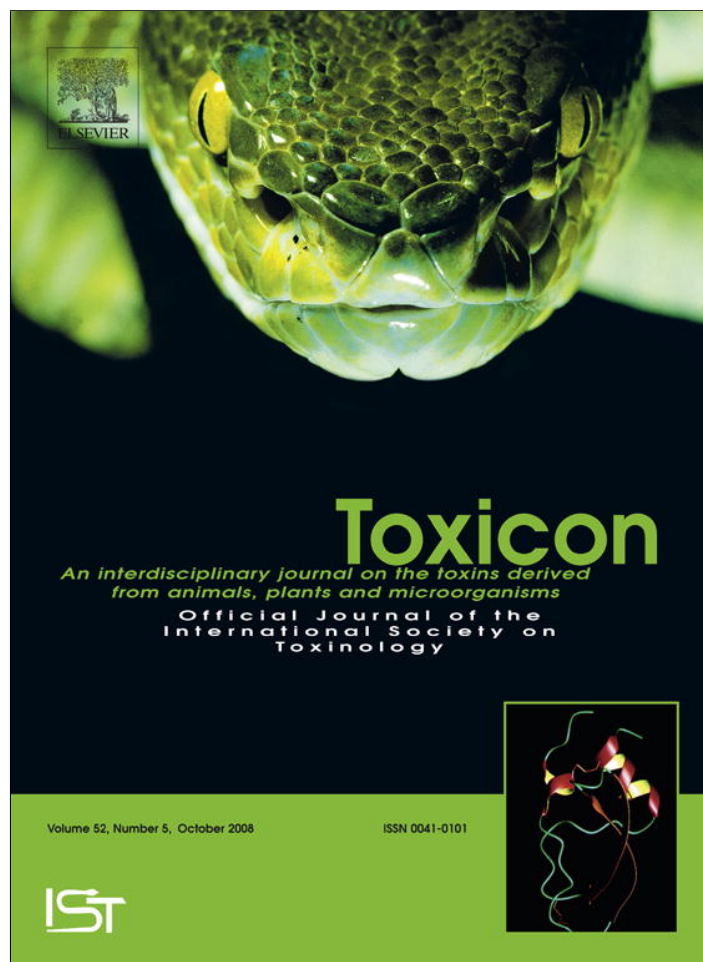


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Physiological resistance of grasshopper mice (*Onychomys* spp.) to Arizona bark scorpion (*Centruroides exilicauda*) venom[☆]

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ABSTRACT

Predators feeding on toxic prey may evolve physiological resistance to the preys' toxins. Grasshopper mice (*Onychomys* spp.) are voracious predators of scorpions in North American deserts. Two species of grasshopper mice (*Onychomys torridus* and *Onychomys arenicola*) are broadly sympatric with two species of potentially lethal bark scorpion (*Centruroides exilicauda* and *Centruroides vittatus*) in the Sonoran and Chihuahuan deserts, respectively. Bark scorpions produce toxins that selectively bind sodium (Na⁺) and potassium (K⁺) ion channels in vertebrate nerve and muscle tissue. We previously reported that grasshopper mice showed no effects of bark scorpion envenomation following natural stings. Here we conducted a series of toxicity tests to determine whether grasshopper mice have evolved resistance to bark scorpion neurotoxins. Five populations of grasshopper mice, either sympatric with or allopatric to bark scorpions, were injected with bark scorpion venom; LD₅₀s were estimated for each population. All five populations of grasshopper mice demonstrated levels of venom resistance greater than that reported for non-resistant *Mus musculus*. Moreover, venom resistance in the mice showed intra- and interspecific variability that covaried with bark scorpion sympatry and allopatry, patterns consistent with the hypothesis that venom resistance in grasshopper mice is an adaptive response to feeding on their neurotoxic prey.

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1. Introduction

Species that must defend themselves from venomous predators or subdue toxic prey often evolve physiological

resistance to their chemically protected adversaries. Evidence that such resistance is the product of selection may be demonstrated by higher levels of tolerance in populations that interact with venomous enemies relative to populations that either do not coexist with the toxic adversary or that interact with only moderately toxic individuals; i.e., level of toxin resistance covaries with level of toxicity or degree of exposure (Brodie and Brodie, 1999; Soong and Venkatesh, 2006). For example, eels (*Gymnothorax* spp.) that are preyed upon by sea kraits (*Laticauda colubrina*) are resistant to larger doses of sea krait venom compared with eels that are either sympatric but do not represent potential prey items or are allopatric to sea kraits (Heatwole and Poran, 1995; Heatwole and Powell, 1998). Similarly, California ground squirrels (*Spermophilus beecheyi*) that have coexisted with northern Pacific rattlesnakes (*Crotalus viridis oreganus*) for thousands of years,

[☆] Ethical statement: the Institutional Animal Care and Use Committee (IACUC) at the Brody School of Medicine, East Carolina University, North Carolina State University and Appalachian State University approved the procedures described below. All appropriate permits were obtained for the collection and transport of grasshopper mice.

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and whose pups comprise a large portion of the diet of these snakes, are highly resistant to the rattlesnake's venom while conspecifics from snake-free regions show greater sensitivity to equivalent doses of venom (Poran et al., 1987). One of the best examples of a predator demonstrating resistance to its venomous prey involves the garter snake, *Thamnophis sirtalis*, dealing with the tetrodotoxin (TTX) secreted by newts in the genus *Taricha* (Brodie, 1968; Brodie and Brodie, 1990). Populations of this garter snake sympatric with the newt demonstrate higher levels of tolerance to TTX than do populations allopatric to *Taricha* (Brodie et al., 2002). Moreover, within the zone of sympatry, there is a significant correlation between the toxicity of the newts and the levels of TTX resistance in the snakes (Hanifin et al., 2008).

