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No safety in the trees: Local and species-level adaptation of an arboreal squirrel to the venom of sympatric rattlesnakes

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ABSTRACT

Within some species, squirrels respond to variable selection from venomous snake predators by showing population-level variation in resistance, while between species, some rattlesnakes possess venom that is more effective at overcoming venom resistance in different species of squirrels. A functional evaluation of resistance variation to venom within and between species of squirrels and snakes can link resistance variation to its evolutionary causes across these different evolutionary scales. To do this, we compared the effectiveness of squirrel sera in inhibiting rattlesnake (*Crotalus* spp.) venom metalloproteinase activity between populations and between species to test for a response to local variation in selection from a single rattlesnake predator and for specialization of two resistant squirrel species to each of their distinct sympatric snake predators. We found that Timber Rattlesnake (*Crotalus horridus*) venom inhibition by Eastern gray squirrels (*Sciurus carolinensis*) is higher at a site where the rattlesnakes are present, which suggests selection may maintain venom resistance in populations separated by short distances. Next, we performed a reciprocal cross of venoms and sera from two rattlesnake and two squirrel species. This showed that squirrel resistance is lower when tested against venom from allopatric compared to sympatric rattlesnake species, demonstrating that squirrel inhibitors are specialized to sympatric venom and suggesting a tradeoff in terms of specialization to the venom of a specific species of rattlesnake predator. This pattern can be explained if inhibitors must recognize venom proteins and resistance evolution tracks venom evolution.

1. Introduction

Diverse animal species participate in ecological interactions where toxic venom and venom-resistance are key traits (Biardi, 2008; Casewell et al., 2012; Perez et al., 1979). Evolved resistance to venom presents ecological opportunities to resistant taxa such as the protection of anemonefish in stinging anemones (Mebs, 2009), the utilization of venomous snakes as a food source by resistant predators (Voss and Jansa, 2012), and the cohabitation of underground burrows by snakes and small mammals (Poran and Coss, 1990). Population-level variation in venom resistance is common (Biardi et al., 2006; Heatwole and Powell, 1998; Poran et al., 1987; Rowe and Rowe, 2008), suggesting that physiological costs of resistance often exist and lead to balancing selection on the resistance phenotype.

Venom is also highly variable (Casewell et al., 2012; Mackessy, 2010), and venoms of closely related venomous species often show prey-specific effects (Gibbs and Mackessy, 2009; Mackessy et al., 2006) but also variable effectiveness due to differences in resistance among prey (Biardi, 2008). Specialization of a resistant species to the challenge of coexisting sympatric venomous enemies might explain cases where a resistant species is less able to overcome venom from a second, geographically distant predator (Biardi and Coss, 2011; Rowe and Rowe, 2008), but strong support for this hypothesis is lacking. It is possible to quantify the functional effects of resistance evolution before and after speciation by comparing population and species-level variation in resistance to sympatric venomous species.

Sciurid rodents have provided most information to date on both population-level variation and species specificity in venom resistance. The California ground squirrel (*Otospermophilus beecheyi*) and rock squirrel (*Otospermophilus variegatus*) show evidence for a direct role of local selection from venomous rattlesnakes (*Crotalus*

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spp.) by exhibiting variable levels of venom resistance across populations (Biardi, 2008; Biardi et al., 2006; Coss et al., 1993; Poran et al., 1987). In *O. beecheyi*, variation in venom resistance is associated with local rattlesnake density across the species range, with populations experiencing higher predation pressure from the Northern Pacific Rattlesnake (*Crotalus oreganus oreganus*) showing higher levels of venom resistance (Biardi, 2008; Poran et al., 1987). Resistance at the level of sympatric versus allopatric rattlesnake species exists in closely related *O. variegatus*. This squirrel is preyed upon by multiple other rattlesnake species, and serum from *O. variegatus* is better able to inhibit the proteolytic and hemolytic activity of sympatric *Crotalus atrox* and *Crotalus viridis* than that of allopatric *C. o. oreganus* (Biardi and Coss, 2011). The combined studies on *Otospermophilus* are consistent with the existence of both balancing selection within a species and specialization between species: intraspecific variation in prey resistance has evolved in response to variable selection pressures from local snake density, while between-species comparisons yield larger effect sizes and a pattern where squirrels may be best adapted to inhibiting local rattlesnake venoms at a cost to inhibition of allopatric venoms.

