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

Abby M. Pomento [1], Blair W. Perry [1], Robert D. Denton, H. Lisle Gibbs, Matthew L. Holding*

Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, 318 W. 12th Avenue, Columbus, OH, 43210, USA

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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*

* Corresponding author. 318 W. 12th Avenue, Rm. 300, Columbus, OH, 43210, USA.
E-mail address: matthewholding28@gmail.com (M.L. Holding).
[1] Joint lead authors.

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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