Here we investigate the physiological tolerance of grasshopper mice (*Onychomys* spp.) to Arizona bark scorpion venom (*Centruroides exilicauda* Wood, formerly known as *Centruroides sculpturatus* Ewing). The genus *Onychomys* includes three species of carnivorous mice (*Onychomys torridus*, *Onychomys arenicola*, and *Onychomys leucogaster*) that are distributed throughout the deserts, shrub steppes, prairies, and intermountain grasslands of western North America (Riddle and Honeycutt, 1990; Riddle, 1995). While dietary analyses indicate that grasshopper mice consume a variety of animal taxa, including birds, reptiles, and other rodents, field and laboratory studies identify these mice primarily as voracious predators of arthropods (Egoscue, 1960; Horner et al., 1965; Horner and Taylor, 1968; Cyr, 1972; Flake, 1973; Hansen, 1975; Langley, 1981). At least one of the three species, *O. torridus* (southern grasshopper mouse), routinely eats scorpions (Horner et al., 1965; Cyr, 1972; Langley, 1981) and was historically referred to as the scorpion mouse (Bailey and Sperry, 1929). However, these studies involved species of scorpion that are not considered potentially lethal to mammals (e.g., *Hadrurus* spp.; Horner et al., 1965; Cyr, 1972; Langley, 1981; *Vaejovis* spp.; Langley, 1989). In fact, it was not known until recently whether grasshopper mice preyed on scorpions possessing neurotoxins that selectively, and often fatally, bind sodium (Na^+) and potassium (K^+) ion channels of vertebrates. Our analyses of the predatory behavior of grasshopper mice demonstrated that at least two species of *Onychomys* (*O. torridus* and *O. arenicola*) unhesitatingly attack, incapacitate and consume potentially deadly bark scorpions, including *C. exilicauda* (Arizona bark scorpion) and *Centruroides vittatus* (striped bark scorpion), as routinely as they attack and subdue crickets and non-lethal species of scorpions (Rowe and Rowe, 2006).

Bark scorpions (*Centruroides* spp.) are a speciose genus of scorpion best known for their production of neurotoxins that selectively bind the Na^+ and K^+ ion channels of vertebrates (Simard et al., 1992; Possani et al., 1999, 2000; Corona et al., 2001, 2002; Nastainczyk et al., 2002; Rodriguez de la Vega et al., 2003; Rodriguez de la Vega and Possani, 2004, 2005; Scorpion database, Tan et al., 2006). *C. exilicauda* is distributed from Sonora, Mexico, throughout Arizona and northward into southern Utah. Its distribution extends westward into southern California and the entire Baja peninsula, and eastward into the

westernmost region of New Mexico (Fet and Lowe, 2000). Recent biochemical, physiological and genetic analyses of the venom of *C. exilicauda* from southern Baja (Mexico) and Arizona (U.S.) show that the venom of Baja *C. exilicauda* is not only less toxic, but also sufficiently distinct from the venom of *C. exilicauda* distributed throughout mainland Mexico and the U.S. to assign the Baja individuals their own specific epithet (Valdez-Cruz et al., 2004). If the taxonomy for *C. exilicauda* is revised, it is likely that the Baja species will retain the listing as *C. exilicauda*, while the species from the mainland will revert to *C. sculpturatus*. Pending this taxonomic revision, we will use *C. exilicauda* as the species designation for the populations of bark scorpion we examined from Arizona.

The distribution of *C. exilicauda* (Fig. 1) is broadly coincident with the distribution of *O. torridus* (southern grasshopper mouse) throughout much of their range in the Sonoran, Sinaloan, and Mojave deserts of the U.S. and Mexico (Riddle and Honeycutt, 1990; Riddle, 1995). *C. exilicauda* (at least populations from mainland Mexico and the U.S.) is included in the group of *Centruroides* species capable of delivering lethal stings to mammals (Stahnke, 1956, 1971; Simard et al., 1992). Differences in toxicity or lethality among *Centruroides* species are largely determined by the unique composition of ion-channel toxins in the venom of each species (Simard et al., 1992; see also Possani et al., 1999; Corona et al., 2002; Rodriguez de la Vega and Possani, 2004, 2005). The median lethal dose (LD_{50}) reported for various populations of *C. exilicauda* from Arizona (U.S.) and Sonora (Mexico) ranges from 0.67 mg/kg to 1.5 mg/kg in *Mus* spp. (Stahnke, 1971; Simard et al., 1992; A. Alagon, unpublished data). The range in LD_{50} values may reflect differences in the strain of *Mus musculus* used in these toxicity tests and/or the route of venom administration; e.g., subcutaneous, intraperitoneal, intravenous, etc. (Simard et al., 1992). Alternatively, the range in LD_{50} values reported for *C. exilicauda* from mainland Mexico and Arizona may represent population-level differences in venom composition (Simard et al., 1992). However, with the exception of the Valdez-Cruz et al. (2004) investigation of geographic differences between Baja and Arizona *C. exilicauda*, there are no detailed studies regarding differences in venom composition and toxicity among *C. exilicauda* populations from either mainland Mexico or the U.S.

