations  
Animal Care and Use Program

OLAW Assurance: D16-00579  
USDA Registration: 84-R-0008



## IACUC Memorandum

To: Dr. Steven Mackessy  
From: Laura Martin, Director of Compliance and Operations  
CC: IACUC Files  
Date: 3/28/2019  
Re: IACUC Protocol 1905D-SM-SBirdsLM-22 Approval

---

The UNC IACUC has completed a final review of your protocol "Toxicity of Venoms and Purified Toxins to Rodents, Lizards and Birds". 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 1905D-SM-SBirdsLM-22.

The next annual review will be due before March 28, 2020.

Sincerely,

A handwritten signature in black ink, appearing to read "Laura Martin", written over a horizontal line.

Laura Martin, Director of Compliance and Operations



UNIVERSITY OF  
**NORTHERN COLORADO**

Institutional Animal Care and Use Committee

To: Dr. Stephen Mackessy and Dr. Todd Castoe  
From: Laura Martin, Director of Compliance and Operations  
CC: IACUC Files  
Date: March 12, 2020  
Re: IACUC Protocol 2004D-SM-S-23 Approval

---

The University of Northern Colorado IACUC has completed a final review of your protocol, *Systematics, Introgression, and Adaptation in Western Rattlesnakes: A Model System for Studying Gene Flow, Selection, and Speciation. Analysis of Venoms from Viperid Snakes - Biochemical Composition and Activities*. The protocol review was based on the requirements of Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the Public Health Policy on Humane Care and Use of Laboratory Animals; and the USDA Animal Welfare Act and Regulations. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. This protocol has been assigned the following number 2004D-SM-S-23.

The next annual review will be due before March 12, 2021.

Sincerely,

A handwritten signature in black ink, appearing to read "Laura Martin".

Laura Martin  
Director of Compliance and Operations  
Animal Care and Use Program

OLAW Assurance: D16-00579  
USDA Registration: 84-R-0008