

ABSTRACT

ROWE, ASHLEE HEDGECOCK. Coevolution between grasshopper mice (*Onychomys* spp.) and bark and striped scorpions (*Centruroides* spp.). (Under the direction of Harold Heatwole.)

Asymmetrical selection has been proposed as the strongest argument for rejecting hypotheses of coevolutionary arms races between predators and prey. In many cases there is evidence of increased investment by the prey in response to the predator, but no evidence of increased investment by the predator in response to the prey, thus producing asymmetry in selection. However, selection against a predator may be increased when the interaction is with a "dangerous" prey. Predators are most likely to respond evolutionarily to potentially lethal prey. This study employs grasshopper mice (*Onychomys* spp.) and bark and striped scorpions (*Centruroides* spp.) as a model to test the hypothesis that interspecific interaction between a predator and a potentially lethal prey will result in behavioral and physiological adaptations that reciprocally mediate their interaction (i.e., coevolution). Bark scorpions (*Centruroides exilicauda*) and striped scorpions (*Centruroides vittatus*) produce a potent venom containing neurotoxins that selectively bind to the ion-channels of vertebrates. Vertebrate-specific neurotoxins may produce lethal effects in mammals, especially small mammals. Southern grasshopper mice (*Onychomys torridus*) and Mearns' grasshopper mice (*O. arenicola*) are known to be voracious predators on scorpions. Southern grasshopper mice are broadly sympatric with bark scorpions in the Sonoran Desert, and Mearns' grasshopper mice are broadly sympatric with striped scorpions in the Chihuahuan Desert. The third species in this genus, the northern grasshopper mouse (*O. leucogaster*) is broadly allopatric with *Centruroides* spp. In a preliminary study, both southern and Mearns' grasshopper mice

demonstrated resistance to bark and striped scorpion neurotoxins. The evolution of toxic venom and resistance to that venom strongly suggests a coevolutionary relationship between *Onychomys* spp. and *Centruroides* spp. To test this hypothesis, I evaluated the predator-prey relationship between grasshopper mice and their toxic scorpion prey during staged feeding trials. Additionally, I compared the geographic patterns of venom resistance in all three species of grasshopper mice with geographic patterns of venom toxicity in bark and striped scorpions. Results from the feeding study demonstrated that grasshopper mice do not distinguish between toxic and non-toxic species of scorpions; mice attacked, incapacitated, and consumed bark and striped scorpions without hesitation and as effectively as they attacked crickets and non-toxic scorpions in the genus *Vaejovis*. The feeding experiments indicate that grasshopper mice have the ability to prey on bark and striped scorpions in habitats where they co-occur. Venom resistance analyses demonstrated that all three species of grasshopper mice have evolved some resistance to the vertebrate-specific neurotoxins produced by *Centruroides* spp. The assays show that patterns of venom toxicity in *Centruroides* and venom resistance in *Onychomys* co-vary geographically, both within and among species; i.e., populations of *Onychomys* interacting with the most toxic populations of *Centruroides* were extremely resistant; populations of *Onychomys* interacting with only moderately toxic populations of *Centruroides* were only moderately resistant; and populations of *Onychomys* not sympatric with *Centruroides* were only weakly resistant. Such systematic covariation between venom toxicity in the scorpions and venom resistance in the mice is consistent with a coevolutionary, arms race hypothesis.

**COEVOLUTION BETWEEN GRASSHOPPER MICE (*ONYCHOMYS* SPP.) AND
BARK AND STRIPED SCORPIONS (*CENTRUROIDES* SPP.)**

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DEDICATION

For Matt and Ellie, my desert companions.

BIOGRAPHY

Ashlee Hedgecock Rowe was born in Raleigh, NC, and grew up in Cary. Ms. Rowe attended Cary Sr. High, and later graduated from Appalachian State University with a B.S. in Biology, a minor in Chemistry and a NC teaching certificate. After completing a desert ecology field course in the Chihuahuan Desert of TX and NM, Ms. Rowe decided to return to Appalachian State to earn her Master's Degree in Biology. Ms. Rowe's thesis research examined the population genetics of the common striped scorpion (*Centruroides vittatus*) throughout its range in Texas. While working toward her degree, Ms. Rowe taught chemistry and biology courses at a public high school. After completing her Master's degree, Ms. Rowe took a position as a research technician in a neurophysiology-pharmacology lab at Bowman Gray Medical School, Wake Forest University. After two years of research on the biological basis of drug addiction, Ms. Rowe returned to Boone, NC where she married Matthew Rowe. Ms. Rowe returned to teaching chemistry and biology at a public high school. In order to pursue both of her interests, research and teaching, Ms. Rowe decided to return to school (once more) and work toward a Ph.D. in Zoology at North Carolina State University. Her fascination with scorpions and the evolution of venom neurotoxins made it easy to choose a research topic. Ms. Rowe plans to continue her study of the evolution of scorpion venom neurotoxins and the evolution of resistance in their predators, as a postdoctoral student.

