Toxicon:X 21 (2024) 100179

Contents lists available at ScienceDirect

Toxicon: X

journal homepage: www.journals.elsevier.com/toxicon-x

A plethora of rodents: Rattlesnake predators generate unanticipated patterns of venom resistance in a grassland ecosystem

Neil R. Balchan [a,b], Cara F. Smith [a,c], Stephen P. Mackessy [a,*]

[a] Department of Biological Sciences, University of Northern Colorado, Greeley, CO, 80639, USA
[b] Department of Integrative Biology, Oklahoma State University, Stillwater, OK, 74078, USA
[c] Department of Biochemistry and Molecular Genetics, 12801 East 17th Avenue, University of Colorado Denver, Aurora, CO, 80045, USA

ARTICLE INFO

Handling Editor: Dr. Ray Norton

Keywords:
Local adaptation
Evolution
Predator-prey dynamics
Geographical variation
Adaptation
Coevolution

ABSTRACT

Predation has the potential to impart strong selective pressures on organisms within their environments, resulting in adaptive changes in prey that minimize risk of predation. Pressures from venomous snakes present an exceptional challenge to prey, as venom represents a unique chemical arsenal evolutionarily tailored to incapacitate prey. In response, venom resistance has been detected in various snake prey species, and to varying 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 trophic ecology, and 2) sympatric and allopatric patterns of venom resistance. Resistance was measured using serum-based metalloproteinase inhibition assays to determine potential inhibition of proteolytic activity, augmented by median lethal dose (LD_{50}) assays on rodent species to assess toxicity of crude venoms. Resistance is present in several rodent species, with strong resistance present in populations of Eastern Woodrat (*Neotoma floridana*), Ord's Kangaroo Rat (*Dipodomys ordii*), and Northern Grasshopper Mouse (*Onychomys leucogaster*). Resistance is less developed in other species, including the House Mouse (*Mus musculus*) and Plains Pocket Mouse (*Perognathus flavescens*). An unexpected differential is present, where Lincoln County Kangaroo Rats are highly resistant to venom of co-occurring Prairie Rattlesnakes yet are sensitive to an allopatric population of Prairie Rattlesnakes in Weld County. Lincoln Co. Northern Grasshopper Mice also demonstrate extremely elevated resistance to Weld Co. Prairie Rattlesnake venoms, and they may possess resistance mechanisms for myotoxin a, an abundant component of Weld Co. *C. v viridis* venoms. This study illustrates the complexity of venom resistance in biological communities that can exist when incorporating multiple species interactions. Future studies aimed at characterizing resistance mechanisms at the molecular level will provide a more detailed physiological context for understanding mechanisms by which resistance to venoms occurs.

1. Introduction

Predation can exert strong selective pressures on organisms that may result in an array of plastic (Goldenberg et al., 2014) or evolved changes (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 altering activity patterns across a landscape (Fortin et al., 2005), or group dynamics changing in the face of increased predation (Thaker et al., 2010). Predation is a strong source of pressure for many organisms, and venomous predators are reliant on toxins to acquire their prey.

Venoms are complex chemical arsenals comprised of various proteins, peptides and other compounds that incapacitate and subdue prey and/or provide protection to an organism in a defensive context (Mackessy, 2010, 2022). Venoms are widespread in many animal phyla (Sunagar and Moran, 2015) such as various invertebrates (Chun et al., 2012), fishes (Kiriake et al., 2017), reptiles (Mackessy, 2010; Mackessy and Saviola, 2016), and even several mammals (Ligabue-Braun et al., 2012). Venomous snakes are distinct among venomous organisms for several reasons, one of which being that their venoms are highly optimized for prey acquisition (Pawlak et al., 2006, 2009; Barlow et al.,

* Corresponding author.
E-mail address: stephen.mackessy@unco.edu (S.P. Mackessy).

https://doi.org/10.1016/j.toxcx.2023.100179
Received 22 August 2023; Received in revised form 5 November 2023; Accepted 20 November 2023
Available online 1 December 2023

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

As a result of strong predation pressures exerted on a prey community by snakes, and more specifically by their venoms, various organisms possess evolved mechanisms to detoxify snake venoms. Frequently, resistance to snake venom metalloproteinases is used as a proxy for overall resistance to venoms, as this toxin family is typically abundant in rattlesnake venoms and has been labeled a "gateway toxin", presumed to facilitate distribution of other toxins via lytic activity toward structural and other proteins of prey (ie, Garcia and Perez, 1984; Biardi et al., 2000, 2011; Phillips et al., 2012; Holding et al., 2016a,b; Ukken et al., 2022). Many prey species have been subjected to intense selective pressures associated with venoms over their evolutionary histories, and as a result, resistance is widespread among them. Varying levels of resistance to rattlesnake venoms have evolved in squirrels (*Sciurus* spp: Pomento et al., 2016; *Otospermophilus* sp.: Holding et al., 2016a,b), small terrestrial rodents (Perez et al., 1979; de Wit, 1982), and various lizards and amphibians (Smiley-Walters et al., 2018; Grabowsky and Mackessy, 2019). This wide diversity of taxa possessing resistance indicates that selection strongly favors this trait in prey species.

With the occurrence of endogenous venom resistance mechanisms comes the potential for co-evolution, as a predator's venom and prey's resistance continue to evolve in tandem. This back-and-forth between predator and prey can create an evolutionary arms-race, as outlined by the Red Queen Hypothesis (Van Valen, 1973). Evolutionary arms races have been explored in various of biological systems ranging from parasite-host dynamics (Turko et al., 2018) to predator-prey relationships (Brodie et al., 2002; Hague et al., 2020).

When considering interactions between rodents and venomous snakes, an arms race dynamic can occur where venom resistance and venom toxicity are pitted against one another in evolutionary time. In one well-characterized example, the Northern Pacific Rattlesnake (*Crotalus o. oreganus*) and California Ground Squirrel (*Otospermophilus beecheyi*) form a predator-prey interaction where venoms and venom resistance co-evolve with one another (Coss et al., 1993). Considerable efforts have been undertaken to understand the arms race in this 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., 2016a). Arms races are dynamic system though, and when studying venom resistance in the present, one gleans a current "snapshot" of an everchanging interaction with constant compensatory adjustments by each partner.

Resistance to snake venoms in rodents has been detected in various species pairings, including the California Ground Squirrel and Pacific Rattlesnake (Holding et al., 2016a), the Southern Plains Woodrat (*Neotoma micropus*) and Western Diamondback Rattlesnake (*C. atrox*; Perez et al., 1978), and the Prairie Vole (*Microtus ochrogaster*) and Copperhead (*Agkistrodon contortrix*; de Wit, 1982). The prevalence of this coevolutionary dynamic across landscapes and taxa suggests that it is a central theme in venom-mediated predation. In spite of this, systems do exist where venom resistance appears to be absent (Phillips et al., 2012), with the Cape Ground Squirrel (*Xerus inauris*) serving as an example by lacking proteolytic resistance to co-occurring Puff Adders (*Bitis arietans*) and Snouted Cobras (*Naja annulifera*). The factors impacting the presence and strength of resistance remain relatively unexplored, and community level analyses offer a means to explore these topics.

The grasslands of eastern Colorado represent an ecosystem where a diverse rodent community interacts with two species of rattlesnake. Common rodents in this system include the Deer Mouse (*Peromyscus maniculatus*), Eastern Woodrat (*Neotoma floridana*), Ord's Kangaroo Rat (*Dipodomys ordii*), Northern Grasshopper Mouse (*Onychomys leucogaster*), introduced House Mouse (*Mus musculus*), and Plains Pocket Mouse (*Perognathus flavescens*). The large-bodied and widespread Prairie Rattlesnake (*Crotalus viridis*) is present throughout the eastern part of state (Hammerson, 1999), while the diminutive Desert Massasauga Rattlesnake (*Sistrurus tergeminus edwardsii*) occupies a restricted distribution in the southeastern corner of the state (Hobert et al., 2004; Wastell and Mackessy, 2016). Together, these two rattlesnakes exert predatory pressures on their respective rodent communities but differences between the two in feeding ecology may impact rodents differentially.