A limitation of previous work on species-specificity in venom resistance is that resistance to multiple venoms is measured for only one resistant species (Biardi, 2008; Biardi and Coss, 2011), or resistance to one venom is measured among multiple resistant species (Heatwole and Poran, 1995; Rowe and Rowe, 2008). While Soto et al. (1988) did test Virginia opossum (*Didelphis virginiana*) and Southern plains woodrat (*Neotoma micropus*) sera for the capacity to neutralize venom hemorrhagic activity of 25 species of snakes, the sera from both mammals completely neutralized all venoms under the experimental conditions used, preventing measurement of possible specificity across a range of inhibition values. Therefore, the collective studies to date do not allow us to rule out the possibility that one species' venom is simply easier to inhibit than another for any resistant animal (a venom main-effect from a statistical perspective) and that the results obtained in previous work support the hypothesis of species-level adaptation of squirrels only by chance due to a main effect of how susceptible a given snake species' venom is to inhibition (Blanquart et al., 2013; Kawecki and Ebert, 2004; Thrall et al., 2002). Full reciprocal crosses of at least two paired, sympatric species of rattlesnake venom and squirrel serum inhibitors represent a more informative test of species-level adaptation of squirrel resistance, because this design has more power to test whether each squirrel species is best at inhibiting its sympatric snake predator's venom, regardless of the average ability of a given venom to avoid serum inhibitors in general (Blanquart et al., 2013; Kawecki and Ebert, 2004).

We quantified inhibition of rattlesnake venom activity by the Eastern gray squirrel (*Sciurus carolinensis*), an arboreal "tree squirrel" distributed over the eastern United States and southern Canada (Reid, 2006). The serum of *S. carolinensis* has been shown to block hemorrhagic activity of Western Diamondback Rattlesnake (*C. atrox*) venom (Perez et al., 1978), suggesting that *S. carolinensis* possesses serum-based inhibitors of snake venom metalloproteinases (SVMPs) as a form of venom resistance. However, *S. carolinensis* only encounters *C. atrox* in the extreme southwestern portion of its range, and is preyed on by sympatric Timber Rattlesnakes (*Crotalus horridus*) across most of its distribution. In fact, *S. carolinensis* can make up a significant portion of the diet of *C. horridus* (Clark, 2002).

Specifically, we assessed serum inhibition of SVMPs, which degrade proteins in the extracellular matrix to perforate blood vessels and allow diffusion of other toxic components out of the bite site (Gutiérrez et al., 2010). These traits make SVMP inhibition an important functional measure of resistance (Biardi, 2008). We first confirmed the ability *S. carolinensis* to resist SVMPs in the venom of a widely-coexisting rattlesnake, *C. horridus*. We then evaluated two hypotheses. First, we hypothesized that local snake predation pressure is necessary to maintain serum-based venom resistance in the face of physiological costs. This leads to the prediction that venom inhibitory capacity of serum from a population of *S. carolinensis* living in the presence of *C. horridus* predators would be higher than that from a second population living outside the geographic range of *C. horridus*. Second, we hypothesized that species-level specialization to inhibit sympatric snake venom occurs in Sciurids, resulting in costs if challenged by an allopatric venom phenotype which they rarely encounter. We tested this hypothesis by conducting a full reciprocal cross of *S. carolinensis* and *O. beecheyi* sera with *C. horridus* and *C. o. oreganus* venoms. We predicted that each squirrel species serum would be most effective as an inhibitor of its sympatric snake predator's venom activity relative to allopatric combinations of squirrel prey and snake predators.

2. Methods

2.1. Study sites and sample collection

We collected *S. carolinensis* blood samples from two sites in Ohio, USA: Shawnee State Park (n = 14; sympatric with *Crotalus horridus* rattlesnakes) and a western suburb of Columbus (n = 10; allopatric with the rattlesnakes; Fig. 1A). We captured *S. carolinensis* with live traps during four trapping days at each site between late April and June 2015. Upon capture, we immediately anesthetized the squirrel with isoflurane gas and drew a blood sample via cardiac puncture. Blood samples were stored on ice overnight to allow the blood to clot, then the serum was removed and centrifuged at 800 rcf for 10 min prior to long-term storage at −80 °C.

We obtained venom from three radio-tagged, adult, male *C. horridus* from Tar Hollow State Park, the northern-most remaining population of the snakes in Ohio. Wecreated a pooled sample with equal amounts of venom protein from each snake for use in all serum inhibition tests. We used the Bradford Protein Assay kit (Bio-Rad) to measure protein concentration of each venom sample, diluted each sample to 0.6125 mg/mL in phosphate-buffered saline, and placed 10 μL of each diluted sample into a final venom pool.