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TABLE OF CONTENTS

	Page
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
1. GENERAL INTRODUCTION.....	1
2. RISK ASSESSMENT BY GRASSHOPPER MICE (<i>ONYCHOMYS</i> SPP.) FEEDING ON POTENTIALLY LETHAL PREY (<i>CENTRUROIDES</i> SPP.).....	9
2.1 Introduction.....	11
2.2 Methods.....	19
2.3 Results.....	27
2.4 Discussion.....	34
2.5 Literature Cited.....	42
2.6 Figures.....	48
3. CONFIRMATION OF RESISTANCE OF GRASSHOPPER MICE (<i>ONYCHOMYS</i> SPP.) TO THE VENOM OF BARK SCORPIONS (<i>CENTRUROIDES EXILICAUDA</i>).....	59
3.1 Introduction.....	60
3.2 Methods.....	61
3.3 Results.....	63
3.4 Conclusion.....	63
3.5 Literature Cited.....	64
4. THE EVOLUTIONARY SIGNIFICANCE OF GEOGRAPHIC VARIABILITY IN VENOM TOXICITY AND VENOM RESISTANCE: COEVOLUTION BETWEEN BARK AND STRIPED SCORPIONS (<i>CENTRUROIDES</i> SPP.) AND GRASSHOPPER MICE (<i>ONYCHOMYS</i> SPP.).....	65
4.1 Introduction.....	66
4.2 Study System.....	71
4.3 Methods.....	76
4.4 Results.....	85
4.5 Discussion.....	87
4.6 Literature Cited.....	93
4.7 Tables.....	103
4.8 Figures.....	105

LIST OF TABLES

	Page
CHAPTER FOUR	
1. Physiological resistance of grasshopper mouse populations to bark scorpion venom.....	103
2. Venom toxicity levels of bark and striped scorpion populations.....	104

LIST OF FIGURES

	Page
CHAPTER TWO	
1. Mean and SE of latency, for each prey type, between a mouse's first attack and the moment the prey is incapacitated.....	48
2. Mean and SE of the number of successful stings landed by either <i>Vaejovis spinigerus</i> or <i>Centruroides</i> spp. during an encounter with <i>Onychomys</i> spp.....	49
3. Mean and SE of the number of times each prey type was dropped during the handling phase of an encounter with <i>Onychomys</i>	50
4. Mean and SE of the number of retreats from each prey type exhibited by <i>Onychomys</i> during the handling phase of an encounter.....	51
5. Mean and SE of the number of attacks required for <i>Onychomys</i> to subdue each prey type during the handling phase of an encounter.....	52
6. Mean and SE of the number of times prey were dropped by juvenile vs. adult <i>Onychomys</i> during the handling phase of an encounter.....	53
7. Mean and SE of the number of times prey were dropped during handling as a function of type of prey and the age of the grasshopper mouse.....	54
8. Mean and SE of the number of successful stings delivered by scorpions to <i>Onychomys</i> from the three different study populations.....	55
9. Mean and SE of the number of attacks required by <i>Onychomys</i> from the three different study populations to subdue their prey during the handling phase of a predatory encounter.....	56
10. Mean and SE of the number of times juvenile and adult mice from each study population had to attack their prey before the prey was either incapacitated or abandoned.....	57
11. Mean and SE of the number of attacks directed by juvenile and adult mice from each population at each of the three prey types.....	58

LIST OF FIGURES (continued)

	Page
CHAPTER FOUR	
1. Species distribution and study sites.....	105
2. Species distribution, study sites, and venom resistance of <i>Onychomys</i> spp.....	106
3. Species distribution, study sites, and venom toxicity of <i>Centruroides</i> spp.....	107

CHAPTER ONE

GENERAL INTRODUCTION

Thompson (1994) pointed out that while Darwin never used the term “coevolution”, his observations of the interaction between bees and the flowers they pollinated might have been the first account of coevolution as a process. Since Darwin, scientists have wondered about the role of species interaction in the evolution of biological diversity. Ehrlich and Raven (1964) are credited with first using the term coevolution to describe the evolution of interactions among groups of ecologically coupled plants and herbivores. With the growing awareness and concern for declining species diversity over the last few decades, many studies of coevolution have emphasized the importance of species interaction in generating biological diversity. The diversity of biological systems depends as much on the diversification of species’ interactions as on the diversification of the species themselves (Thompson, 1996). Therefore, conservation of interaction diversity is as important to the maintenance of community and ecosystem structure and function as is the conservation of species. The loss of a species from a community results in the loss of interactions, which might have serious consequences for other species in the community. For example, Thompson (1996) discussed the importance of coevolved plant-pollinator systems and how the loss of their interaction could result in a reduction of plant biomass, which might impact nutrient recycling, thereby resulting in reduced growth rates of the herbivores in that community. Such studies, most of which have appeared over just the last two decades, on the evolution of species interactions provide hints of the ecological and evolutionary consequences that may result from the loss of interaction diversity. A deeper appreciation for the dynamics of coevolution will be crucial to developing novel strategies for conserving species interaction diversity and, thus, of preserving biodiversity (Thompson, 1996, 1997, 1999).

Most coevolutionary studies concentrate on interactions between plants and their herbivores, pathogens and pollinators (Thompson and Pellmyr, 1992; Futuyma and Mitter, 1996; Benkman, 1999; Burden and Thrall, 1999; Parker, 1999). Other investigations involve host-pathogen or host-parasite relationships (Kraaijeveld and Godfray, 1999; Lively, 1999). Brodie and Brodie (1999) pointed out that because predation is considered a fundamental force responsible for generating a plethora of adaptations (Cott, 1940; Edmunds, 1974), interactions between predators and prey should yield countless examples of coevolution. However, well-supported examples of coevolved predator-prey systems are relatively few. In many cases researchers are able to successfully demonstrate that an adaptation in one species is the direct result of selection by a second species. However, most studies are not successful in demonstrating the reciprocal; i.e., that counter selection by the first species has produced an adaptation in the second species in response to the adaptation in the first species (Futuyma and Slatkin, 1983). The failure to detect reciprocal selection in predator-prey systems is most often attributed to escalation, diffuse coevolution, or asymmetrical selection. Escalation is described as a process in which an adaptation in one species evolves in response to the collective selective pressures exerted by all of the species' enemies; i.e., its competitors, predators, and armed prey (Vermeij, 1994). For example, the adaptation of increased speed might enable a species to out-compete an interspecific rival, to out-run a predator, and to out-manuever a well-armed prey. Futuyma and Slatkin (1983) described diffuse coevolution as the development of non-specific adaptations as a consequence of interactions among groups of ecologically related species, such as the interactions among plants and their non-specific pollinators and herbivores. Asymmetrical selection has been proposed by Abrams (1986) as the strongest argument for rejecting hypotheses of