This study system serves as an ideal model to explore venom resistance at the community level in a multi-predator and multi-prey context. Here, we explore the roles of predation pressures and geography on the strength of venom resistance and test the hypotheses that A) rodents will be more resistant to the rattlesnake species exerting greater predation on them, and B) rodents will have greater resistance to venoms of sympatric snake populations rather than allopatric snake populations. Considering these hypotheses, we predict that: 1) All rodent population will possess resistance to Prairie Rattlesnake venoms, given that the large body size and broad geographic distribution of this snake allows it to apply predation pressure throughout the tested rodent community. 2) Only small rodent species (those within gape constraints of the Desert Massasauga Rattlesnake) will possess resistance to Desert Massasauga venom, as larger rodent species (exceeding gape constraints) do not incur high predation pressure from this rattlesnake. 3) Rodents will possess greater resistance to venoms of sympatric snake populations and reduced resistance to venoms of allopatric snake populations. Further, rodents from the Weld County site should possess minimal resistance to the Desert Massasauga, as this species does not occur at this location.

2. Methods

2.1. Study design and sample collection

Sample collection occurred at two field sites on privately owned lands in Colorado in Weld County (hereafter WC) and in Lincoln County (hereafter LC, approx. 120 miles due south; Fig. 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 sympatry and allopatry on the presence and strength of resistance among interacting species.

Two species of venomous snakes are present at the field sites. The (northern) WC field site is inhabited by only the larger Prairie Rattlesnake, while the (southern) LC field site is inhabited by both the Prairie Rattlesnake and the more diminutive Desert Massasauga Rattlesnake. Each of the three rattlesnake populations studied herein display variation in toxin composition and relative abundance in their respective venom proteomes (Fig. 2). Venoms of both species were collected at the respective sites via sampling of animals at den sites, opportunistic sampling of day-active snakes, and driving roads during evening and night for active snakes. Venoms were manually extracted from snakes, centrifuged at 9.5 k x g to pellet cellular debris and frozen at −80 °C. Following freezing, samples were lyophilized and stored at −20 °C. All sample collections were conducted under permits issued by the Colorado Parks and Wildlife (to SPM: 19HP0974).

The following species of rodents were trapped at field sites (Fig. 3): Deer Mouse (*Peromyscus maniculatus*), House Mouse (*Mus musculus*), Northern Grasshopper Mouse (*Onychomys leucogaster*), Ord's Kangaroo Rat (*Dipodomys ordii*), Eastern Woodrat (*Neotoma floridana*), and Plains Pocket Mouse (*Perognathus flavescens*) 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

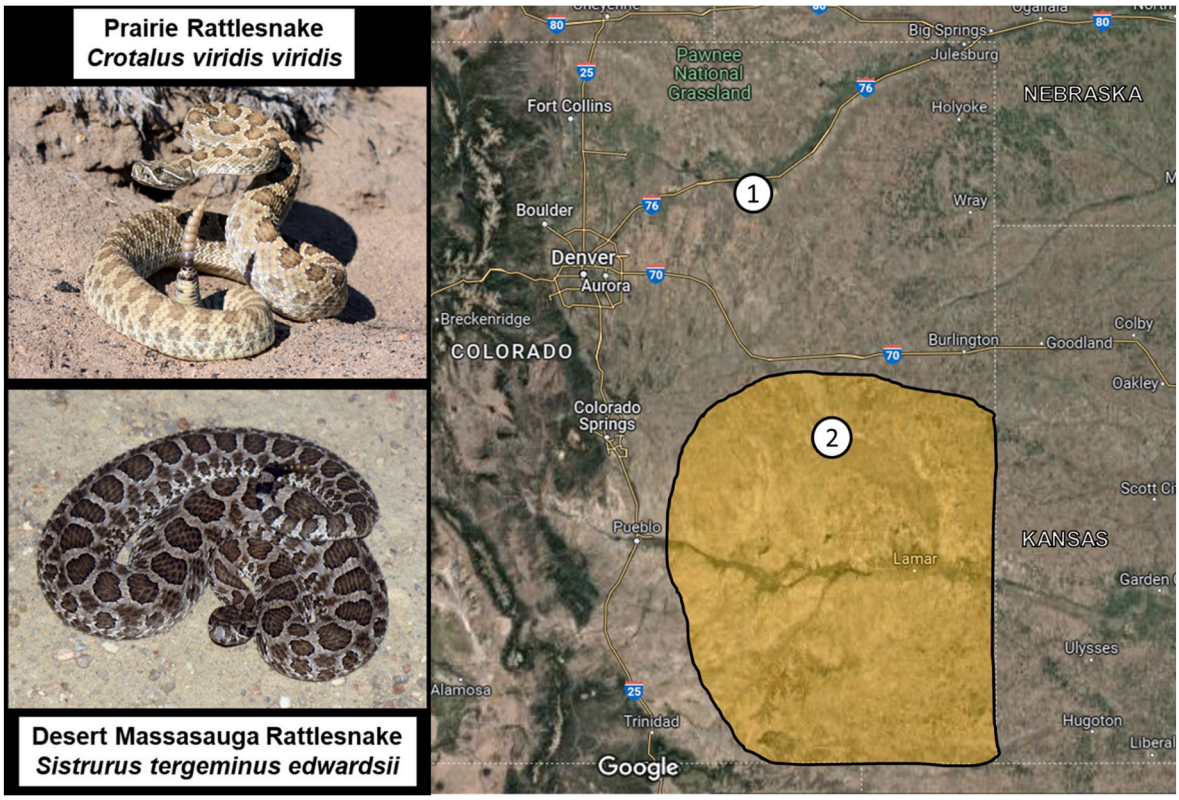

Fig. 1. Map of study sites and rattlesnake species present, indicating 1) Weld Co. And 2) Lincoln Co. field sites. Only *Crotalus v. viridis* is present at the Weld Co. field site (1), but both *Crotalus v. viridis* (photo: SPM) and *Sistrurus tergeminus edwardsii* (photo: Kaye Holman) are present at the Lincoln Co. field site (2). Yellow overlay represents the approximate geographic distribution of *S. t. edwardsii* in Colorado (based on Hammerson, 1999), and *C. v. viridis* is broadly distributed across the eastern plains. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

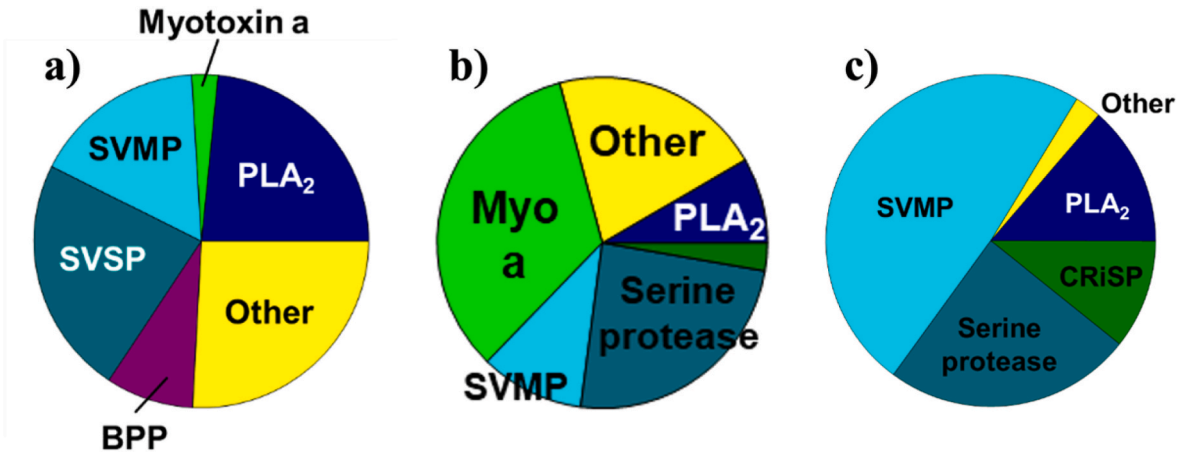

Fig. 2. Simplified venom proteomes of a) *Crotalus v. viridis* (Lincoln Co.) b) *Crotalus v. viridis* (Weld Co.) and c) *Sistrurus t. edwardsii* (Lincoln Co.), depicting major toxin groups present in venoms of respective populations. (From a) Mackessy, unpubl. Data; b) Saviola et al. (2015); c; Sanz et al. (2006). See also Smith et al. (2023) for geographic trends in *C v. viridis* venom composition.

retrieved the following morning. Live rodents were transported back to the laboratory for use in assays (IACUC protocol 1905D-SM-SBirdsLM-22, to SPM). Individual rodents were housed in laboratory caging on Carefresh bedding and were supplied with rodent lab chow diet, bird seed, and water *ad libitum*.