2.2. Measuring venom activity and testing for inhibition

We followed Biardi et al. (2011b) for quantifying SVMP activity. The Enz Chek Gelatinase assay (Life Technologies, Carlsbad, CA) measures SVMP activity to the exclusion of other venom protease enzymes. We employed the microassay format of this enzyme assay, measuring the activity of 0.3125 ng of venom in each assay well. We followed standard product protocols and used a 1:100 dilution of the gelatinase substrate. Enzymatic reaction rate was obtained by measuring the change in fluorescence expressed in Relative Fluorescence Units (RFU) in each well in a Fluostar Omega microplate reader (BMC Labtech, Ortenberg, Germany) and calculating the blank-corrected linear slope between 9 and 49 min into the reaction. We ran all assays in triplicate.

We measured serum inhibition of SVMPs by incubating one part venom with nine parts 2.5 mg/mL squirrel serum for 30 min prior to initiation of the gelatinase reaction, after which we obtained a venom activity measure as above, using 0.3125 ng venom in each well. To test for significant inhibition of SVMP activity by *S. carolinensis* serum, we performed a one-sample, two-sided *t*-test on the total set of SVMP activity measurements obtained from all *S. carolinensis* individuals (N = 24), using the baseline activity of the *C. horridus* venom sample (493.9 RFU/min) as the hypothesized

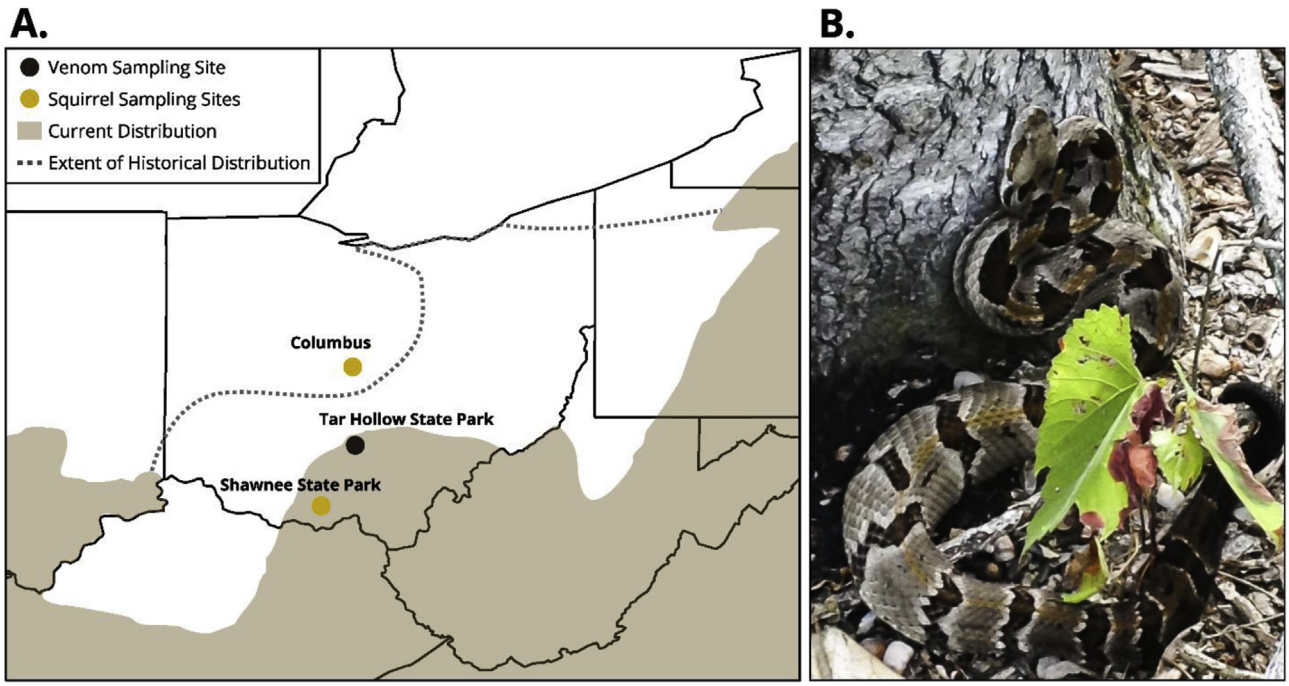

Fig. 1. A) The historical and current distribution of the Timber Rattlesnake (*Crotalus horridus*) in Ohio based on information from D. Wynn (pers. comm.) and from Conant and Collins (1998). The historical distribution closely matches the extent of Pleistocene glaciation in Ohio that flattened the land and made it unsuitable habitat for *C. horridus* (Szabo and Chanda, 2004). The allopatric site in Columbus is located north of the historical range boundary in central Ohio, while the sympatric site in Shawnee State Park is located in the current range on the border of southern Ohio. Venom was collected from *C. horridus* at Tar Hollow State Park. B) A Timber Rattlesnake waits in ambush posture, facing up the trunk of a large tree (photo by Craig Lind). This position is hypothesized to be a squirrel-hunting posture.

mean.