coevolutionary arms races between predators and prey. In many cases there is evidence of increased investment by the prey in response to the predator, but no evidence of increased investment by the predator in response to the prey. The absence of adaptive response by the predator is interpreted as the result of asymmetry in selection (Abrams, 1986). However, Brodie and Brodie (1990, 1991, 1999) proposed that selection against a predator and subsequent investment in its response to the prey might be increased when the interaction is with a “dangerous” prey. Interactions with prey that have the potential to cause death represent “severe and predictable” outcomes for the predator. Brodie and Brodie argued that severe and predictable consequences increase the strength of selection against the predator. Results from these authors’ investigations into the relationship between the newt, *Taricha granulosa*, and its predator the garter snake, *Thamnophis sirtalis*, are consistent with this suggestion and more generally with an arms race perspective. Geographic patterns of variability in tetrodotoxin production by the newt and toxin resistance by the garter snake suggest that each species has responded adaptively to the other.

This study employed grasshopper mice of the genus *Onychomys* and bark and striped scorpions of the genus *Centruroides* as a model to test the hypothesis that interspecific interaction between a predator and a toxic prey will result in coevolution. Bark scorpions (*C.exilicauda*) of the Sonoran Desert and striped scorpions (*C.vittatus*) of the Chihuahuan Desert produce venom with neurotoxins that selectively bind to the sodium (Na^+) and potassium (K^+) ion-channels of vertebrates as well as invertebrates. Bark and striped scorpions are known to feed only on invertebrate species, thus, vertebrate-specific neurotoxins are thought to be the product of selection due to vertebrate predators. The vertebrate-specific Na^+ channel neurotoxins are capable of producing lethal effects in

mammals (Simard et al., 1992). Southern grasshopper mice (*O.torridus*) of the Sonoran Desert and Mearns' grasshopper mice (*O.arenicola*) of the Chihuahuan Desert are voracious predators of scorpions. More importantly, both species of grasshopper mouse demonstrate physiological resistance to the neurotoxins in bark and striped scorpion venoms. The development of vertebrate-specific neurotoxins and resistance to those neurotoxins strongly suggests a coevolutionary relationship between bark scorpions and southern grasshopper mice in the Sonoran Desert, and striped scorpions and Mearns' grasshopper mice in the Chihuahuan Desert. Here, I test the hypothesis that neurotoxin resistance in grasshopper mice evolved in response to vertebrate-specific neurotoxins produced by bark and striped scorpions. I also test the hypothesis that bark and striped scorpions have evolved increased venom toxicity in response to resistance in grasshopper mice. In order to test these hypotheses, I had four project goals. The first goal was to identify habitat where *Onychomys* and *Centruroides* co-occur, as well as habitat where they are allopatric to one another. The second goal was to determine whether grasshopper mice actually feed on these potentially lethal scorpions and, if so, whether the mice used behavioral adaptations to avoid being stung. The third and fourth goals were to gather data on the geographical patterns of expression in venom toxicity and venom resistance from both sympatric and allopatric populations of mice and scorpions. The reciprocal selection or arms race hypothesis predicts that patterns of venom toxicity in the scorpions should covary with patterns of venom resistance in the mice.

All appropriate permits were obtained from national, state, and local government agencies to collect and house the specimens required for this project. The University Animal Care and Use Committee (IACUC) approved the protocols for all the procedures conducted

during this project. Captive grasshopper mice were maintained in USDA approved animal facilities according to USDA guidelines for wild mice.

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CHAPTER TWO

RISK ASSESSMENT BY GRASSHOPPER MICE (*ONYCHOMYS* SPP.) FEEDING ON POTENTIALLY LETHAL PREY (*CENTRUROIDES* SPP.)

RISK ASSESSMENT BY GRASSHOPPER MICE (*ONYCHOMYS* SPP.) FEEDING ON POTENTIALLY LETHAL PREY (*CENTRUROIDES* SPP.)

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Abstract: Predators should benefit from assessing the risks posed by prey that differ in the dangerousness of their defenses. Assessment, coupled with adaptive adjustment of the predator's behavior, could come at any phase of the predatory sequence: search/recognition; pursuit/attack; and handling/subjugation. Southern grasshopper mice are voracious carnivores in North American deserts, feeding on a diversity of insects, other rodents, and birds. In certain regions of the Sonoran Desert, grasshopper mice co-occur with extremely neurotoxic bark scorpions, the only scorpion in the U.S. whose sting can prove lethal to humans. Given the density of these scorpions and the large home ranges of the mice, encounters between the two are a certainty. I presented three different prey items to wild-caught grasshopper mice in a small enclosure at three different field sites in the southwestern U.S. The three prey items, listed in increasing order of their presumed dangerousness, were: lab crickets, possessing few if any defenses; *Vaejovis spinigerus*, a noxious but non-lethal scorpion; and two species of toxic *Centruroides*. Grasshopper mice made no distinctions among the prey items in either the recognition or pursuit phases of the encounter, attacking crickets and both genera of scorpions with little hesitation. There were, however, significant differences in how the mice handled the three different prey types, with *Centruroides* requiring dramatically more time and effort to subdue. The difficulties mice had in subduing *Centruroides* were not related to the neurotoxic components of these scorpions' venoms, to which the mice are extremely resistant. Instead, the difficulties appear to be the result of