2.2. Metalloproteinase inhibition assays

Metalloproteinase assays were used to determine the inhibitory potential of rodent serum against rattlesnake venom metalloproteinases, as this assay has been used commonly in resistance studies. Rodents were humanely euthanized via cervical dislocation and exsanguinated immediately after via the ventricles, orbital sinus, and/or jugular vein. Whole blood was collected in 1.5 mL Eppendorf tubes held on ice during collection. Following collection, whole blood was spun at 8.0 k x g for 10 min 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 from each study population (three individuals per pooled venom) were solubilized in MilliQ ultra-pure water

Fig. 3. UNC Museum of Natural History specimens of rodent species commonly encountered on study sites. Arranged by size: A, *Neotoma floridana*; B, *Dipodomys ordii*; C, *Onychomys leucogaster*; D, *Peromyscus maniculatus*; E, *Mus musculus*; F, *Perognathus flavescens*.

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 individual rodents per population 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 (total volume 250 μL) depending on trial were incubated together at room temperature (approximately 20 °C) for 30 min. Tubes were placed in an ice bath for 5 min, after which 250 μL of substrate solution (azocasein, in buffer, 2.0 mg/mL buffer; Sigma) was added to each culture tube. Tubes were immediately incubated for 30 min at 37 °C. Following this incubation period, 125 μL of TCA stop solution was added (to terminate the reaction), held at RT for 10 min and tubes were centrifuged at 2000 rpm 125 μL of supernatant was drawn up from each and transferred to a well plate, mixed with 125 μL of 0.5 M NaOH, and allowed to sit at room temperate for approximately 5 min. Absorbance readings were then taken with a plate reader at 450 nm.

2.3. Median lethal dose (LD_{50}) assays

Median lethal dose (LD_{50}) assays were used to assess toxicity of a venom to each population of rodents. Lyophilized venoms from three individual snakes of the same population were reconstituted at equal amounts at a concentration of 10 μg/μL in MilliQ ultra-pure water. 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 (50 μL for small species or 100 μL for large species) in their lower right quadrant, replaced in their cages, and mortality was recorded at 24 h 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).

2.4. Statistical analysis

Rodent serum inhibition values were evaluated for significance using an unpaired *t*-test to determine differences of means between rodent populations (of the same species) from each field site against a single rattlesnake venom. Serum inhibition values for *Neotoma floridana* and *Perognathus flavescens* were excluded from analysis due to inclusion of only one sample of each in the study. *p*-values <0.05 were considered statistically significant.

3. Results

3.1. Metalloproteinase assays

The highest snake venom metalloproteinase (SVMP) activity was detected in the venom of the LC Desert Massasauga population (0.872 $A_{450\ nm/min}$/mg; T1able 3.1), somewhat lower SVMP activity in Lincoln County Prairie Rattlesnake venom (0.752 $A_{342\ nm/min}$/mg; T1able 3.1), and much lower activity in Weld County Prairie Rattlesnake venom (0.495 $A_{342\ nm/min}$/mg; T1able 3.1). All venoms tested exhibited considerable metalloproteinase activity, and thus metalloproteinases are an important component in each of these venoms to facilitate prey incapacitation (see also Sanz et al., 2006; Saviola et al., 2015). Further, these data are directly comparable to previously published papers evaluating resistance in prey species (i.e., Ukken et al., 2022; Holding et al., 2016a,b).

Inhibition of snake venom metalloproteinase (SVMP) activity is highly variable across rodent species and populations, and some amount of variation is also present 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 WC Deer Mice are challenged with the venom of LC Prairie Rattlesnakes. Deer Mice may exhibit some local adaptation (metalloproteinase resistance) to the presence of Desert Massasauga, though this difference is not statistically significant ($p = 0.0723$).

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 SVMPs of the Lincoln County Prairie Rattlesnake than they were to the SVMPs of this species from Weld County. Interestingly, reduced inhibition was detected with co-occurring snake-mouse pairings. It does not appear that differential resistance is present between populations to Desert Massasauga venom.

Ord's Kangaroo Rats (*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 effects against Desert Massasauga SVMP activity.

Eastern Woodrat (*Neotoma floridana*) serum displays the greatest metalloproteinase inhibition to co-occurring Prairie Rattlesnakes and are only weakly inhibitory to Desert Massasauga SVMPs. Plains Pocket Mouse (*Perognathus flavescens*) sera from the two field sites do not appear to display differential resistance to Desert Massasauga SVMPs. However, sample sizes were very limited for both rodent species.

3.2. Median lethal dose (LD_{50}) assays

Dose-response curves generated for potential prey species using WC Prairie Rattlesnake, LC Prairie Rattlesnake, and LC Desert Massasauga venoms revealed considerable differences in toxicity amongst snake venoms, prey species, and field sites. Venom of the WC Prairie Rattlesnake was generally more toxic to all rodent populations (where both populations were assayed) than that of the LC Prairie Rattlesnake (Table 3). In a naïve *Mus musculus* population (NSA; non-Swiss albino

lab strain) WC Prairie Rattlesnake venom is approximately twice as toxic (1.4 mg/kg) as LC Prairie Rattlesnake venom (2.4 mg/kg). Wild WC *M. musculus* display a similar sensitivity to their domestic conspecifics when challenged with WC Prairie Rattlesnake venom (1.6 mg/kg). Unfortunately, additional wild *M. musculus* from either county were unavailable for comparative toxicity values. Differential toxicity between Prairie Rattlesnake populations is most dramatically apparent with respect to LC *Dipodomys ordii*, which are sensitive to the venom of allopatric WC Prairie Rattlesnakes (4.2 mg/kg) yet highly resistant to the venom of sympatric LC Prairie Rattlesnakes (125.0 mg/kg). Conversely, both populations of Prairie Rattlesnake are equally toxic to WC *D. ordii* (15.0 mg/kg for both populations). WC Prairie Rattlesnakes are again more toxic to both WC (2.3 mg/kg) and LC (6.3 mg/kg) *Peromyscus maniculatus* than the LC Prairie Rattlesnake (10.5 mg/kg for WC mice and 10.6 mg/kg for LC mice). The greatest median lethal dose value recovered from our analyses came from LC *Onychomys leucogaster* tested against WC Prairie rattlesnake venom (127.7 mg/kg). Additional *O. leucogaster* were unavailable to conduct assays for additional pairings. Fewer LD_{50} assays were conducted with respect to LC Desert Massasauga venom, but sympatric LC *P. maniculatus* were less sensitive to this venom (4.3 mg/kg) than allopatric WC *P. maniculatus* (3.3 mg/kg). Desert Massasauga venom has been previously characterized as more toxic to a murine model than either population of Prairie Rattlesnake studied here (Gibbs and Mackessy, 2009).

4. Discussion

4.1. Rattlesnake venom compositional variation

Snake venoms have the potential for immense variation among species (Modahl et al., 2020), across the range of a single species (Strickland et al., 2018; Smith et al., 2023), within populations of a species (Rashmi et al., 2021), and even ontogenetically within the lifetime of a single individual (Mackessy, 1988; Saviola et al., 2015). The three populations of rattlesnakes used in this study (WC Prairie Rattlesnake, LC Prairie Rattlesnake, and LC Desert Massasauga) produce venoms that vary considerably from each other in both composition and toxicity. Our results suggest that both the LC Desert Massasauga and LC Prairie Rattlesnake possess highly degradative venoms that may be less optimized for rapid incapacitation than the venom of the WC Prairie Rattlesnake, consistent with trends more broadly observed in rattlesnake venom phenotypes (Mackessy, 2010).

Whole venom toxicity serves as 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 rapidly should be selected for when prey is released following envenomation, a feeding strategy typical of many vipers (Saviola et al., 2013). LD_{50} values produced using NSA strain *Mus musculus* allow for comparisons of whole venom toxicity toward a naïve model rodent that does not possess co-evolved resistance mechanisms to snake venoms. 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; Mason et al., 2022; Ochoa et al., 2023). From the standpoint of crude venom lethality, LC Desert Massasaugas possess the most toxic venom against a murine model out of the three tested venoms. This may be attributable at least in part to the relative importance of small rodents in the diet of this rattlesnake, as congeners with a reduced dietary dependence on rodent prey have less rodent-toxic venoms (Gibbs and Mackessy, 2009).