2.3. Local and species-level adaptation tests

To evaluate the importance of predation pressure by local snakes on *S. carolinensis*, we compared squirrel serum inhibition of SVMPs of *C. horridus* venom pool between the Shawnee State Park and Columbus squirrel populations. We expected stronger selection pressures from rattlesnakes at the southern site to lead to local adaptation in the form of sera with a higher capacity to inhibit venom relative to the more northern site. Because the inhibition of the same venom pool was assessed for each *S. carolinensis* population and individual, we can compare the raw enzymatic rates of SVMP activity obtained in the presence of each serum sample. The SVMP activity of the *C. horridus* venom pool was tested in the presence of serum from each individual squirrel captured at each site. We used a two-sample *t*-test without assuming equal variances to compare venom activity in the presence of sera of these two squirrel populations.

To test for adaptation of sciurid serum inhibitors at the level of different rattlesnake and squirrel species, we took advantage of available serum and venom samples from the well-studied system of venom-resistant California ground squirrels (*O. beecheyi*) and the Northern Pacific Rattlesnakes (*C. o. oreganus*) that feed on them, collected by M.L.H. in the Sutter Buttes area of north-central California (Holding et al., 2016). We produced a 0.6125 mg/mL pool of *C. o. oreganus* venom, derived from 3 individual snakes. We also produced a pool of serum from each squirrel species, combining equal parts of the sera of 5 individual squirrels from each species to produce separate 2.5 mg/mL pools of *S. carolinensis* and *O. beecheyi* serum. The *S. carolinensis* in the pool were randomly selected from the Shawnee State Park samples.

We performed a fully reciprocal cross of these squirrel and rattlesnake species' sera and venom pools. Thus, the sera of each squirrel species was tested with the venom of its sympatric rattlesnake predator as well as the allopatric rattlesnake species. The response variable in this analysis was relative activity: the serum-incubated value of SVMP activity divided by the activity of a venom and expressed as a percentage of baseline activity (Biardi et al., 2000; Biardi et al., 2006). This relative activity score was interpreted as the percent of a venom's baseline SVMP activity maintained when in the presence of squirrel venom inhibitor molecules. This standardized the measurement of resistance which was necessary for cross species comparisons given the different baseline activities of *C. horridus* and *C. oreganus* venom. We logit transformed the percentage data prior to parametric analysis.

The pooled samples we used are expected to represent average venom and serum phenotypes from the populations in which they were collected, allowing us to evaluate the relative metalloproteinase inhibition by sciurid sera of these samples in sympatry and allopatry. Our reciprocal cross quantified performance of these specific pooled samples, and the triplicate wells assayed for each combination of venom and serum can be used as replicates in testing for these species-level performance differences. We caution that this test does not provide any information about possible levels of variance in performance among venoms or sera at the level of other populations of these species. However, population pools approximate population-mean inhibition measurements (M. Holding, unpublished data).

If the venom-inhibitors in squirrel sera evolve to track diverging venoms of their sympatric snake predators, then we would expect each squirrel species to best inhibit the venom of its sympatric rattlesnake. We assessed this prediction with a two-way ANOVA, with squirrel species, rattlesnake species, and a rattlesnake × squirrel species interaction as factors. Species-level adaptation would be indicated by a significant interaction where

each squirrel was best at inhibiting its sympatric rattlesnake species' venom. The pooled samples are expected to represent average venom and serum phenotypes from the populations in which they were collected, allowing us to evaluate the relative metalloproteinase inhibition by sciurid sera of these pooled samples specifically in sympatry and allopatry. This test quantifies performance of these specific pooled samples, and the triplicate wells assayed for each combination of venom and serum can be used as replicates in testing for these species-level performance differences. We caution that this test does not provide any information about possible levels of variance in performance among venoms or sera at the level of other populations of these species. However, as mentioned above population pools approximate population-mean inhibition measurements (M. Holding, unpublished data).

3. Results

The activity of *C. horridus* venom was significantly reduced in the presence of *S. carolinensis* serum proteins ($t_{22} = -34.5$, $P < 0.001$, Fig 2); no trials involving individual serum samples from either Ohio population yielded venom activity values within 150 RFU/min of the activity of the venom by itself. Snake venom metalloproteinase activity was reduced by 77 percent, on average, in these trials. Therefore, *S. carolinensis* shows considerable ability to inhibit SVMP activity in *C. horridus* venom, a pre-requisite for our additional hypothesis tests.