C. vittatus is distributed from Mexico (Chihuahua, Coahuila, Nuevo Leon, Tamaulipas, and Zacatecas) northward through Texas and Oklahoma, westward through New Mexico to the Rio Grande, and eastward through Louisiana, Arkansas and Missouri (Fet and Lowe, 2000). The distribution of *C. vittatus* (Fig. 1) overlaps with the distribution of *O. arenicola* (Mearns' grasshopper mice) throughout most of their range in the Chihuahuan Desert of the U.S. and Mexico (Riddle and Honeycutt, 1990; Riddle, 1995). The venom of *C. vittatus* has not been as well characterized at the biochemical or physiological level as *C. exilicauda* because *C. vittatus* does not pose a health risk to humans (Stahnke, 1956, 1971). However, high performance liquid chromatography (HPLC) of the venom shows that *C. vittatus* produces both Na^+ and K^+ ion-channel neurotoxins and toxicity tests demonstrate that the venom is potentially dangerous to mice (A. Rowe, unpublished data).

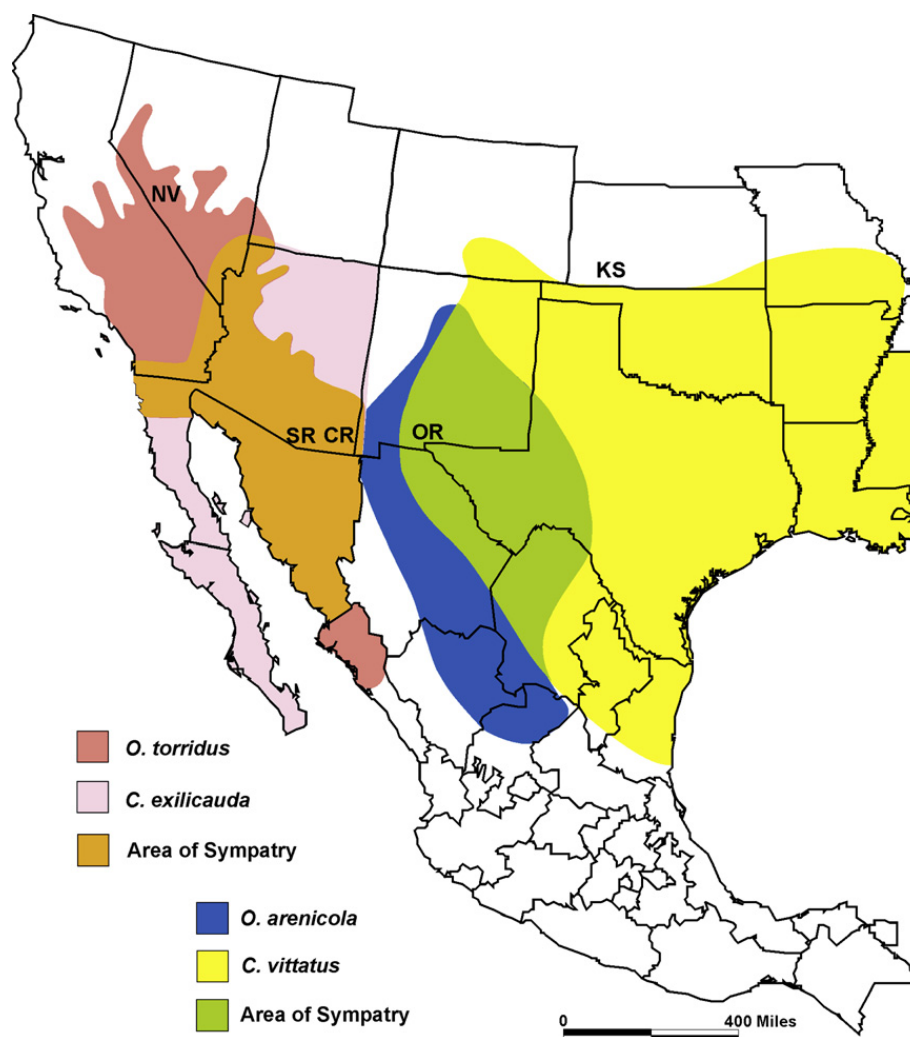


Fig. 1. Geographical distributions of the southern grasshopper mouse (*Onychomys torridus*), Mearns' grasshopper mouse (*O. arenicola*), the Arizona bark scorpion (*Centruroides exilicauda*), and the striped bark scorpion (*C. vittatus*) in the southwestern U.S. Not shown is the distribution of the northern grasshopper mouse (*O. leucogaster*), which inhabits the Great Plains and the intermountain grasslands of the western and northwestern U.S. and south-central Canada. The five populations of grasshopper mice tested for their resistance to *C. exilicauda* venom are identified by two-letter abbreviations at their respective locations on the map: NV = Lyon and Nye Counties, NV; SR = Santa Rita Mountains, AZ; CR = Chiricahua Mountains, AZ; OR = Organ Mountains, NM; KS = Finney County, KS.

The manner in which grasshopper mice disabled and then consumed *C. exilicauda* and *C. vittatus* during feeding trials suggests that these mice routinely encounter and prey on bark scorpions in communities where they coexist (Rowe and Rowe, 2006). During feeding trials, the mice were usually unable to evade stings by bark scorpions. Most mice sustained at least one sting, while some mice were stung several times during an interaction. However, none of the grasshopper mice exhibited any of the toxic or lethal effects associated with *Centruroides*' venom. These observations suggest that grasshopper mice have developed physiological mechanisms for detoxifying *C. exilicauda* and *C. vittatus* neurotoxins.

Our first objective was to determine if grasshopper mice sympatric with either *C. exilicauda* or *C. vittatus* have developed physiological resistance to bark scorpion venoms. If grasshopper mice have developed resistance to the cocktail of Na⁺ and K⁺ ion-channel neurotoxins produced by bark scorpions, we predicted that the LD₅₀ of venom reported for non-resistant *Mus* spp. should be non-

toxic or only mildly toxic to grasshopper mice. Our second objective was to estimate the LD₅₀ for populations of grasshopper mice sympatric with and allopatric to bark scorpions to determine if venom resistance covaries with the distribution of this group of scorpions. If venom resistance is the product of natural selection by bark scorpions, then population-level resistance (measured by an LD₅₀ using *Centruroides* venom) should correspond at least coarsely, though not necessarily perfectly (see Thompson, 2005; Hanifin et al., 2008), with regions of scorpion sympatry and allopatry.