LD_{50} values for the two Prairie Rattlesnake populations differed considerable, with the WC Prairie Rattlesnake venom being approximately twice as toxic to lab mice as LC population venom. Recent work characterizing distribution-wide venom variation across the range of the Prairie Rattlesnake (Smith et al., 2023) has identified divergent venom phenotypes in this species, similar in some respects to the Type I and Type II venom dichotomy characterized previously (Mackessy, 2010). LC Prairie Rattlesnakes possess a Type I venom characterized by high metalloprotease activity but reduced toxicity, resulting in a more degradative venom requiring more time to incapacitate prey items. Conversely, WC Prairie Rattlesnake venom shows reduced metalloprotease activity yet heightened toxicity, resulting in quicker incapacitation of prey with reduced tissue degradation. Unlike other highly toxic venoms, however, the main contributor to rapid rodent tetanic immobility is the abundant myotoxin a, an inhibitor of the calcium pump of skeletal muscle sarcoplasmic reticulum (Utaisincharoen et al., 1991). The LD_{50} assays conducted upon the venoms of these two rattlesnake populations are consistent with the expected nature of the two venom phenotypes, but it is worth noting that these outcomes may not be as predictable when considering co-evolved wild prey items with endogenous defense mechanisms.

4.1.1. Predator-prey dynamics

The study system herein reveals the variation in toxicity and resistance present within a food web, incorporating multiple venom-producing predators, multiple prey species, and distributed across two geographically distant sites. To date, studies have generally characterized biochemical interactions between predator-prey pairings in a relatively isolated context (e.g., Perez et al., 1978, 1979; Poran et al., 1987; Phillips et al., 2012), though several investigations have incorporated geographic and phylogenetic components (e.g., Poran and Heatwole, 1995; Pomento et al., 2016; Smiley-Walters et al., 2018; Mason et al., 2022). These studies, coupled with investigations into physiological processes (e.g., Khan et al., 2020) have allowed formation of a relatively well-resolved image of the biochemical ecology of toxins and toxin resistance in the natural world (e.g., van Thiel et al., 2022).

However, the exploration of venom resistance dynamics within food webs (and throughout ecosystems more broadly) remains a largely unaddressed gap in the coverage of research to date. Robinson et al. (2021) explored this topic in a system containing two rattlesnakes, two rodent prey species, and four field sites to tease apart the roles sympatry and allopatry play in snake venom resistance dynamics. They found that venoms from Red Diamond Rattlesnakes (*Crotalus ruber)* were locally adapted to co-occurring Bryant's Woodrat (*Neotoma bryanti*) prey, and that sympatry between the Red Diamond and Southern Pacific Rattlesnake (*Crotalus o. helleri*) may result in character displacement in venoms. These dynamics illustrate the complex nature of the chemically mediated interactions created by venoms and highlight the need to look beyond isolated predator-prey pairings. Our results indicate a variety of outcomes for each tested species/population and underscore the complex nature of biochemical interactions in an ecosystem.

Peromyscus maniculatus are some of the most abundant rodents at the study sites and are an important prey item for both rattlesnakes (Holycross and Mackessy, 2002) and other predators. It is apparent that WC *C. viridis* are more toxic to WC than LC *P. maniculatus*, indicating that the snake appears to be the locally adapted partner in this interaction. Ecological interactions may explain the lack of obvious adaptation against snake venom in *P. maniculatus*. Being abundant and important in the diet of many grassland predators, Deer Mice are subject to a wide array of selective pressures (e.g., Clarke, 1983; Connolly and Orrock, 2018). This plethora of pressures, coupled with the large population sizes of mice may effectively dilute the pressure exerted by snake venoms to the point where there is no longer strong selection for resistance. More detailed work is needed to tease apart the finer scale patterns of resistance in *P. maniculatus*, but our study provides the first exploration on resistance to venoms in this rodent species.

The House Mouse (*Mus musculus*) is a relatively new arrival to North America, having existed on the continent for approximately five centuries (Tichy et al., 1994; Suzuki et al., 2013). Despite this, House Mice can be important components of snake diets in their introduced range (Slip and Shine, 1988; Wolfe et al., 2018). This raises an interesting question: in the short time that House Mice have been on the continent,

has resistance to snake venom evolved? Because lab mice are a naïve model organism and have no coevolutionary history with North American rattlesnakes, they serve as the ideal model to compare venom toxicities, providing a general baseline of toxicity for the three snakes indicated prior. When compared to the LD_{50} obtained with lab strain mice, it is apparent that feral *Mus musculus* have not evolved resistance in the short time that they have been present in North America.

Various other rodents are present in the study system that warrant further investigation, but many of these were outside of the scope of this study due to sampling difficulties. We were able to source only a limited number of *Perognathus flavescens*, a small heteromyid rodent that is particularly suitable as a prey item for *S. t. edwardsii* due to its small size and ecology. Serum protease inhibition assays (Table 1) revealed WC *P. flavescens* (existing in the absence of *S. t. edwardsii*) and LC *P. flavescens* (co-occurring with *S. t. edwardsii*) to be equally inhibitory against snake venom metalloprotease. Consequently, there appears to be no local adaptation for predation pressures exerted by *S. t. edwardsii* in *P. flavescens*, though larger sample sizes are needed to understand better the relationship between these two species.

4.1.2. Rodents with high levels of resistance

Three of the rodents assayed herein showed exceptionally high resistances to rattlesnake venoms. Woodrats (*Neotoma* spp.) are well characterized in their ability to detoxify pitviper venoms, and this theme has been recapitulated in various studies (Perez et al., 1978; de Wit, 1982; Robinson et al., 2021). Garcia and Perez (1984) identified an antihemorrhagic factor in the serum of *Neotoma micropus* that provided protection against the venom of *Crotalus atrox*. It is likely that serum proteins are broadly distributed throughout woodrat diversity, as various *Neotoma* spp. All appear to exhibit resistance when challenged with pitviper venoms. Woodrats are an important prey item for rattlesnakes (e.g., Dugan and Hayes, 2012), and consequently selection pressures exerted upon them may be elevated compared to rodents that are less abundant in rattlesnake diets. The large body size of adult woodrats also restricts their consumption to large bodied (mature) snakes. Many rattlesnake venoms are known to shift ontogenetically from a highly toxic phenotype to a highly degradative (proteolytic) phenotype (Mackessy, 1985, 1988), and woodrats would presumably be interacting with the most degradative venoms present within the population of snakes. Thus, woodrats further narrow down the scope of variability in venoms that they interact with through their large body size, potentially strengthening the selection pressures for a highly degradative venom. The single individual of *Neotoma floridana* (Lincoln County) tested herein more effectively neutralized proteolytic activity (by approx. 2-fold) from co-occurring LC *C. viridis* than from WC *C. viridis*. Consequently, the woodrat appears to be locally adapted to the venom of co-occurring *C. viridis*, which is more protease-rich in composition when compared to venoms of WC rattlesnakes. Surprisingly, woodrat serum was only weakly inhibitory to the proteolytic activity of co-occurring LC *S. t. edwardsii* venom (8.4% inhibition). This may be attributable to the constraints of body size described above, and corresponding strength of predation pressures. *Sistrurus t. edwardsii* cannot be considered a typical predator of *N. floridana* due to its diminutive adult body size, and consequently, envenomation is likely an uncommon event. Additional robust sampling of *N. floridana* is needed to understand better the biochemical ecology of these interacting species.

We present the first exploration of snake venom resistance in *Onychomys leucogaster* and find surprisingly high levels of resistance in this species. Serum metalloprotease inhibition for both Grasshopper Mice populations against all rattlesnake venoms showed high inhibition values across all treatments. Both LC and WC mice were approximately equally inhibitory against proteolytic activity of the venoms of LC *S. t. edwardsii* and WC *C. viridis*, but WC mice were significantly more resistant to the proteolytic activity of *C. viridis* than LC mice. As such, it appears that the venom of LC *C. viridis* is locally adapted for increased toxicity for co-occurring grasshopper mice, while the other pairings do not suggest local adaptation for either partner. While we were unable to collect appropriate numbers of *O. leucogaster* needed to conduct LD_{50} tests across all pairings, a single assay revealed remarkably high resistance to the crude venom of WC *C. viridis* by LC *O. leucogaster* (127.7 mg/kg). Given that the rattlesnake-rodent pairing in this case is mismatched (WC snake and LC mouse), we would not have an *a priori* expectation for evolved resistance due to co-evolutionary pressures. Additionally, venoms of WC snakes are myotoxin a-dominated (and correspondingly more toxic) compared to the metalloprotease-dominated venoms of LC snakes (Smith et al., 2023). Consequently, we would expect LC *O. leucogaster* to have a higher sensitivity to WC *C. viridis* than LC *C. viridis* venom. It is possible that *O. leucogaster* also possesses resistance mechanisms to detoxify myotoxin a, along with those that inhibit proteolytic activity, as indicated by the serum-based assays, but additional sampling and analyses are needed.