other venom constituents causing acute, short-term pain. Thus, the cautious handling of *Centruroides* by southern grasshopper mice need not imply that mice are employing sophisticated, prey specific attack strategies. A parsimonious interpretation is that, during the subjugation phase of predation, grasshopper mice simply drop *Centruroides* more than they drop other prey, including scorpions in the genus *Vaejovis*, because the stings of *Centruroides* are more painful.

INTRODUCTION

Adversaries engaged in intraspecific conflict should attempt to assess their opponent's strengths and weaknesses and modify their aggressive behavior accordingly (e.g., Parker & Rubenstein 1981). This "assessment strategy" should also apply to predator-prey interactions, especially in the context of predator harassment (Swaigood et al. 1999a). Female California ground squirrels (*Spermophilus beecheyi*), for example, use the rattling sounds they provoke in rattlesnakes to assess both the snake's body size and body temperature; when defending pups, the females are more aggressive when harassing smaller, cooler, and thus less dangerous rattlesnakes (Swaigood et al. 1999b; Swaigood et al. 2003).

The importance of decision-making by predators has received less attention (Lima 2002), although they too should benefit from assessment, especially when dealing with potentially dangerous prey. An appraisal of the costs and benefits of attacking might occur at any point during a predatory sequence, which historically has been divided into three phases: detection/recognition, pursuit/attack, and subjugation (see Elliott et al. 1977; Holling 1966; Vermeij 1982). Assessment of the dangerousness of a given prey might determine whether or not the predator attacks, how it attacks, and how it handles the prey following the initial

attack. Southern toads (*Bufo terrestris*), for example, quickly learn to associate the sight and sound of honeybees (*Apis mellifera*) with the bee's painful sting, and ignore the bees after just a few laboratory trials (Brower & Brower 1965; Brower & Brower 1962). When dealing with potentially lethal prey, rejection by the predator may require no learning at all; hand-reared birds from several taxa show innate avoidance of highly toxic coral snakes (*Micrurus* spp.; Smith 1975, 1977) and yellow-bellied sea snakes (*Pelamis platurus*; Caldwell & Rubinoff 1983).

Assessment can influence not only whether a predator attacks, but how. Lions (*Panthera leo*), for example, attack dangerous prey such as buffalo (*Syncerus caffer*) in larger groups than when attacking weaker prey such as warthog (*Phacochoerus aethiopicus*; Packer & Pusey 1997). The spider-eating jumping spider, *Portia labiata*, shows very sophisticated assessment and attack modification behavior when hunting *Scytodes pallidus*, a spider that spits a sticky gum it uses in turn to hunt jumping spiders (Jackson et al. 2002). *Portia* normally stalks *Scytodes* slowly and stealthily from behind; however, when a *Scytodes* female is carrying her egg sac in her mouth, and thus cannot spit effectively, *Portia* attacks directly and without hesitation (Jackson et al. 2002).

A predator may benefit by assessing the dangerousness of its prey even after the prey has been captured. Black-backed orioles (*Icterus galbula*) attack and feed on monarch butterflies (*Danaus plexippus*) at the monarchs' overwintering sites in Mexico (Brower & Calvert 1985; Fink & Brower 1981), even though the butterflies are thought to be protected from avian predation by the cardenolides they incorporate from the milkweeds (*Asclepias* spp.) they fed on as caterpillars (Brower 1969). The orioles do not appear to possess morphological or physiological adaptations that might render the toxins ineffective; rather,

the birds simply bite the wings and thorax of a torpid butterfly, assess (taste) the level of toxins it contains, and rejects those with high concentrations of cardenolides (Fink and Brower 1981; Brower and Calvert 1985). Garter snakes (*Thamnophis sirtalis*) that feed on extremely toxic rough-skinned newts (*Taricha granulosa*) show similar post-capture assessment and occasional rejection of toxic newts, but with a noteworthy twist (Williams et al. 2003). The snakes have evolved physiological resistance to the newts' toxins, although such resistance exhibits considerable inter- and intrapopulation variability (Brodie & Brodie 1990). Remarkably, the threshold for rejecting a newt as too toxic depends on the level of resistance in the snake; in laboratory trials, snakes with high levels of resistance took longer to reject newts, and were more likely to survive after eating a newt, than did snakes with lower levels of resistance (Williams et al. 2003).