Our comparison of serum-based inhibition in two *S. carolinensis* populations yielded results consistent with the prediction that venom inhibition will be higher in sites where rattlesnake predators are present. The *C. horridus* venom pool showed 37 percent lower metalloproteinase activity in the presence of serum from *S. carolinensis* collected where snakes are present (90.9 ± 11.2 RFU/min, 95% C.I.) than when serum was collected where snakes are absent (145.1 ± 40.9 RFU/min; $t_{10.5} = 2.5$, $P = 0.03$, Fig. 2). Expressed in terms of percent inhibition of the *C. horridus* venom, the squirrels at Shawnee State Park where the snakes occur reduced venom activity by 81.6 percent, whereas Columbus squirrels living in the absence of rattlesnake predators reduced activity by only 70.6 percent.

Finally, our species-level reciprocal cross produced a result consistent with species-level adaptation, where each squirrel species was best at inhibiting the SVMPs of its sympatric rattlesnake predator. The rattlesnake species × squirrel species interaction was significant ($F_{1,8} = 1259.2$, $P < 0.001$), with each squirrel species being much more effective at reducing the metalloproteinase activity of its sympatric rattlesnake species' venom (Fig. 3). *Sciurus carolinensis* reduced sympatric venom activity by an additional 20 percent compared to its performance on allopatric venom, while *O. beecheyi* serum reduced its sympatric venom by an additional 35 percent compared to allopatric venom. These functional differences from the interspecific reciprocal crosses are of larger magnitude than in the intraspecific comparisons of the populations of *S. carolinensis* in Ohio, only one of which was exposed to rattlesnakes.

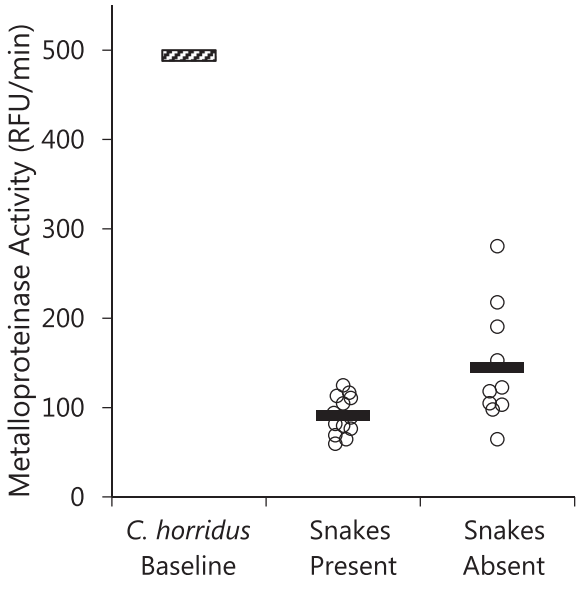

Fig. 2. Snake venom metalloproteinase activity of a pooled sample of Timber Rattlesnake (*Crotalus horridus*) venom alone or in the presence of serum from two populations of Eastern Gray Squirrels (*Sciurus carolinensis*): a southern Ohio population that is sympatric with and preyed upon by Timber Rattlesnakes (n = 14 squirrels, middle) and a central Ohio population that exists outside of the distribution of the snakes (n = 10 squirrels, far right). The serum of all squirrels used (circles) resulted in venom activity lower than the venom-only baseline activity (hashed bar). Mean venom activity in the presence of each squirrel population is indicated by black bars.

4. Discussion

4.1. Inhibition of SVMPs by gray squirrels

Our analysis of enzymatic activity demonstrated that *S. carolinensis* has serum that is capable of extensive inhibition of metalloproteinases in *C. horridus* venom, reducing this activity by 77 percent on average. This high resolution *in vitro* assessment of serum inhibition of the venom of a sympatric rattlesnake species complements the *in vivo* anti-hemorrhagic assays of Perez et al. (1978) and strengthens the conclusion that *S. carolinensis* has evolved resistance to SVMPs despite spending much of their time high in the trees where they can avoid predation by rattlesnakes (Bowers and Breland, 1996). The SVMPs are thought to be important proteins involved in the killing prey, because their hemorrhagic and tissue-degrading effects facilitate movement of other venom components, such as myotoxic or neurotoxic PLA_2s within the prey and allow coagulopathic lectins and serine proteases to