Grasshopper Mice (*Onychomys* spp.) have been the subject of intense study regarding interactions with scorpions, and investigators have characterized behavioral elements of predation on scorpions (Rowe and Rowe, 2006; Niermann et al., 2020), the presence of physiological resistance to scorpion venoms (Rowe and Rowe, 2008), and the physiological mechanism conferring scorpion venom resistance (Rowe et al., 2013). The biochemical compositions of scorpion and rattlesnake venoms differ markedly, with the former being comprised primarily of smaller peptides (Carcamo-Noriega et al., 2018), while the latter is comprised more heavily of larger enzymatic molecules (Mackessy, 2008). It thus appears that Grasshopper Mice may have a broad spectrum of resistance mechanisms to a variety of venoms, and much of this may be attributable to their unique ecology among rodents. While grasshopper mice do fall prey to rattlesnakes (e.g., Holycross and Mackessy, 2002), they are carnivores themselves and have been recorded predating upon other rodents, lizards, and birds (Sherbrooke, 1991; Rowe and Rowe, 2015). It is expected that Grasshopper Mice also prey upon small snakes, and in the context of our study system this may include young *C. viridis* and *S. t. edwardsii*, exposing themselves to envenomation in a defensive context. This bidirectional predation dynamic between Grasshopper Mouse and rattlesnake may be a driving force in facilitating the evolution of strong venom resistance in Grasshopper Mice.

Ord's Kangaroo Rats (*D. ordii)* also showed variable resistance to rattlesnake venoms across proteolytic assays. Rat sera showed considerably lower resistance to proteolytic activity from the venom of *S. t. edwardsii* compared to the venoms of either *C. viridis* population. This result aligns with our initial predictions that resistance capacities will relate to predation pressures, and since *S. t. edwardsii* is expected to feed on *D. ordii* only rarely, we would expect selective pressures exerted by this venom to be low in comparison to that of *C. viridis*. The remaining four pairings of LC and WC *D. ordii* with LC and WC *C. viridis* are relatively consistent in proteolytic inhibition potential. Unpaired *t* tests did not find significant differences for inhibition between either population, suggesting that rats do not exhibit local adaptation to the venom metalloproteases of their co-occurring *C. viridis*. A much more interesting picture of resistance dynamics emerges when comparing LD_{50} values across population pairings for *D. ordii* and *C. viridis*. We find that WC

Table 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.

Rattlesnake Population	Metalloproteinase Activity ($\Delta A_{450\ nm}$/minute/mg venom protein)
Crotalus viridis (Weld Co.)	0.495
Crotalus viridis (Lincoln Co.)	0.752
Sistrurus tergeminus edwardsii (Lincoln Co.)	0.872

D. ordii are approximately equally resistant to the crude venoms of both WC and LC *C. viridis,* but a dramatic differential in resistance is present in LC *D. ordii*. When challenged with the crude venom of co-occurring *C. viridis*, LC *D. ordii* are incredibly resistant (125.0 mg/kg), yet they are highly sensitive to WC *C. viridis* venom (4.2 mg/kg). These median lethal dose values suggest remarkable local adaptation on behalf of the rat and raise questions about venom compositional variation in *C. viridis*. With LC snakes possessing a metalloprotease-rich venom, is appears that LC *D. ordii* may have sufficient endogenous mechanisms to detoxify SVMPs and other toxins. Conversely, rats from the same population showed high susceptibility to venoms of allopatric WC *C. viridis*, which possess a venom phenotype dominated by myotoxin a (Smith et al., 2023). This paradigm suggests that the metalloprotease/myotoxin a dichotomy may be the main factor driving resistance patterns in this population of *D. ordii*. Further, these results raise questions about the ecological significance of a myotoxin a-rich venom phenotype, and the impact that kangaroo rats (and other prey items) may play in selecting for these venom phenotypes. *Dipodomys ordii* may be an important prey species for understanding the relatively dramatic pattern of venom variation in *C. viridis*.

4.1.3. The biological relevance of resistance

To understand fully the utility (and selective advantage) of venom resistance in rodents, it becomes important to contextualize values derived in a laboratory setting with the ecology of species on a landscape. While still debated in the literature (Pintor et al., 2010; Smith et al., 2014), the consensus among researchers is that venom is not metabolically costly for a snake to produce. Yet, constraints still exist that encourage the modulation of venom expenditure, specifically the time associated with replenishment of the venom gland after expulsion (Mackessy, 2022). Consequently, venom use can be influenced by behavioral context (Hayes, 1995; Young and Zahn, 2001), where snakes modulate venom output depending on the nature of the interaction. Additionally, the venom bolus delivered may be impacted by other factors, including strike accuracy and prey behavior (Schraft and Clark, 2017; Whitford et al., 2017, 2019; Freymiller et al., 2017, 2019; Whitford et al., 2019a,b). Considering the complexity of interacting factors associated with prey envenomation, an envenomated rodent may be subjected to a variety of dosages, meaning that even low levels of resistance have the potential to offer considerable fitness advantages.

When we consider the median lethal doses of two rodents determined in this study, the LC *D. ordii* (against LC *C. viridis* venom) and the LC *O. leucogaster* (against WC *C. viridis*), 125.0 mg/kg and 127.0 mg/kg respectively, it is immediately apparent that these rodents are far more venom-resistant than other co-occurring species. But what are the ecological ramifications of these values? With an average adult mass of ~70 g for *D. ordii* and ~35 for *O. leucogaster*, the LD_{50} values above translate to 8.75 mg and 4.45 mg of venom, respectively. An adult *C. viridis* may possess 50–75 mg of venom (dry weight; unpub. data), and only a fraction of this volume is expelled in a typical strike. When one factors in additional potential uncertainties associated with strike accuracy and strike avoidance behavior of prey, it becomes clear that the ability to detoxify venom at this magnitude can confer a significant survival advantage to rodents. Further, even relatively minor levels of venom resistance may offer considerable advantages, considering the proportion of strikes that fail to deliver a sufficient bolus.

4.1.4. Future directions

Evaluations of venom resistance at the community level via *in vitro* and *in vivo* methods provide unrivaled insight on the roles that additional selective pressures play upon biological communities outside of isolated predator-prey interactions. Unfortunately, it is exceedingly difficult to factor all trophic interactions in a food web into experimental analyses. In part, our limited understanding of both predators and prey of rattlesnakes hinders our capacity to design comprehensive studies, but logistical constraints associated with assaying many of these interactions adds additional complication. Explorations on venom resistance in snake predators remain relatively sparse compared to those conducted on prey species. While select snake predators, like opossums (Voss and Jansa, 2012) and mongoose (Barchan et al., 1992) have been studied extensively, little work has occurred elsewhere, especially with respect to predators that incorporate venomous snakes as only a part of a varied diet. In the study system presented herein, birds of prey and mesopredatory mammals presumably provide considerable predation pressure on rattlesnakes, and consequently, the biochemical ecology associated with these interactions should be studied. Further, investigation into venom resistance capabilities of snake predators and prey at the same study site would provide unique insight into simultaneously occurring offensive and defensive interactions. Future work in biochemical ecology should consider food webs more holistically when attempting to tease apart venom-mediated interactions, as isolated predator-prey pairings provide only a single snapshot of an entire scene occurring in one place and time.