We used a series of toxicity tests to determine the effects of *C. exilicauda* venom on grasshopper mice. We estimated the LD₅₀ of venom for populations of grasshopper mice both sympatric with and allopatric to *C. exilicauda* and *C. vittatus*. We chose to use *C. exilicauda* venom for all of the assays because it is more toxic than *C. vittatus* venom and because the physiological effects and LD₅₀ of the venom are well characterized in *Mus* spp. (Stahnke, 1971; Simard et al., 1992; Valdez-Cruz et al., 2004).

Here we report that grasshopper mice demonstrate physiological tolerance to *C. exilicauda* venom as compared with non-resistant *Mus* spp. We also show that geographic variability in venom resistance for several populations of mice covaries with the distribution of *C. exilicauda* and *C. vittatus*.

2. Materials and methods

2.1. Study sites and grasshopper mouse populations

Grasshopper mice were collected from geographical locations either sympatric with or allopatric to bark scorpions (Fig. 1). *O. torridus* were collected from two study sites in Arizona (AZ) and two sites in Nevada (NV). The first Arizona site represents a population of mice that occur sympatrically with *C. exilicauda* in the Santa Rita Mountains (SR) of Pima County in south-central AZ ($N=16$ *O. torridus*). The second location, near the Chiricahua Mountains (CR) of Cochise County in southeastern AZ, represents a site where the mice are parapatric with a nearby population of *C. exilicauda* ($N=9$ *O. torridus*). The nearest *C. exilicauda* population is located approximately 15 to 20 miles to the east of the Chiricahuas in the Peloncillo Mountains. Study sites in NV were located near the Carson River in Lyon County of southwestern NV ($N=6$ *O. torridus*), and in the mountains of Nye County in south-central NV ($N=7$ *O. torridus*). The distribution of bark scorpions does not extend from AZ into NV beyond the Colorado River. Thus, populations of *O. torridus* from Lyon and Nye Counties are allopatric to *Centruroides*. *O. torridus* from NV are, however, sympatric with several species of non-toxic scorpion, including members of the genus *Vaejovis*. *O. torridus* from the two study sites in Lyon and Nye Counties were pooled into one population (NV). A single population of *O. arenicola* was collected from the Organ Mountains (OR) of Doña Ana County in south-central New Mexico (NM) ($N=8$ *O. arenicola*). In this region, *O. arenicola* occur sympatrically with *C. vittatus*. A single population of *O. leucogaster* (northern grasshopper mice) was collected from a sand-sage prairie community located in Finney County of southwestern Kansas (KS) ($N=13$ *O. leucogaster*). *O. leucogaster* from this region are allopatric to all species of *Centruroides*; moreover, no scorpions of any species were detected during nocturnal surveys using black-lights.

Grasshopper mice were collected using Sherman live traps baited with a mixture of dry cat food and peanut butter. The traps were baited prior to sunset and checked the following morning before sunrise. Mice were maintained in standard mouse cages with bedding and water bottles, and provided commercial mouse chow as a food source. With the exception of the KS population, wild grasshopper mice were transported to a USDA-approved animal facility to establish breeding colonies (Department of Comparative Medicine, Brody School of Medicine, East Carolina University, Greenville, NC). In captivity, grasshopper mice were maintained in large mouse cages on a 12/12, light/dark cycle at a room temperature of 22–23 °C. Mouse chow and water were provided *ad libitum*. Grasshopper mice from the KS population were collected and

immediately tested in the field. Survivors were released at their site of capture.

2.2. Scorpions and venom

C. exilicauda venom used to test the SR, CR, OR and NV populations was provided by Dr. Dean D. Watt (College of Medicine, Creighton University, Omaha, NE) by way of Dr. Paul Fletcher (Department of Microbiology and Immunology, Brody School of Medicine, East Carolina University, Greenville, NC). The scorpions were collected from Pinal County in central AZ by Lorin Honetschlagger. The venom was extracted using electrical stimulation of the telson, lyophilized and stored frozen. Prior to bioassays, lyophilized venom was reconstituted in a small amount of distilled water and centrifuged (4 °C) to remove insoluble components. The supernatant was stored at –20 °C. Scorpions collected from this geographic location over a 15-yr period produced venom samples consistent in composition and toxicity ($LD_{50}=1.0–1.5$ mg/kg for *Mus* sp., Simard et al., 1992). The venom sample used to test the KS population was obtained from *C. exilicauda* collected by the authors from Pima County in south-central AZ ($LD_{50}=0.8$ mg/kg for *Mus* sp.; A. Rowe unpublished data). After extracting the venom using electrical stimulation of the telson, the samples were dissolved in distilled water and centrifuged (4 °C) to remove insoluble components. The supernatant was lyophilized and stored at –20 °C. Lyophilized samples were reconstituted in a small amount of distilled water prior to bioassays.