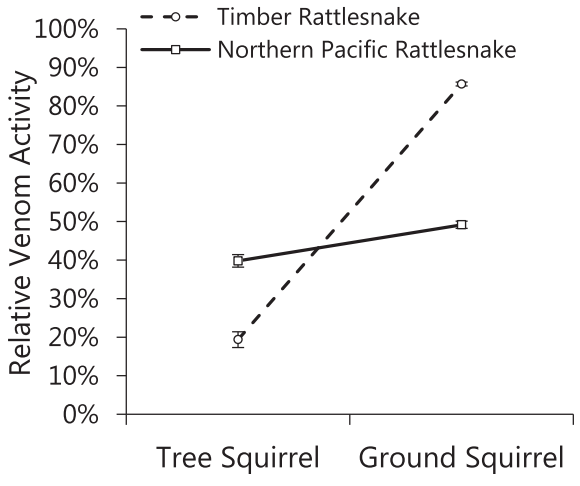

Fig. 3. Relative activity (percent of venom activity maintained when challenged with prey serum inhibitors, ± 95% confidence interval) of snake venom metalloproteinases in pooled samples of Timber Rattlesnake (*Crotalus horridus*) venom and Northern Pacific Rattlesnake (*Crotalus oreganus oreganus*) venom in the presence of serum from the fossorial California ground squirrel (*Otospermophilus beecheyi*) and tree-living Eastern gray squirrel (*Sciurus carolinensis*). Tree squirrels were most effective at inhibiting Timber Rattlesnake venom, while ground squirrels were most effective at inhibiting Northern Pacific Rattlesnake venom, suggesting that squirrel species possess inhibitors adapted to dealing with the venom of the rattlesnake they encounter at home.

more rapidly invade target tissues (Biardi, 2008). As such, rapid and lasting inhibition of SVMP enzymes is predicted to be crucial to venom resistance, and all circulating inhibitor proteins discovered to date bind SVMPs, save one PLA_2 inhibitor from the opossum *Didelphis aurita* (Rocha et al., 2002).

Sciurus carolinensis appears to possess such resistance and, moreover, there is evidence for evolutionary specialization of the capacity of resistance within and between species of squirrels (see below). To our knowledge, *S. carolinensis* is the only non-fossorial squirrel to be shown to have resistance to venom. Given that the southern African ground squirrel (*Xerus inauris*) does not show a capacity for serum based inhibition of venom proteases despite abundant snake predators (Phillips et al., 2012), the factors selecting for and evolutionary history of venom resistance across family Sciuridae is likely complex.

4.2. Local variation in resistance

In southern Ohio, *S. carolinensis* that are sympatric with *C. horridus* have serum with a significantly higher capacity for metalloproteinase inhibition than conspecifics only 135 km north, where the snakes are absent. As such, we would expect that *in vivo* assays of hemorrhagic activity or lethal dose measurement would reveal increased resistance to venom by the southern Ohio squirrel population (Bernardoni et al., 2014; Perez and Sanchez, 1999; Soto et al., 1988). One important implication of this result is that populations of *S. carolinensis* are harboring intraspecific variation in venom resistance. This variation could result from the presence of isoforms of the inhibitor proteins, or through variable levels of expression of the same inhibitor in different populations. In the latter case, genetic variation would be present in the regulatory machinery governing expression, instead of in the resistance protein gene itself (Stranger et al., 2007).

Importantly, higher venom inhibition by squirrels where rattlesnakes are present supports the hypothesis that local selection pressure from rattlesnake predation is the driving force for maintaining this variation. Since one goal of our paper is to promote an explicitly statistical approach to studies of variation in venom resistance, we caution that our comparison of only two populations of *S. carolinensis* leaves open the possibility that factors intrinsic to each site and unassociated with rattlesnakes could be responsible for the observed differences, and a study with population-level replication is required to confirm our interpretation. Nonetheless, our study design parallels others on resistance in *O. beecheyi*, *O. variegatus*, and the Southern grasshopper mouse (*Onychomys torridus*) that all document the trend of higher venom resistance to the venom of a locally-abundant venomous enemy (Biardi et al., 2000; Biardi et al., 2006; Biardi and Coss, 2011; Rowe and Rowe, 2008). Further, our result shows significant population-level variation consistent with our *a priori* prediction that the southern population will be more effective at SVMP inhibition.