The strong resistance capabilities of two rodents examined herein, *D. ordii* and *O. leucogaster*, raises many questions, the most interesting of which relate to myotoxin a. This peptide comprises the major toxic component of *C. viridis* venom and may comprise >50% of total venom protein in some populations (Smith et al., 2023), quickly inducing tetanic paralysis and immobilization in envenomed prey items (Saviola et al., 2015). As illustrated by LD_{50} results (cf. Table 2), LC *D. ordii* are incredibly resistant to LC *C. viridis* venom (125.0 mg/kg), but highly sensitive to WC *C. viridis* venom (4.2 mg/kg). The main compositional difference here is that of myotoxin a, which is essentially absent in the former but abundant in the latter. Is myotoxin a specifically exceptionally toxic to *D. ordii*? Conversely, LC *O. leucogaster* were highly resistant to this same venom of WC *C. viridis*, resulting in a median lethal dose of 127.7 mg/kg. At present, at least in Weld Co., *C. viridis* appears to be in the lead in the arms race with *D. ordii*. Future work should more deeply explore the role of myotoxin a specifically in influencing venom

Table 2
Contingency table of inhibitory effect (percent inhibition) of rodent serum (mean ± standard deviation) against snake venom metalloproteinase activity of three rattlesnake venoms. The two populations of the same rodent species for each rattlesnake venom type (indicated by matching superscript number) were compared with an unpaired *t*-test to evaluate significant differences in inhibitory effect.

	Lincoln County *S. t. edwardsii*	Lincoln County *C. viridis*	Weld County *C. viridis*
Weld County *P. maniculatus*	2.4% ± 2.04 n = 3 $^{1}p = 0.0723$	5.2% ± 2.22 n = 3 $^{2}p = 0.1230$	12.1% ± 8.22 n = 3 $^{3}p = 0.9472$
Lincoln County *P. maniculatus*	25.0% ± 16.01 n = 3 $^{1}p = 0.0723$	10.3% ± 3.95 n = 3 $^{2}p = 0.1230$	12.5% ± 5.39 n = 3 $^{3}p = 0.9472$
Weld County *O. leucogaster*	41.8% ± 24.45 n = 3 $^{4}p = 0.8922$	76.2% ± 1.44 n = 3 $^{5}\mathbf{p = 0.0101}$	46.1% ± 9.96 n = 3 $^{6}p = 0.8430$
Lincoln County *O. leucogaster*	39.4% ± 15.21 n = 3 $^{4}p = 0.8922$	25.8% ± 18.96 n = 3 $^{5}\mathbf{p = 0.0101}$	43.8% ± 16.01 n = 3 $^{6}p = 0.8430$
Weld County *D. ordii*	8.7% ± 6.46 n = 3 $^{7}p = 0.5509$	29.2% ± 4.25 n = 3 $^{8}p = 0.8261$	15.3% ± 0.31 n = 3 $^{9}p = 0.1943$
Lincoln County *D. ordii*	5.6% ± 5.14 n = 3 $^{7}p = 0.5509$	27.9% ± 8.61 n = 3 $^{8}p = 0.8261$	22.6% ± 8.11 n = 3 $^{9}p = 0.1943$
Lincoln County *Neotoma floridana*	8.4% n = 1	56.6% n = 1	25.2% n = 1
Weld County *Perognathus flavescens*	16.3% n = 1	Not determined	Not determined
Lincoln County *Perognathus flavescens*	14.7% n = 1	Not determined	Not determined

Statistically significant p-values are indicated in **bolded** font.

Table 3
Contingency table of intraperitoneal median lethal dose (IP LD_{50}) of rattlesnake venoms against tested rodent populations.

	Lincoln County *S. t. edwardsii*	Lincoln County *C. viridis*	Weld County *C. viridis*
Weld County *P. maniculatus*	3.3 mg/kg n = 18	10.5 mg/kg n = 18	2.3 mg/kg n = 15
Lincoln County *P. maniculatus*	4.3 mg/kg n = 12	10.6 mg/kg n = 12	6.3 mg/kg n = 18
Lincoln County *O. leucogaster*	Not determined	Not determined	127.7 mg/kg n = 15
Weld County *D. ordii*	Not determined	15.0 mg/kg n = 12	15.0 mg/kg n = 12
Lincoln County *D. ordii*	Not determined	125.0 mg/kg n = 12	4.2 mg/kg n = 18
Weld County *M. musculus*	Not determined	Not determined	1.6 mg/kg n = 12
NSA Strain (inbred) *M. musculus*	0.60 mg/kg[a]	2.4 mg/kg n = 15	1.3 mg/kg n = 15

[a] From Gibbs and Mackessy, 2009).

resistance dynamics with respect to this system of interacting species.

Deeper understanding of venom resistance will come from pointed investigation into the diversity of molecular mechanisms for resistance. Our understanding of the physiological basis of venom resistance remains relatively preliminary, with only a handful of resistance pathways and mechanisms being identified for venom toxins (reviewed in Holding et al., 2016b; Gibbs et al., 2020; van Thiel et al., 2022). Given the array of activities among venom toxins, it is unsurprising that a correspondingly diverse spectrum of resistance mechanisms exists (Tarvin et al., 2023). Molecular mechanisms broadly categorized as circulating venom-inhibiting proteins (e.g., Perez et al., 1978; Perez and Sanchez, 1999; Voss and Jansa, 2012; Gibbs et al., 2020; Ukken et al., 2022), altered receptors inhibiting venom protein binding (e.g., Barchan et al., 1992; Jansa and Voss, 2011; Drabeck et al., 2015; Khan et al., 2020), venom toxin repurposing (e.g., Rowe and Rowe, 2006, 2008; Rowe et al., 2013), and acquired immunity (e.g., Metz et al., 2006) have all been identified in various studies of venom resistance. Even within these mechanisms, there exists an assortment of pathways, and future research should endeavor to identify and describe these various pathways to venom resistance that are present throughout organismal diversity.

5. Conclusions

Venom resistance is a complex interaction involving chemically mediated predation and corresponding physiological responses in prey, 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 instance in ecological systems where prey may shape predator phenotype, and vice versa. As a result, studies of venom 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 to 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.

Ethical statement

All venoms and animals utilized in this study were sampled in accordance with protocols 1701D-SM-S-20 and1905D-SM-SBirdsLM-22 approved by the University of Northern Colorado Institutional Animal Care and Use Committee (to SPM) and under scientific collecting permits from Colorado Parks and Wildlife (19HP0974 and 19TR3327 to SPM).

CRediT authorship contribution statement

Neil R. Balchan: Writing - review & editing, Writing - original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Cara F. Smith:** Writing - review & editing, Writing - original draft, Methodology, Formal analysis. **Stephen P. Mackessy:** Writing - review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Neil Balchan reports financial support was provided by University of Northern Colorado.

Data availability

Data will be made available on request.

Acknowledgements

We thank private landowners in Weld and Lincoln Counties for allowing us to sample snakes and rodents from their properties and for continued support of our fieldwork and research program. Numerous individuals assisted with fieldwork, sample collection, and lab work, and we are grateful for their much-appreciated help and good company. This work was financially supported by the University of Northern Colorado (NHS Student Research Funds and Graduate Student Association Grants to NRB) and the Society for the Study of Amphibians and Reptiles (Roger Conant Grants in Herpetology and Dean Metter Memorial Award to NRB).

References

Aird, S.D., da Silva Jr., N.J., 1991. Comparative enzymatic composition of Brazilian coral snake (*Micrurus*) venoms. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 99, 287–294.

Akcali, C.K., Pfennig, D.W., 2017. Geographic variation in mimetic precision among different species of coral snake mimics. J. Evol. Biol. 30, 1420–1428.

Barchan, D., Kachalsky, S., Neumann, D., Vogel, Z., Ovadia, M., Kochva, E., Fuchs, S., 1992. How the mongoose can fight the snake: the binding site of the mongoose acetylcholine receptor. PNAS USA 89, 7717–7721.

Barlow, A., Pook, C.E., Harrison, R.A., Wüster, W., 2009. Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. Proc. Royal Soc. B 276, 2443–2449.

Biardi, J.E., Coss, R.G., Smith, D.G., 2000. California ground squirrel (*Spermophilus beecheyi*) blood sera inhibits crotalid venom proteolytic activity. Toxicon 38, 713–721.

Biardi, J.E., Nguyen, K.T., Lander, S., Whitley, M., Nambiar, K.P., 2011. A rapid and sensitive fluorometric method for the quantitative analysis of snake venom metalloproteases and their inhibitors. Toxicon 57, 342–347.

Brodie, E.D., Ridenhour, B.J., Brodie, E.D., 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.

Carcamo-Noriega, E.N., Olamendi-Portugal, T., Restano-Cassulini, R., Rowe, A., Uribe-Romero, S.J., Becerril, B., Possani, L.D., 2018. Intraspecific variation of *Centruroides sculpturatus* scorpion venom from two regions of Arizona. Arch. Biochem. Biophys. 638, 52–57.