Grasshopper mice (*Onychomys* spp.) provide an excellent system for studying assessment strategies in a predator that must, at least occasionally, deal with dangerous and potentially lethal prey. The genus contains three species of carnivorous mice distributed throughout the deserts, shrub steppes, and prairies of western North America (Riddle & Honeycutt 1990). Grasshopper mice are extremely aggressive predators that consume a diverse array of animal taxa including other small mammals, reptiles, amphibians, birds, and numerous arthropods (Egoscue 1960; Flake 1973; Hansen 1975; Horner & Taylor 1968; Horner et al. 1965). Dietary analyses have demonstrated that arthropods constitute the majority of the grasshopper mouse diet (Horner et al. 1965; Flake 1973; Hansen 1975). Some of the arthropods that grasshopper mice routinely consume possess potent morphological and/or biochemical anti-predator defenses. For example, stink beetles (*Eleodes* sp.) stand on their anterior end and spray a noxious solution of quinone from the tip

of their posterior end to deter their predators, while whip scorpions (*Mastigoproctus* sp.) spray their opponents with an irritating secretion of acetic acid (Eisner & Meinwald 1966). Lubber grasshoppers (*Brachystola magna*) possess powerful spine-tipped jumping legs, which they thrust and jab at their predators in order to escape capture (Whitman et al. 1985). Scorpions attempt to repel or escape from their would-be predators by lashing out with their stinger-tipped tails and injecting painful venom (Horner et al. 1965). It has been reported that grasshopper mice successfully mediate interactions with these well-defended prey by modifying their attack behavior to counteract each specific defense mechanism. For example, grasshopper mice avoid being sprayed with noxious quinone secretions during interactions with stink beetles by grasping and biting the head of the beetle, while pushing the quinone-producing posterior end of the beetle into the ground (Cyr 1972; Eisner and Meinwald 1966; Langley 1981). Grasshopper mice disable the spine-tipped jumping legs of the lubber grasshopper by grasping and biting the appendages until they break (Whitman et al. 1985). Similarly, grasshopper mice are reported to disarm scorpions by persistently focusing their attacks on the stinger-tipped tail. Mice grasp and attempt to pin the tail so that they may bite the tail repeatedly without being stung (Cyr 1972; Eisner & Meinwald 1966; Langley 1981). Collectively, these reports suggest that grasshopper mice assess their prey prior to attack, match each particular species with its associated defenses, and adjust their attack strategy to compensate for prey-specific defense mechanisms. Rather than a generalized predatory response, these reports imply that attack behavior of grasshopper mice is highly specific to each prey type.

Paradoxically, just as many published studies, by some of the same investigators, suggest that sophisticated assessment of a prey's dangerousness is not part of a grasshopper

mouse's predatory repertoire. Rather, these studies indicate that attack behavior in grasshopper mice is a generalized, inflexible response elicited by any small, moving object (Cyr 1972; Horner et al. 1965; Langley 1981, 1983a, 1983b, 1989, 1991; Langley & Knapp 1982; Ruffer 1968). Langley (1989), for example, showed that models of most any shape, whether representing relevant prey items or abstract geometric designs, would elicit an immediate attack from a grasshopper mouse as long as the model was animated; latencies to attack were significantly longer for both stationary models and for large models (>50 mm in length). Moreover mice, whether or not they had previous experience feeding on crickets or scorpions, attacked models of both prey types indiscriminately (Langley 1989). The lack of prey-specific attack behavior in this study by Langley contrasts dramatically to the definitive statement he made in an earlier publication (Langley 1981, pg. 115); i.e., "Grasshopper mice attack crickets, stink beetles, and scorpions differently."

What explains this paradox in the literature created by contradictory interpretations of grasshopper mouse predatory behavior? The confusion may be a product of three weaknesses common to the earlier studies. The first is sample size; all the aforementioned studies are primarily descriptive and lack statistical rigor. For example, the number of mice (and, presumably, the number of scorpions) used in the classic studies (Cyr 1972; Horner et al. 1965; Langley 1981) purporting to show that grasshopper mice preferentially attack a scorpion's tail are one, three, and four, respectively; small sample size explains the paucity of inferential statistical analyses reported by these authors.

The second weakness/explanation is that many of the earlier studies did not make a clear distinction between the different phases of a predatory sequence – search, attack, and subdue. Conflating one of these phases with another at best may lead to confusion and, at

worst, may confer a level of sophisticated assessment to a predator that it does not truly deserve. Langley (1981), for example, appeared to conflate subjugation with attack. His conclusion that grasshopper mice differentially attack prey species that differ in their dangerousness shows, upon close inspection, that the mice simply *handle* the different prey items differently. When stink beetles had their quinone-squirting abdominal glands sealed with wax, inexperienced grasshopper mice failed to concentrate their bites on the beetle's head and focused instead on the beetles posterior end; similarly, grasshopper mice interacting with a scorpion whose stinger had been removed retreated less often than mice interacting with scorpions that could sting (Langley 1981). Sophisticated assessment strategies and complex cognitive skills may have little explanatory power here, as anyone who has inadvertently picked up a hot skillet could attest. What is needed is a statistically rigorous analysis of whether grasshopper mice make cost/benefit adjustments in their behavior at each phase of the predatory sequence including, but not limited to, assessing the dangerousness of a prey item prior to its capture.

Finally, no study to date has examined the interactions of the mice with potentially lethal prey. All of the noxious prey studied, thus far, are species with biochemical or mechanical defenses capable of causing only short-term discomfort (e.g., the quinone sprays of *Eleodes* sp., or the acetic acid mists of *Mastigoproctus* sp.), but none are capable of producing death in small mammals. Perhaps there is little evidence that grasshopper mice make pre-capture assessments of the dangerousness of their prey because such distinctions have been unnecessary for the mice; attacking a stink beetle may involve no more risk than attacking a cricket. Indeed, even the scorpions used in previous investigations have been relatively benign; e.g., *Hadrurus* spp. (Cyr 1972; Horner et al. 1965; Langley 1981) and

Vaejovis spp. (Langley 1989). Although the stings from both genera of scorpions are probably painful, they are not known to be fatal to mammals, even to small ones like mice. Yet *Onychomys* may routinely interact with scorpions in a third genus, *Centruroides*, whose toxins are potentially lethal and, thus, a genus for whom pre-capture assessment could be imperative.