2.3. Initial toxicity tests to confirm resistance

In order to confirm the physiological resistance of grasshopper mice to bark scorpion venom, groups of grasshopper mice were tested with a dose of Arizona *C. exilicauda* venom equivalent to the LD_{50} reported for *Mus* spp. (1.00–1.50 mg/kg, Simard et al., 1992). Three groups of grasshopper mice representing populations sympatric with bark scorpions (SR, CR, and OR) were injected with a single dose of venom ($N=8$ mice per group, venom = 1.56 mg/kg). Vehicle control groups from each population received an equivalent volume of phosphate buffered saline ($N=3$ mice per group, PBS, pH 7.3). Grasshopper mice in the venom and vehicle groups were born in captivity and had never been exposed to scorpion venom. The SR group consisted of two males and nine females (F_1 generation, avg. wt. = 26.11 g). The CR group consisted of four males and seven females (F_1 generation, avg. wt. = 25.83 g). The OR group consisted of six males and five females (F_1 and F_2 generations, avg. wt. = 21.5 g). In order to show that the batch of *C. exilicauda* venom used was biologically active, two *M. musculus* (strain CD-1) were each injected with a single dose of venom (first mouse = 25.9 g, venom = 1.56 mg/kg; second mouse = 26.0 g, venom = 2.03 mg/kg). Mice from all groups were weighed and venom doses were adjusted to each individual mouse's weight. Aliquots of venom were diluted in PBS (pH 7.3) to the final concentration and injected into the intraperitoneal (IP) cavity of the mice (volume of venom injected ranged from 0.2 ml to 0.4 ml). The mice were observed continuously for the first 4 h and again at 24 h.

The result was designated as: “non-toxic” if the mice exhibited no effects of the venom; “toxic” if the mice demonstrated increased salivation, repetitive head movements, slight seizures or twitching, respiratory distress, loss of motor control, or hyper- or hypo-activity; and “lethal” if the mice showed some or all of the “toxic” effects culminating in ataxia and respiratory failure. Mice that demonstrated ataxia and respiratory failure were designated as “morbid” and were humanely euthanized as required by our IACUC guidelines.

2.4. UDP toxicity tests to estimate LD₅₀

The Up-and-Down Procedure (UDP) was used to estimate the dose of *C. exilicauda* venom that would be lethal to 50% (LD₅₀) of a population of grasshopper mice (Dixon, 1965; Bruce, 1985; NIEHS, 2001; USEPA, 2002). The UDP technique was selected because it generates results indistinguishable from the conventional method while reducing the number of animals needed to estimate the LD₅₀ (Bruce, 1987; Lipnick et al., 1995). Individual mice were injected with a single dose of venom in a sequential order. If the mouse survived, the dose administered to the next mouse was increased. If the mouse died, the dose administered to the next mouse was decreased. Doses were increased and decreased incrementally by a dose progression factor of 1.3. Dosing was continued until a clear pattern emerged where at least five reversals occurred in dose direction. Median lethal doses were estimated for each population of mice using the maximum-likelihood method; and confidence intervals were estimated using the profile-likelihood procedure and probit analysis (Dixon, 1965; AOT425-StatPgm, USEPA, 2002).

The LD₅₀ was estimated for five geographically distinct populations of grasshopper mice (Table 1). The SR, CR, NV and OR groups included both captive born and wild-caught mice, while the KS group included only wild-caught mice. The SR population consisted of 11 female mice ($N=6$ wild caught, $N=5$ captive born, avg. wt. = 28.3 g). The CR population consisted of one male and 10 female mice ($N=6$ wild caught, $N=5$ captive born, avg. wt. = 29.6 g). The NV population consisted of 11 male mice ($N=7$ wild caught, $N=4$ captive born, avg. wt. = 16.8 g). The OR population

consisted of six male and four female mice ($N=1$ wild caught, $N=9$ captive born, avg. wt. = 24.8 g). The KS population consisted of five male and three female mice ($N=8$ wild caught, avg. wt. = 39.7 g). Mice were weighed and each dose of venom was adjusted to the test animal's weight. Venom aliquots were diluted in PBS (pH 7.3) to the final concentration and injected IP (volume of venom injected ranged from 0.2 ml to 0.4 ml). Mice were observed continuously for 3–4 h and again at 24 h (see above description of “non-toxic”, “toxic” and “lethal” effects).

3. Results

3.1. Initial toxicity test to confirm resistance

All of the grasshopper mice in the SR, CR and OR test populations survived a single dose of *C. exilicauda* venom. Moreover, none of the grasshopper mice demonstrated any of the toxic effects typically associated with *C. exilicauda* neurotoxins. Grasshopper mice in the vehicle control groups that received an equivalent volume of PBS exhibited no physiological effects of either the saline or the injection. *M. musculus* in the venom control group exhibited all of the toxic and lethal effects typically associated with *C. exilicauda* neurotoxins, thus demonstrating that the batch of venom used in this assay was biologically active. Both *M. musculus* reached a stage of morbidity between 25 and 35 min post-injection and were humanely euthanized as required by IACUC guidelines.

3.2. UDP toxicity tests and estimates of median lethal doses

The results of the UDP indicate that while all five populations of grasshopper mice demonstrate physiological resistance to *C. exilicauda* venom when compared with non-resistant *Mus* spp., intra- and interspecific differences exist among grasshopper mice populations in their degree of resistance (Table 1). Moreover, these patterns of resistance covary with the presence of *C. exilicauda* and *C. vittatus*. Venom resistance for each grasshopper mouse population is expressed as the dose of *C. exilicauda* venom that produced mortality in 50% of the grasshopper mice tested (LD₅₀). Differences among LD₅₀ values are

Table 1

Physiological resistance of five different grasshopper mouse populations to *Centruroides exilicauda* venom