As a human commensal common in neighborhoods and parks (Reid, 2006), there is likely a continuous distribution between and gene flow in *S. carolinensis* between our two study sites. Predation-related selection has been linked to phenotypic variation among closely spaced sites in other squirrels. For instance, camouflaging coat coloration shows a cline associated with canopy cover in red squirrels (*Tamiasciurus douglasi)* over short distances despite confirmed high gene flow (Chavez and Kenagy, 2014). Additionally, population-level variation in venom resistance of *O. beecheyi* covaries with local snake density, and over even shorter distances than those separating our current study sites (Biardi, 2008). Crucially, early studies in *O. beecheyi* showed that resistance was innately expressed, confirming that variation is an evolutionary response to selection from snakes (Poran and Coss, 1990). The steepness of clines over which venom resistance varies in both *S. carolinensis* and *O. beecheyi* suggests that maintaining resistance comes at a physiological cost. As serum proteins, resistance factors may interact with other key regulatory and homeostatic functions in squirrels, leading to evolutionary trade-offs and possibly threshold limits for the amount of these proteins that can be dissolved in the serum (Hereford, 2009; Towers and Coss, 1990).

4.3. Evolutionary history and the functional outcomes of envenomation

We have demonstrated functional adaptation of squirrels to rattlesnake species with which they are sympatric and currently interacting. The *S. carolinensis* serum pool was most effective at inhibiting sympatric *C. horridus*, while the *O. beecheyi* was best at inhibiting the venom of sympatric *C. o. oreganus*. Although our species-level reciprocal cross was limited to one pooled sample from each squirrel species, pooled samples incorporate individual variation in each phenotype of interest. Pooled samples combined with the large differences in the ability of each squirrel species to inhibit sympatric versus allopatric venoms convince us that our limited and unreplicated test still provides useful information about the existence of species-level adaptation of venom inhibitors. Our work demonstrates the value of a reciprocal crossing study design, while emphasize that more statistically powerful studies will replicate populations in each venomous and resistant species.

Species-level, reciprocal differences in venom performance imply complexity and specificity in the ways serum inhibitor proteins interact with isoforms of SVMPs, since there is no clear mechanism whereby the concentration of inhibitors alone could generate the pattern of specificity observed in our experiment (Nuismer et al., 2005; Ridenhour and Nuismer, 2007). A likely scenario is that serum-based resistance involves molecular matching of resistance molecule to target venom (Holding et al., 2016). If instead there was a “general resistance” factor that both species possess that could target multiple SVMPs, and only the concentration of this factor varied, only overall levels of resistance and venom activity in each species would matter. The squirrel species by snake species interaction in Fig. 3 would be absent. Instead, the evidence suggests squirrels may be able to evolve resistance mechanisms specific to the SVMP isoforms present in the rattlesnakes species that prey on them. Specialization of this sort is not surprising given ecology of these interactions. *Sciurus carolinensis* is the most frequently encountered diurnal prey by *C. horridus* (Clark, 2006) and makes up 5 percent of the food items recovered from adult snakes (Clark, 2002) despite a likely sampling bias against snakes with large meals in museum studies. The interactions of *O. beecheyi* and *C. o. oreganus* appear even more specialized, as the ground squirrels can comprise 33 percent of the snake’s diet (Fitch and Twining, 1946) and 96 percent of diurnal encounters with potential prey (Putman et al., 2016).

Species-level adaptation of inhibitors is consistent with the hypothesized mechanism of SVMP inhibition in squirrels and other mammals and, consequently, the existence of costs of specialization. Serum-inhibitors are molecular scavengers that bind and inactivate venom proteins (Biardi et al., 2011a; Voss and Jansa, 2012). Changes to surface residues or overall structure of a venom protein could reduce the capacity of an inhibitor to bind its target venom protein. Squirrel species may then evolutionarily track these alterations to venom proteins with evolution of the serum-based inhibitors. Such specificity produces a close match to a sympatric snake’s venom, while simultaneously leading to increasing mismatch with allopatric snakes and producing the functional outcome observed here (Fig. 4A). In terms of inhibition of SVMP activity, this species-level specialization resulted in larger