Bark scorpions (*Centruroides exilicauda*) and striped scorpions (*Centruroides vittatus*), in particular, may be excellent models for examining the adaptations used by grasshopper mice to mediate their interactions with potentially lethal prey. Of the numerous species of scorpion available to grasshopper mice throughout their distribution in the southwestern U.S., only bark and striped scorpions produce venom with potentially lethal effects (LoVecchio & McBride 2003; Russell & Madon 1984; Simard et al. 1992). Scorpion species in the genus *Centruroides* produce neurotoxins that selectively bind to the voltage-gated sodium (Na^+) and potassium (K^+) ion-channels of vertebrates (Corona et al. 2002; Corona et al. 2001; Possani et al. 2000; Simard et al. 1992). Some of the neurotoxins selectively target the ion-channels of mammals (SCORPION, molecular database of neurotoxins). These neurotoxins have the potential to produce fast paralysis and death, especially in small mammals (Simard, Meves et al., 1992). In sum, scorpions in the genus *Centruroides* represent potentially more dangerous prey for grasshopper mice than other scorpion genera that have been tested.

Bark and striped scorpions are biologically relevant prey for grasshopper mice. Not only do *Centruroides* spp. and *Onychomys* spp. overlap considerably in their extant geographical ranges, they also share similar biogeographic histories throughout the warm deserts of North America (Fet & Lowe 2000; Hinesley 1979; Riddle 1995; Riddle &

Honeycutt 1990; Shelley & Sissom 1995; Sullivan et al. 1986; Williams 1980), suggesting the potential for a long evolutionary contact between these two groups. Indeed, fossil records of both *Centruroides* and *Onychomys* indicate these two genera may have had a close ecological association on an evolutionary time scale (approximately 6-3.6 MYA) sufficient to generate reciprocal adaptations (Baskin 1979; Carleton & Eshelman 1979; Sullivan et al. 1995; Fet & Lowe 2000; Gantenbein et al. 2001). Moreover, bark and striped scorpions are extremely abundant, especially when compared to *Hadrurus*; indeed, *C. exilicauda* is the most abundant scorpion species in particular regions of the Sonoran and Mojave Deserts of Arizona (AZ) where they coexist with southern grasshopper mice (*Onychomys torridus*; Rowe & Rowe, unpublished data). Similarly, striped scorpions are extremely abundant in regions of the Chihuahuan Desert in New Mexico (NM) where they are sympatric with Mearns' grasshopper mice (*Onychomys arenicola*; Rowe & Rowe, unpublished data). Indeed, at a study site in west Texas physiographically similar to my study sites in NM and AZ, striped scorpions comprise more than 90% of the scorpions active on the desert surface at night (Brown et al. 2002). Thus, bark and striped scorpions may represent an important food resource for grasshopper mice in regions of the southwestern United States (U.S.) and Mexico (MX) where both co-occur; given the density of the scorpions and the huge home ranges of the mice (Frank 1992), interactions between the two are a certainty.

The purpose of this study is to investigate the predator-prey relationship between grasshopper mice and their potentially lethal prey, bark and striped scorpions in the genus *Centruroides*. Feeding experiments were used to determine if grasshopper mice do indeed prey on these highly toxic scorpions, and if so, what behavioral and/or physiological adaptations the mice employ to mediate the interaction during each phase of the predatory

sequence. Grasshopper mice may simply recognize scorpions in the genus *Centruroides* as lethal and avoid them. Avoidance is not expected, however, as many of the studies cited previously show little hint of assessment by grasshopper mice prior to contact with their prey; the general rule of thumb appears to be “if it is small and moves, attack”. If, however, grasshopper mice do indeed prey on bark and striped scorpions in regions of sympatry, then the mice should exhibit behavioral and/or physiological adaptations to mediate their interaction with this potentially lethal prey. Scorpion stings can be painful (Simard et al., 1992), so grasshopper mice may modify their attack behavior such that they minimize the risk of being stung. Complete evasion of scorpion stings is not expected, however, because many of the studies cited earlier (using less noxious genera such as *Hadrurus*) show that mice, regardless of any behavioral adjustments they may be attempting, are in fact usually stung at some point during an interaction with a scorpion. The potentially lethal neurotoxins of *Centruroides* could thus impose strong selection on grasshopper mice, resulting in alternative, non-behavioral mechanisms for mitigating interaction with toxic prey. If grasshopper mice cannot easily distinguish between highly toxic and less toxic species of scorpions, and if being stung is unavoidable, then one would predict the mice to have evolved physiological mechanisms for detoxifying *Centruroides*' potent neurotoxins.

METHODS

Study Sites and Animals:

Three species of prey items were selected to represent different degrees of dangerousness to predatory grasshopper mice: scorpions in the genus *Centruroides*, a potentially lethal prey; scorpions in the genus *Vaejovis*, representing a noxious but non-lethal prey, and lab crickets (*Acheta domesticus*), representing a palatable prey lacking any

formidable defenses. Feeding trials were conducted using grasshopper mice from three different geographic locations where grasshopper mice are sympatric with either bark (*Centruroides exilicauda*) or striped (*C. exilicauda*) scorpions. Specimens of bark scorpion and southern grasshopper mouse (*Onychomys torridus*) were collected from two locations in Arizona where both species occur in the same habitat (i.e., biotic sympatry). The first study site was located near the Virgin River Gorge in the Beaver Dam Mountains Wilderness Area of Mohave County in northwestern AZ. The second study site was located at the Santa Rita Experimental Range, in the Santa Rita Mountains of Pima County, in south-central AZ. Specimens of striped scorpion and Mearns' grasshopper mouse (*O. arenicola*) were collected from a third site where the species are biotically sympatric. This study site was located in the Organ Mountains of Doña Ana County, in southern NM.