Study Site	Species of Grasshopper Mouse [<i>Scientific name</i> (common name)]	Co-occurrence with Bark Scorpions [distribution; <i>Scientific name</i> (common name)]	Toxicity LD ₅₀ (mg/kg) (95% C.I.)
SR	<i>Onychomys torridus</i> (southern grasshopper mouse)	Sympatric with <i>Centruroides exilicauda</i> (Arizona bark scorpion)	18.38 (15.2–19.8)
CR	<i>O. torridus</i> (southern grasshopper mouse)	Parapatric to <i>C. exilicauda</i> (Arizona bark scorpion)	12.49 NA ^a
NV	<i>O. torridus</i> (southern grasshopper mouse)	Allopatric to all <i>Centruroides</i> spp.	10.27 (9.0–11.7)
OR	<i>O. arenicola</i> (Mearns' grasshopper mouse)	Sympatric with <i>C. vittatus</i> (striped bark scorpion)	8.60 (7.5–9.8)
KS	<i>O. leucogaster</i> (northern grasshopper mouse)	Allopatric to all <i>Centruroides</i> spp.	3.91 (3.4–4.5)

Values are reported as the dose of *C. exilicauda* venom that produces mortality in 50% of the grasshopper mouse population (LD₅₀ mg/kg). The median lethal dose previously reported for *C. exilicauda* venom in a non-resistant mammal model (*Mus* sp.) is equivalent to 0.67–1.5 mg/kg (Stahnke, 1971; Simard et al., 1992; A. Alagon, unpublished data).

^a Not assessable due to high inter-individual variation; see text for details.

considered significant if the confidence intervals (C.I.) for different populations do not overlap. See Appendix 1 for UDP raw data for each population.

3.2.1. Intraspecific patterns

While all three populations of *O. torridus* (SR, CR, and NV) demonstrated higher levels of venom resistance than the populations of *O. arenicola* (OR) and *O. leucogaster* (KS), toxicity tests revealed differences among *O. torridus* populations that covary with the presence of *C. exilicauda* (Table 1). The SR population of *O. torridus* that coexist with an abundant population of *C. exilicauda* in the Santa Rita Mountains expressed the highest level of resistance in the assay. The CR population of *O. torridus*, parapatric to a population of *C. exilicauda* in the Peloncillo Mountains, expressed a level of resistance lower than the sympatric SR population. It was not possible to assess whether the difference between the SR and CR population LD₅₀s was significant because individual measures of toxicity within the CR population were too variable to generate a confidence interval (AOT₄₂₅StatPgm, USEPA, 2002). The NV population of *O. torridus*, allopatric to all *Centruroides*, expressed a level of resistance significantly lower than the sympatric SR population, and lower than the parapatric CR population. While the NV population of *O. torridus* is allopatric to all *Centruroides* species, they do coexist with other genera of scorpion such as the *Vaejovis* group.

3.2.2. Interspecific patterns

The UDP revealed that all three species of grasshopper mice demonstrate a level of physiological resistance to *C. exilicauda* venom greater than that reported for non-resistant *Mus* spp. (Table 1). However, differences exist in the level of resistance expressed among the three species of grasshopper mice. Populations of *O. torridus* sympatric (SR), parapatric (CR) and allopatric (NV) to the highly toxic *C. exilicauda* demonstrated the highest levels of resistance. The population of *O. arenicola* (OR) that coexist with *C. vittatus* in the Organ Mountains expressed a level of resistance lower than that of all three populations of *O. torridus*, and significantly so when compared to the LD₅₀ for SR mice. It is important to note that the venom of *C. vittatus* (LD₅₀ = 7.0 mg/kg, measured in *M. musculus*, strain CD-1, A. Rowe, unpublished data) is less toxic than the venom of *C. exilicauda* (LD₅₀ = 0.67–1.5 mg/kg, measured in *Mus* spp., Stahnke, 1971; Simard et al., 1992; A. Alagon, unpublished data). The population of *O. leucogaster* (KS), allopatric to all *Centruroides* species, exhibited a level of resistance significantly lower than either the SR or NV populations of *O. torridus*, and significantly less than the resistance shown by the single OR population of *O. arenicola*. *O. leucogaster* from the KS population are not only allopatric to all *Centruroides* species, but also to all other species of scorpion.

4. Discussion

The initial toxicity test demonstrated that a single dose of *C. exilicauda* venom (1.56 mg/kg) equivalent to the LD₅₀ reported for *Mus* spp. had no effect on two populations of *O. torridus* (SR and CR) and one population of *O. arenicola* (OR).

These results suggest that grasshopper mice have evolved physiological resistance to the Na⁺ and K⁺ ion-channel neurotoxins produced by these scorpions. However, subsequent estimates of the median lethal dose of *C. exilicauda* venom for five populations of grasshopper mice reveal intra- and interspecific variation in the expression of venom resistance. These patterns of venom resistance covary geographically with the distribution of *C. exilicauda* and *C. vittatus* and suggest that venom resistance in grasshopper mice may have evolved due to selection pressure from *Centruroides*.

4.1. Interspecific patterns

Results from the LD₅₀ assays suggest that grasshopper mice have evolved specific counter defenses in response to the potent venoms of *C. exilicauda* and *C. vittatus*. Strong support for this conclusion is provided by the geographic patterns of expression in venom resistance among the three species of grasshopper mice inhabiting regions sympatric with or allopatric to *Centruroides*. Populations of *O. torridus* and *O. arenicola*, two species whose distributions closely coincide with those of *C. exilicauda* and *C. vittatus*, respectively, exhibited levels of venom resistance significantly greater than that of a population of *O. leucogaster*, a species whose extensive range in the north-central U.S. is primarily, though not exclusively, allopatric to *Centruroides*. Specifically, the LD₅₀s for three populations of *O. torridus* ranged from 10.3 to 18.4 mg/kg; for *O. arenicola*, the dose was 8.6 mg/kg; and for *O. leucogaster*, the concentration was only 3.9 mg/kg. Thus, species of grasshopper mice that routinely interact with *Centruroides* appear to have evolved physiological defenses against these toxins.