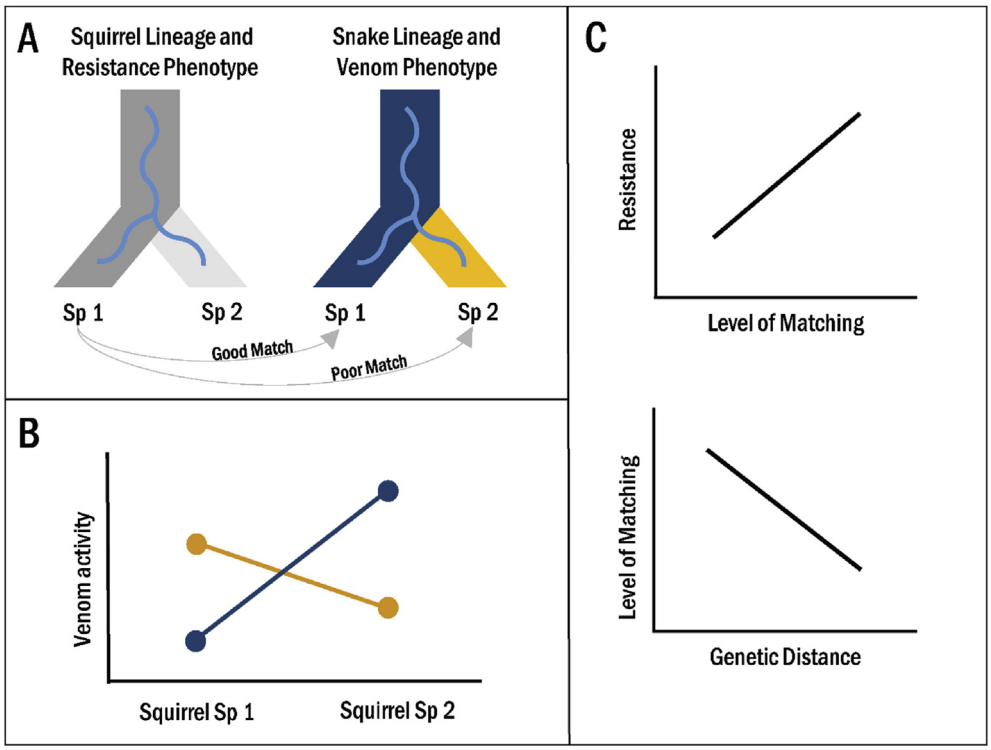

Fig. 4. Conceptual process by which serum scavenger proteins that inhibit snake venom track venom evolution of sympatric snakes at a cost to their ability to inhibit allopatric venoms. A) Phenotypic variation for venom and resistance phenotypes is shown for a snake and squirrel species, respectively. Squirrel species 1 (dark gray) is sympatric with snake species 1 (blue). The serum phenotype tracks or coevolves with the venom phenotype of the sympatric snake. A snake species that shared a common ancestor with the local snake at some point in the past (yellow) but is now allopatric with squirrel species 1 diverges phenotypically from its sister species' venom in phenotype space. A squirrel species will be more poorly matched with the allopatric snake's venom over time due to both tracking the local snake and independent divergence of the allopatric snake. Theoretical relationships are shown for B) matching of serum to venom vs serum-based resistance and C) genetic distance between sympatric and allopatric snake predators and the level of matching between squirrel serum and snake venom. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reductions in function than those seen within *S. carolinensis* living in the absence of rattlesnake predators. Therefore, just as a parasite evolves to infect its own host at a cost of general ability to infect other organisms (Antonovics et al., 2013), squirrels may be evolutionarily tracking local snake venoms at the expense of their ability to resist allopatric snake species' venom.

The squirrels' mismatch with allopatric snake predators should be accelerated by evolution of the allopatric snake species' venom, occurring by both neutral forces and possible coevolution with yet other prey (Holding et al., 2016), and may generate a relationship between functional variation in envenomation outcomes and phylogenetic distance of an allopatric snake from the sympatric snake predator (Fig. 4B,C). It would be of interest to conduct these types of tests on western gray squirrels (*Sciurus griseus*) that are broadly sympatric with *C. o. oreganus* but closely related to *S. carolinensis* (Herron et al., 2004; Reid, 2006), to begin to disentangle to roles of phylogeny and convergence in the observed functional adaptation of different squirrel species to dealing with the threat of envenomation. Because functional interactions between species are key to community formation, range limits, and the costs of migration (Becerra and Venable, 1999; Dyer et al., 2010; Mougi and Kondoh, 2012a, b; Naeem et al., 2012), our results lead to the prediction that migration and possibly range expansion will be easier for venomous predators than for their prey, at least with respect to the fitness consequences of interactions with novel antagonists.

5. Conclusions

We have provided further evidence for venom-inhibiting serum in an unexpected place: arboreal squirrels fed on by terrestrial snakes. Moreover, there is intraspecific variation in this functional trait consistent with expected local selection strength and possibly trade-offs that lead to loss of resistance when snakes are absent. Finally, squirrel prey may evolve serum-based inhibitors that closely complement the divergent venom phenotypes of the particular rattlesnakes that feed on them. Evolved venom resistance seems to be common in animals that interact frequently with venomous species, making it feasible to replicate ecologically relevant studies of functional variation across phylogenies of venomous and resistant enemies. Such future studies will help to elucidate the complex roles of coevolution and constraints in producing the variable venom and resistance arsenals of predators and prey.

Ethical statement

All aspects of this manuscript and its production conform to the Elsevier guidelines for Ethics in Publishing.

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