A total of seven southern grasshopper mice, four adults and three juveniles, were collected from the Beaver Dam Mountains in mid-July of 1999. I also collected approximately 50 specimens of bark scorpion, and approximately 20 specimens of a local, non-toxic (i.e., non-lethal to vertebrate species) scorpion, *Vaejovus spinigerus*. The average weight of juvenile mice was 25.9 grams, and the average weight of adult mice was 34.6 grams. I collected six southern grasshopper mice (three adults and three juveniles), approximately 50 bark scorpions, and approximately 20 *V. spinigerus* from the Santa Rita Mountains in late July of 1999. The average weight of juvenile mice was 21.6 grams, and the average weight of adult mice was 31.2 grams. I collected nine Mearns' grasshopper mice (six adults and three juveniles), approximately 50 striped scorpions, and approximately 20 *V. spinigerus* from the Organ Mountains in early August of 1999. The average weight of juvenile mice was 20.5 grams, and the average weight of adult mice was 24.3 grams. In

order to increase the sample size and assess the generality of the results from 1999, I collected six southern grasshopper mice (four adults, two juveniles), bark scorpions, and *V. spinigerus* from the Santa Rita Mountains in July of 2000; the average weight of juvenile mice was 18 grams, and the average weight of adult mice was 23.1 grams. I collected an additional ten southern grasshopper mice (five adults and five juveniles), bark scorpions, and *V. spinigerus* from the Santa Rita Mountains in June of 2003. The average weight of juvenile mice was 22.8 grams, and the average weight of adult mice was 27.2 grams. Severe drought and wildfires prevented obtaining additional specimens of grasshopper mice from either the Beaver Dam Mountains or the Organ Mountains study sites following my collecting trip in 1999.

Mice were captured using Sherman live traps baited with a mixture of dry cat food and peanut butter. Traps were placed in a line approximately 15-20 meters apart, adjacent to either small mammal burrows or clumps of vegetation, such as shrubs and cacti. Traps were baited just before sunset and checked the following morning before sunrise. Specimens were removed from their traps and identified; species other than grasshopper mice were released immediately. Grasshopper mice were sexed, weighed, measured, and categorized as either juvenile or adult based on their dorsal pelage color. Juvenile grasshopper mice have a dark-gray coat until approximately three months of age. Molting typically occurs when mice are between 80 to 140 days old, resulting in a cinnamon-color coat (Horner and Taylor, 1968). Immediately after gathering these morphometric data, the mouse was placed in a standardized mouse cage with bedding. Mouse cages were maintained in portable ice chests inside a temporary shelter, and a maximum/minimum thermometer was used to monitor the ambient temperature inside the chests. Water was provided *ad libitum*, but food was withheld prior to

the feeding trials (with the exception of the bait in the trap). Grasshopper mice were maintained in captivity less than eighteen hours. All the mice were marked and released at their site of capture during the early evening hours of that same day, with the exception of voucher specimens. Two voucher specimens from each geographic location were retained and preserved.

Scorpions were collected from in and around the habitat where grasshopper mice were trapped. Collections were limited to specimens of either *Centruroides* spp. or *Vaejovis spinigerus*. Scorpions were collected using UV illumination, usually between the hours of 9:00 pm and 12:00 am. Forceps were used to grasp specimens by the tail and place them in large zip-lock freezer bags. Care was taken not to damage the tail or stinger-bearing telson of scorpions during their collection. Scorpion specimens with either damaged tails or stingers were eliminated from the feeding trials. Specimens were identified as either *Centruroides* spp. (bark or striped depending on geographic location) or *V. spinigerus* and maintained in separate one-gallon plastic bags. No more than 20 specimens were placed in each plastic bag. Several paper towels were placed in each bag to provide substrate and refuge for the scorpions. A small amount of water was sprinkled over the paper towels to provide a source of moisture for the scorpions. Scorpions were not provided food prior to the feeding trials. The plastic bags containing scorpion specimens were maintained in the same shelter, but separate ice chests, as the mouse cages. Instead of releasing the scorpion specimens that were not consumed during the feeding trials, excess specimens were maintained for venom analyses.

I obtained house crickets (*Acheta domesticus*) from local commercial sources (pet stores and feed shops) for all the feeding trials.

Apparatus and Procedure:

Feeding trials were conducted in an 8 cm (width) X 38 cm (length) X 26 cm (height) terrarium. The bottom, top lid, and three sides of the chamber were constructed of plexiglass, while the fourth side was constructed of glass to improve clarity during filming. The bottom of the terrarium was filled to a height of 4-5 cm with odorless, natural clay kitty litter that provided a substratum for the animals. Feeding trials were filmed using a digital video camcorder (Canon XL1 mini DV) equipped with a 3X wide-angle zoom lens (Canon XL 3.4-10.2 mm).