What hypotheses might account for the fact that *O. leucogaster*, at least those from the KS population we tested, show partial resistance to *C. exilicauda* venom? After all, the LD₅₀ for *O. leucogaster* to *C. exilicauda* venom was 3.9 mg/kg, approximately three times the LD₅₀ reported for domestic mice, *Mus* spp., at 0.67–1.5 mg/kg. One possibility is that a low level of resistance is simply an ancestral characteristic of sigmodontine rodents, a physiological preadaptation common in the clade, similar to the low levels of TTX resistance reported for all species of garter snakes, even those distantly allopatric to *Taricha* (Motychak et al., 1999). Alternatively, the partial resistance we observed in *O. leucogaster* may represent relaxed selection. Vestigialization of previously adaptive traits under relaxed selection can take an extremely long time (Fong et al., 1995). For example, the loss of venom resistance and functional antsnake behavior in California ground squirrels (*S. beecheyi*) no longer experiencing predation from rattlesnakes (*Crotalus* spp.) has been shown to require thousands to millions of years (Poran et al., 1987; Towers and Coss, 1990; Coss, 1999). Molecular and fossil evidence suggests that all three species of *Onychomys* diverged from a common ancestor inhabiting the desert regions of the southwestern U.S. sometime between 2.5 and 3.5 mya (Riddle and Honeycutt, 1990; Riddle, 1995), were already feeding on insects (Carleton and Eshelman, 1979), and were almost certainly interacting with *Centruroides* (Gantenbein et al., 2001). We hope to test the “preadaptation” vs.

“vestigialization” hypotheses explaining venom resistance in *O. leucogaster* by expanding resistance analyses to include additional species of rodents in the subfamily Sigmodontinae.

Comparisons of venom resistance in the two populations of grasshopper mice tested that still coexist with *Centruroides*, namely *O. torridus* (SR) with *C. exilicauda* and *O. arenicola* (OR) with *C. vittatus*, suggest that bark scorpions and grasshopper mice may have acted as reciprocal sources of selection on each other during their ~3 million-plus years of sympatry. When applied to predator–prey interactions, reciprocal selection requires that the traits observed in predators be the products of selection imposed by its prey, with the prey's traits having been produced by selection from the predator; i.e., the defensive and offensive traits of the prey and the predator should appear linked or matched (i.e., coevolved). That is exactly what is seen when the venom resistance levels in SR mice (18.4 mg/kg) and OR mice (8.6 mg/kg) are compared with the corresponding toxicities of the venoms they must deal with; *C. exilicauda* is more toxic ($LD_{50} = 0.67\text{--}1.5$ mg/kg) than *C. vittatus* ($LD_{50} = 7.0$ mg/kg). We know from our behavioral work (Rowe and Rowe, 2006) that neither *O. torridus* nor *O. arenicola* show any signs of envenomation even when stung multiple times during interactions with the species of bark scorpion they normally encounter (*C. exilicauda* and *C. vittatus*, respectively). Coevolution between venom resistance in the mice and venom toxicity of the scorpions is further illustrated by estimates of the quantity of venom that could be injected by a scorpion during a natural sting – a maximum 0.1–0.3 mg of toxin protein (estimated from venom extracted via electrostimulation; Simard et al., 1992; Rowe and Rowe, unpublished data). Based on these admittedly coarse estimates, the quantity of venom protein required to incapacitate even a small (20 g) *O. torridus* (~0.37 mg from the more toxic *C. exilicauda* which SR mice encounter) or *O. arenicola* (~1.20 mg from the less toxic *C. vittatus* with which OR mice are sympatric) is more than either species of scorpion could deliver.

4.2. Intraspecific patterns

Within-species comparisons of resistance provide additional evidence that grasshopper mice have responded evolutionarily to *Centruroides*' toxic venom. For example, *O. torridus* sympatric with a dense population of *C. exilicauda* in the Santa Rita Mountains of AZ exhibited the highest level of venom resistance observed in this study. *O. torridus* from the Chiricahua Mountains of AZ, a region lacking but in close geographic proximity to a population of *C. exilicauda* in the Peloncillo Mountains of NM, demonstrated an intermediate level of resistance. And finally, exhibiting the lowest level of resistance of *O. torridus* to *C. exilicauda*'s toxins was a population of *O. torridus* from northwestern NV, a region distantly allopatric to all known populations of *Centruroides*. Thus, intraspecific patterns of variability in venom resistance within *O. torridus* parallel the broader interspecific patterns; i.e., high levels of venom resistance in regions

where grasshopper mice are sympatric with *Centruroides*, low levels of resistance in regions where they are not. This pattern suggests that venom resistance in *Onychomys torridus* is the product of interactions with *C. exilicauda*. In regions where southern grasshopper mice are attacking, and being stung by, chemically protected scorpions (e.g., SR mice), the rodents have countered with physiological resistance to those venoms. In the absence of continued selection by the scorpion (e.g., CR and NV mice), grasshopper mice lose resistance to neurotoxins. Thus, NV mice, which left *Centruroides* behind when they first colonized the Great Basin Desert in northwestern Nevada approximately 7–10 thousand years ago (Riddle, personal communication), exhibit significantly lower levels of venom resistance (10.3 mg/kg) than SR mice (18.4 mg/kg) who probably interact nightly with *C. exilicauda*. Mice from the CR population, which do not currently overlap with *C. exilicauda*, show intermediate levels of venom resistance (12.5 mg/kg) coupled with extreme levels of inter-individual variability in resistance (see raw data, Appendix 1); these results suggest that there may be gene flow between grasshopper mice from the CR population and grasshopper mice who, less than 15 miles east, are interacting with *C. exilicauda*.