Chun, J.B.S., Baker, M.R., Kim, D.J., LeRoy, M., Toribo, P., Bingham, J.-P., 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*). Behav. Ecol. Sociobiol. 13, 205–209.

Connolly, B.M., Orrock, J.L., 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., Smith, D.G., 1993. Development of antisnake defenses in California Ground Squirrels (*Spermophilus beecheyi*): II. microevolutionary effects of relaxed selection from rattlesnakes. Behaviour 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.H., Dean, A.M., Jansa, S.A., 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., Hayes, W., 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., Mao, J., 2005. Wolves influence elk movements: behavior shapes a trophic cascade in Yellowstone National Park. Ecol. 86, 1320–1330.
Freymiller, G.A., Whitford, M.D., Higham, T.E., Clark, R.W., 2019. Escape dynamics of free-ranging desert kangaroo rats (Rodentia: heteromyidae) evading rattlesnake strikes. Biol. J. Linn. Soc. 127, 164–172.
Freymiller, G.A., Whitford, M.D., Higham, T.E., Clark, R.W., 2017. Recent interactions with snakes enhance escape performance of Desert Kangaroo Rats (Rodentia: heteromyidae) during simulated attacks. Biol. J. Linn. Soc. 122, 651–660.
Garcia, V.E., Perez, J.C., 1984. The purification and characterization of an antihemorrhagic factor in woodrat (*Neotoma micropus*) serum. Toxicon 22, 129–138.
Gibbs, H.L., Mackessy, S.P., 2009. Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. Toxicon 53, 672–679.
Gibbs, H.L., Sanz, L., Pérez, A., Ochoa, A., Hassinger, A.T.B., Holding, M.L., Calvete, J.J., 2020. The molecular basis of venom resistance in a rattlesnake-squirrel predator-prey system. Mol. Ecol. 29, 2871–2888.
Goldenberg, S.U., Borcherding, J., Heynen, M., 2014. Balancing the response to predation—the effects of shoal size, predation risk and habituation on behaviour of juvenile perch. Behav. Ecol. Sociobiol. 68, 989–998.
Grabowsky, E.R., Mackessy, S.P., 2019. Predator-prey interactions and venom composition in a high elevation lizard specialist, *Crotalus pricei* (Twin-spotted Rattlesnake). Toxicon 170, 28–40.
Hague, M.T.J., Stokes, A.N., Feldman, C.R., Brodie Jr., E.D., Brodie 3rd, E.D., 2020. The geographic mosaic of arms race coevolution is closely matched to prey population structure. Evol. Lett. 4, 317–332.
Hammerson, G.A., 1999. Amphibians and Reptiles in Colorado, second ed. University Press of Colorado, Niwot, Colorado.
Hayes, W.K., 1995. Venom metering by juvenile prairie rattlesnakes, *Crotalus v. viridis*: effects of prey size and experience. Anim. Behav. 50, 33–40.
Hobert, J.P., Montgomery, C.E., Mackessy, S.P., 2004. Natural history of the Massasauga, *Sistrurus catenatus edwardsii*, in southeastern Colorado. SW. Nat. 49, 321–326.
Holding, M.L., Biardi, J.E., Gibbs, H.L., 2016a. Coevolution of venom function and venom resistance in a rattlesnake predator and its squirrel prey. Proc. Royal Soc. B. 283, 20152841.
Holding, M.L., Drabeck, D.H., Jansa, S.A., Gibbs, H.L., 2016b. Venom resistance as a model for understanding the molecular basis of complex coevolutionary adaptations. Integr. Comp. Biol. 56, 1032–1043.
Holycross, A.T., Mackessy, S.P., 2002. Variation in the diet of *Sistrurus catenatus* (Massasauga), with emphasis on *Sistrurus catenatus edwardsii* (Desert Massasauga). J. Herpetol. 36, 454–464.
Jansa, S.A., Voss, R.S., 2011. Adaptive evolution of the venom-targeted vWF protein in opossums that eat pitvipers. PLoS One 6, e20997.
Jayne, B.C., Voris, H.K., Ng, P.K.L., 2002. Snake circumvents constraints on prey size. Nature 418, 143.
Khan, M.A., Dashevsky, D., Kerkkamp, H., Kordis, D., de Bakker, M.A.G., Wouters, R., van Thiel, J., Op den Brouw, B., Vonk, F., Kini, R.M., Nazir, J., Fry, B.G., Richardson, M.K., 2020. Widespread evolution of molecular resistance to snake venom alpha-neurotoxins in vertebrates. Toxins 12, 1–20.
Kiriake, A., Ishizaki, S., Nagashima, Y., Shiomi, K., 2017. Occurrence of a Stonefish toxin-like toxin in the venom of the Rabbitfish *Siganus fuscescens*. Toxicon 140, 139–146.
Lee, C.-Y., Yo, S.-P., Clark, R.W., Hsu, J.-Y., Liao, C.-P., Tseng, H.-Y., Huang, W.-S., 2018. The role of different visual characters of weevils signalling aposematism to sympatric lizard predators. J. Zool. 306, 36–47.
Ligabue-Braun, R., Verli, H., Carlini, C.R., 2012. Venomous mammals: a review. Toxicon 59, 680–695.
Mackessy, S.P., 1985. Fractionation of red diamond rattlesnake (*Crotalus ruber ruber*) venom: protease, phosphodiesterase, L-amino acid oxidase activities and effects of metal ions on protease activity. Toxicon 23, 337–340.
Mackessy, S.P., 1988. Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *Crotalus viridis oreganus*. Copeia 92–101, 1988.
Mackessy, S.P., 2008. Venom composition in rattlesnakes: trends and biological significance. In: Hayes, W.K., Beaman, K.R., Cardwell, M.D., Bush, S.P. (Eds.), The Biology of Rattlesnakes. Loma Linda University Press, Loma Linda, CA, pp. 495–510.
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., 2022. Venom production and secretion in reptiles. J. Exp. Biol. 225 jeb227348.
Mackessy, S.P., Saviola, A.J., 2016. Understanding biological roles of venoms among the Caenophidia: the importance of rear-fanged snakes. Integr. Comp. Biol. 56, 1004–1021.
Mason, A.J., Holding, M.L., Rautsaw, R.M., Rokyta, D.R., Parkinson, C.L., Gibbs, H.L., 2022. Venom gene sequence diversity and expression jointly shape diet adaptation in pitvipers. Mol. Biol. Evol. 39, msac082.
Metz, M., Piliponsky, A.M., Chen, C.C., Lammel, V., Abrink, M., Pejler, G., Tsai, M., Galli, S.J., 2006. Mast cells can enhance resistance to snake and honeybee venoms. Science 313, 526–530.
Modahl, C.M., Mrinalini, Frietze, S.E., Mackessy, S.P., 2018. Adaptive evolution of prey-specific three-finger toxins in the Amazon Puffing Snake, *Spilotes sulphureus*. Proc. Royal Soc. B. 285, 20181003.
Modahl, C.M., Roointan, A., Rogers, J., Currier, K., Mackessy, S.P., 2020. Interspecific and intraspecific venom enzymatic variation among Cobras (*Naja* sp. and *Ophiophagus hannah*). Comp. Biochem. Physiol. 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., Rowe, M.P., 2020. Defensive venoms: is pain sufficient for predator deterrence? Toxins 12, 260. https://doi.org/10.3390/toxins12040260.
Ochoa, A., Hassinger, A.T.B., Holding, M.L., Gibbs, H.L., 2023. Genetic characterization of potential venom resistance proteins in California ground squirrels (*Otospermophilus beecheyi*) using transcriptome analyses. J. Exp. Zool. B Mol. Dev. Evol. 340, 259–269.
Pawlak, J., Mackessy, S.P., Fry, B.G., Bhatia, M., Mourier, G., Fruchart-Gaillard, C., Servent, D., Ménez, R., Stura, E., Ménez, A., Kini, R.M., 2006. Denmotoxin: a three-finger toxin from the colubrid snake *Boiga dendrophila* (Mangrove Catsnake) with bird-specific activity. J. Biol. Chem. 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., Kini, R.M., 2009. Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. Faseb. J. 23, 534–545.
Perez, J.C., Sanchez, E.E., 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., Hatch, C.H., 1978. Resistance of woodrats (*Neotoma micropus*) to *Crotalus atrox* venom. Toxicon 16, 198–200.
Perez, J.C., Pichyangkul, S., Garcia, V.E., 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., Bennett, N.C., 2012. No evidence for proteolytic venom resistance in southern African ground squirrels. Toxicon 60, 760–763.
Pintor, A.F., Krockenberger, A.K., Seymour, J.E., 2010. Costs of venom production in the common death adder (*Acanthophis antarcticus*). Toxicon 56, 1035–1042.
Pomento, A.M., Perry, B.W., Denton, R.D., Gibbs, H.L., Holding, M.L., 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., Heatwole, H., 1995. Resistances of sympatric and allopatric eels to sea snake venoms. Copeia 136–147, 1995.
Poran, N.S., Coss, R.G., Benjamini, E., 1987. Resistance of California ground squirrels (*Spermophilus beecheyi*) to the venom of the northern pacific rattlesnake (*Crotalus viridis oreganus*): a study of adaptive variation. Toxicon 25, 767–777.
Rashmi, U., Khochare, S., Attarde, S., Senji Laxme, R.R., Suranse, V., Martin, G., Sunagar, K., 2021. Remarkable intrapopulation venom variability in the monocellate cobra (*Naja kaouthia*) unveils neglected aspects of India's snakebite problem. J. Proteonomics 242, 104256.
Robinson, K.E., Holding, M.L., Whitford, M.D., Saviola, A.J., Yates, J.R., Clark, R.W., 2021. Phenotypic and functional variation in venom and venom resistance of two sympatric rattlesnakes and their prey. J. Evol. Biol. 34, 1447–1465.
Rowe, A.H., Rowe, M.P., 2006. Risk assessment by grasshopper mice (*Onychomys* spp.) feeding on neurotoxic prey (*Centruroides* spp.). Anim. Behav. 71, 725–734.
Rowe, A.H., Rowe, M.P., 2008. Physiological resistance of grasshopper mice (*Onychomys spp.*) to Arizona Bark Scorpion (*Centruroides exilicauda*) venom. Toxicon 52, 597–605.
Rowe, A.H., Rowe, M.P., 2015. Predatory grasshopper mice. Curr. Biol. 25, R1023–R1026.
Rowe, A.H., Xiao, Y., Rowe, M.P., Cummins, T.R., Zakon, H.H., 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., Calvete, J.J., 2006. Venom proteomes of closely related *Sistrurus* rattlesnakes with divergent diets. J. Proteome Res. 5, 2098–2112.
Saviola, A.J., Chiszar, D., Busch, C., Mackessy, S.P., 2013. Molecular basis for prey relocation in viperid snakes. BMC Biol. 11, 20.
Saviola, A.J., Pla, D., Sanz, L., Castoe, T.A., Calvete, J.J., Mackessy, S.P., 2015. Comparative venomics 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. J. Proteonomics 121, 28–43.
Schraft, H.A., Clark, R.W., 2017. Kangaroo rats change temperature when investigating rattlesnake predators. Physiol. Behav. 173, 174–178.
Sherbrooke, W., 1991. Behavioral (predator-prey) interactions of captive Grasshopper Mice (*Onychomys torridus*) and horned lizards (*Phrynosoma cornutum* and *P. modestum*). Am. Midl. Nat. 126, 187–195.
Slip, D., Shine, R., 1988. Feeding habits of the Diamond Python, *Morelia s. spilota*: ambush predation by a boid snake. J. Herpetol. 22, 323–330.
Smiley-Walters, S.A., Farrell, T.M., Gibbs, H.L., 2018. The importance of species: pygmy Rattlesnake venom toxicity differs between native prey and related non-native species. Toxicon 144, 42–47.
Smith, C.F., Nikolakis, Z.L., Perry, B.W., Schield, D.R., Balchan, N.R., Parker, J., Ivey, K., Hansen, K.C., Saviola, A.J., Castoe, T.A., Mackessy, S.P., 2023. Snakes on a plain: biotic and abiotic factors determine venom compositional variation in a wide-ranging generalist rattlesnake. BMC Biol. 21, 136. https://doi.org/10.1186/s12915-023-01626-x.
Smith, M.T., Ortega, J., Beaupre, S.J., 2014. Metabolic cost of venom replenishment by Prairie Rattlesnakes (*Crotalus viridis viridis*). Toxicon 86, 1–7.
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., Parkinson, C.L., 2018. Evidence for divergent patterns of local selection driving venom variation in Mojave Rattlesnakes (*Crotalus scutulatus*). Sci. Rep. 8, 1–15.

Sunagar, K., Moran, Y., 2015. The rise and fall of an evolutionary innovation: contrasting strategies of venom evolution in ancient and young animals. PLoS Genet. 11, e1005596.

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., Moriwaki, K., 2013. Evolutionary and dispersal history of Eurasian House Mice *Mus musculus* clarified by more extensive geographic sampling of mitochondrial DNA. Heredity 111, 375–390.

Tarvin, R.D., Pearson, K.C., Douglas, T.E., Ramírez-Castañeda, V., Navarrete, M.J., 2023. The diverse mechanisms that animals use to resist toxins. Annu. Rev. Ecol. Evol. Systemat. 54.

Thaker, M., Vanak, A.T., Owen, C.R., Ogden, M.B., Slotow, R., 2010. Group dynamics of Zebra and Wildebeest in a woodland savanna: effects of predation risk and habitat density. PLoS One 5, e12758.

Tichy, H., Zaleska-Rutczynska, Z., O'Huigin, C., Figueroa, F., Klein, J., 1994. Origin of the North American House mouse. Folia Biol. 40, 483–496.

Turko, P., Tellenbach, C., Keller, E., Tardent, N., Keller, B., Spaak, P., Wolinska, J., 2018. Parasites driving host diversity: incidence of disease correlated with *Daphnia* clonal turnover. Evolution 72, 619–629.

Ukken, F.P., Dowell, N.L., Hajra, M., Carroll, S.B., 2022. A novel broad spectrum venom metalloproteinase autoinhibitor in the rattlesnake *Crotalus atrox* evolved via a shift in paralog function. Proc. Natl. Acad. Sci. U.S.A. 119 (51), e2214880119 https://doi.org/10.1073/pnas.2214880119.

Utaisincharoen, P., Baker, B., Tu, A.T., 1991. Binding of myotoxin a to sarcoplasmic reticulum Ca2+-ATPase: a structural study. Biochemistry 30, 8211–8216.

van Thiel, J., Khan, M.A., Wouters, R.M., Harris, R.J., Casewell, N.R., Fry, B.G., Kini, R. M., Mackessy, S.P., Vonk, F.J., Wüster, W., Richardson, M.K., 2022. Convergent evolution of toxin resistance in animals. Biol. Rev. 97, 1823–1843.

Van Valen, L., 1973. A new evolutionary law. Evol. Theor. 1, 1–30.

Voss, R.S., Jansa, S.A., 2012. Snake-venom resistance as a mammalian trophic adaptation: lessons from didelphid marsupials. Biol. Rev. 87, 822–837.

Ward-Smith, H., Arbuckle, K., Naude, A., Wüster, W., 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., Mackessy, S.P., 2016. Desert Massasauga Rattlesnakes (*Sistrurus catenatus edwardsii*) in southeastern Colorado: life history, reproduction, and communal hibernation. J. Herpetol. 50, 594–603.

Whitford, M.D., Freymiller, G.A., Clark, R.W., 2017. Avoiding the serpent's tooth: predator-prey interactions between free-ranging sidewinder rattlesnakes and desert kangaroo rats. Anim. Behav. 130, 73–78.

Whitford, M.D., Freymiller, G.A., Clark, R.W., 2019a. Managing predators: the influence of kangaroo rat antipredator displays on sidewinder rattlesnake hunting behavior. Ethology 125, 450–456.

Whitford, M.D., Freymiller, G.A., Higham, T.E., Clark, R.W., 2019b. Determinants of predation success: how to survive an attack from a rattlesnake. Funct. Ecol. 33, 1099–1109.

Wolfe, A.K., Bateman, P.W., Fleming, P.A., 2018. Does urbanization influence the diet of a large snake? Curr. Zool. 64, 311–318.

Young, B.A., Zahn, K., 2001. Venom flow in rattlesnakes: mechanics and metering. J. Exp. Biol. 204, 4345–4351.