In sum, both the inter- and intraspecific patterns we observed in grasshopper mouse resistance to bark scorpion venom are consistent with a selection hypothesis; i.e., venom resistance in the mice appears to be an adaptive response to feeding on their neurotoxic prey. Future work will explore the mechanism of this resistance, as well as examining whether or not selection by the mice has led to counter-adaptations in the scorpions.

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Conflict of interest

The authors of this manuscript, Ashlee H. Rowe and Matthew P. Rowe, declare that there are neither professional nor financial conflicts of interest regarding their research.

Appendix I

Up-and-Down Procedure (UDP) raw data. Abbreviations: F = female, M = male, O = survival, X = death. See Section 2 for description of venom effects.

Table 1

UDP toxicity tests for SR population (*Onychomys torridus*, Santa Rita Mountains, AZ)

Mouse	Wild or captive born	Gender	Weight (g)	Dose (mg/kg)	Effects of venom	Outcome
SR01	Captive	F	21.00	2.03	Non-toxic	O
SR02	Captive	F	28.30	2.64	Non-toxic	O
SR03	Captive	F	31.40	3.43	Non-toxic	O
SR04	Wild	F	33.64	9.01	Toxic	O
SR05	Wild	F	26.24	11.71	Toxic	O
SR06	Wild	F	27.33	15.22	Toxic	O
SR07	Wild	F	33.56	19.79	Lethal	X
SR08	Wild	F	23.49	15.22	Toxic	O
SR09	Captive	F	27.64	19.79	Lethal	X
SR10	Captive	F	26.30	15.22	Toxic	O
SR11	Wild	F	31.83	19.79	Lethal	X

LD₅₀ = 18.38 mg/kg (C.I. = 15.22–19.8).

Table 2

UDP toxicity tests for CR population (*Onychomys torridus*, Chiricahua Mountains, AZ)

Mouse	Wild or captive born	Gender	Weight (g)	Dose (mg/kg)	Effects of venom	Outcome
CR01	Captive	F	27.1	2.03	Non-toxic	O
CR02	Captive	M	28.4	2.64	Non-toxic	O
CR03	Captive	F	24.7	3.43	Non-toxic	O
CR04	Captive	F	22.76	15.22	Toxic	O
CR05	Captive	F	26.54	19.79	Lethal	X
CR06	Wild	F	34.06	15.22	Lethal	X
CR07	Wild	F	34.11	11.71	Lethal	X
CR08	Wild	F	45.21	9.01	Toxic	O
CR09	Wild	F	28.45	11.71	Lethal	X
CR10	Wild	F	32.11	9.01	Toxic	O
CR11	Captive	F	22.47	11.71	Toxic	O

LD₅₀ = 12.49 mg/kg (C.I. not assessed due to high inter-individual variability).

Table 3

UDP toxicity tests for NV population (*Onychomys torridus*, Lyon and Nye Counties, NV)

Mouse	Wild or captive born	Gender	Weight (g)	Dose (mg/kg)	Effects of venom	Outcome
NV01	Wild	M	15.5	2.64	Non-toxic	O
NV02	Wild	M	17.9	3.43	Toxic	O
NV03	Wild	M	15.8	4.10	Toxic	O
NV04	Wild	M	19.9	5.33	Toxic	O
NV05	Wild	M	14.1	6.93	Toxic	O
NV06	Captive	M	14.5	9.01	Toxic	O
NV07	Captive	M	15.3	11.71	Lethal	X
NV08	Captive	M	14.7	9.01	Toxic	O
NV09	Captive	M	15.0	11.71	Lethal	X
NV10	Wild	M	24.67	9.01	Toxic	O
NV11	Wild	M	17.5	11.71	Lethal	X

LD₅₀ = 10.27 mg/kg (C.I. = 9.01–11.7).

Table 4

UDP toxicity tests for OR population (*Onychomys arenicola*, Organ Mountains, NM)

Mouse	Wild or captive born	Gender	Weight (g)	Dose (mg/kg)	Effects of venom	Outcome
OR01	Wild	F	26.63	2.64	Toxic	O
OR02	Captive	F	28.46	3.43	Toxic	O
OR03	Captive	M	22.59	4.46	Toxic	O
OR04	Captive	F	21.20	5.79	Toxic	O
OR05	Captive	M	21.40	7.54	Toxic	O
OR06	Captive	M	22.45	9.80	Lethal	X
OR07	Captive	F	19.37	7.54	Toxic	O
OR08	Captive	M	26.67	9.80	Lethal	X
OR09	Captive	M	27.08	7.54	Toxic	O
OR10	Captive	M	31.96	9.80	Lethal	X

LD₅₀ = 8.596 mg/kg (C.I. = 7.54–9.8).

Table 5

UDP toxicity tests for KS population (*Onychomys leucogaster*, Finney County, KS)

Mouse	Wild or captive born	Gender	Weight (g)	Dose (mg/kg)	Effects of venom	Outcome
KS01	Wild	M	41.00	7.54	Lethal	X
KS02	Wild	F	41.50	5.80	Lethal	X
KS03	Wild	F	39.00	4.46	Lethal	X
KS04	Wild	M	36.00	3.43	Toxic	O
KS05	Wild	M	40.00	4.46	Lethal	X
KS06	Wild	M	35.50	3.43	Toxic	O
KS07	Wild	F	44.50	4.46	Lethal	X
KS08	Wild	M	40.00	3.43	Toxic	O

LD₅₀ = 3.911 mg/kg (C.I. = 3.43–4.46).

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