The feeding trials were conducted on the same day grasshopper mice were captured. All feeding trials were filmed just after sunrise, using ambient light only. Each grasshopper mouse was placed in the terrarium prior to its feeding trial and given time to habituate to the arena. Typically, the mice explored the terrarium for a few minutes and then began to groom; they did not appear to be distracted by the presence of investigators. Each mouse was allowed to habituate to the terrarium for a maximum of five minutes before receiving the first prey item. If the mouse began to groom before five minutes had elapsed, the feeding trial was initiated and the first prey item was introduced. Each mouse was tested once with each of the three different food items (prey species). A single prey item was introduced into the terrarium at the start of each trial. Consecutive prey items were not introduced until the previous trial was completed. The order of introduction for prey items was systematically counter-balanced to avoid trial effects. Prey were added to the terrarium through an opening in the top of the container. A prey item was introduced while the mouse was either grooming or exploring, so that the mouse would not observe the addition of the test species. Mice usually discovered the prey item within a few minutes of its introduction, either through the

mouse's exploration of the terrarium, or through attraction to the movement of the prey. Each mouse was allowed to interact with each prey species in the terrarium until the trial was terminated. A feeding trial on a specific prey item was terminated when one of the following situations occurred: 1) the mouse incapacitated and consumed the prey item; 2) the mouse incapacitated the prey item but failed to consume it within a period of ten minutes; 3) the mouse attacked but failed to incapacitate the prey, then retreated from the prey and demonstrated no interest in the prey for a period of ten minutes; 4) the mouse failed to attack the prey after noticing it, and continued to avoid contact with the prey for a period of ten minutes. The remains of each prey item were removed from the terrarium at the end of each feeding trial for an individual mouse. The terrarium was cleaned and the kitty litter replaced at the completion of a given mouse's three feeding trials to eliminate odors that might distract the subsequent mouse during its feeding trials. All trials were videotaped. The tapes were later analyzed using real time, slow motion, and frame-by-frame playback modes on a Panasonic AG-DV2000 Pro-Line digital cassette recorder/player.

Behavioral Analyses and Measurement Variables:

Using the videotapes, I evaluated the predatory behavior of the grasshopper mice at each of the three phases of a predatory sequence: detection/recognition; pursuit/attack; and subjugation. Because the trials were staged, I obviously could not measure behaviors the grasshopper mice might use to search for and locate potential prey. I could, however, determine whether or not the mice recognized a prey item, once detected, as "palatable"; i.e., a mouse either attacked the item, or it did not.

Three measures of duration lifted from the video tapes were used to determine whether grasshopper mice adjust their behavior to the dangerousness of a potential prey item

during the pursuit/attack phase of a predatory sequence. These three measures were: total prey assessment time; prey sniff time; and total sniff time. Total prey assessment time was measured as the time that elapsed between the instant a mouse first attended to a prey item and the instant it attacked. Prey sniff time was measured as the amount of time a mouse spent sniffing within 5 mm of a prey item prior to attacking. Total sniff time was measured as the total amount of time, prior to its first attack, that a mouse spent sniffing the track deposited by a prey species on the substrate plus the amount of time it spent sniffing the prey. A fourth dependent variable was employed to gauge whether or not mice adjust their attack behavior to offset the defenses of their prey; from the tapes, I determined which region (anterior, body, or posterior/tail) of the prey item drew the initial attack by the mouse.

Differential handling of the different prey species during the subjugation phase of the predatory sequence was quantified using a single measure of duration along with several measures based on counts. The duration variable, handling time, was measured as the total elapsed time between a mouse's initial attack and the moment the prey was incapacitated. Dependent variables based on counts included: the number of times a mouse attacked a prey item; the number of times a mouse retreated (following an attack) from the prey; the number of times prey were dropped; and (for scorpion prey only), the number of times a mouse was stung.

Operational Definitions:

- 1) Attend: the discovery and/or recognition of introduced prey items; attention focused on prey; measured as a discernible change in the behavior of the test mouse that suggests the mouse has focused its attention on the prey (e.g., a shift in the orientation

- of the mouse's head, eyes, and ears toward the prey; twitching of the mouse's nares and whiskers indicating sniffing directed at the prey).
- 2) Sniffing: using the olfactory system to gather information about the prey and its surroundings through chemical cues; measured as any observable change in the orientation of the mouse's nares and whiskers that suggests the mouse is using its olfactory system (e.g., twitching of the mouse's nostrils and whiskers while the mouse's face is in close proximity to the prey, or in close proximity to the substrate with which the prey has made contact).
 - 3) First attack: first attempt to capture the prey; measured as the mouse's first physical act of aggression toward the prey (e.g., the point at which the mouse first lunges toward the prey while using its claws to grasp the prey and/or opening its mouth to bite the prey).
 - 4) Subsequent attacks: additional attempts to capture and subdue the prey following an unsuccessful initial attack; measured as additional acts of physical aggression toward the prey (e.g., subsequent lunges toward the prey while using claws to grasp and/or attempting to bite).
 - 5) Sting: scorpion's stinger is observed making physical contact with the mouse and the mouse reacts (e.g., the mouse flinches and/or grooms the area on its body where the scorpion's stinger made contact), indicating that the scorpion successfully injected the mouse with venom.
 - 6) Drop: loss of control of prey; prey escapes subjugation as defined, operationally, by the mouse losing physical contact with the prey (e.g., the mouse releases the prey by

withdrawing its claws and/or releasing its bite, and the prey moves out of the immediate grasp of the mouse).

- 7) Incapacitation: mouse kills or immobilizes the prey; recorded as the moment the prey makes no further attempt to resist subjugation, escape, or cause harm to the mouse (e.g., prey stops struggling because it is severely injured or dead).

Experimental Design:

A total of 38 grasshopper mice from the three different populations were tested with each of the three prey species. Of the 38 mice, 22 were adults and 16 were juveniles. Thus, data were analyzed (SPSS, version 11.0) using a three-factor ANOVA that included a single within subjects, repeated-measures factor (prey type) and two between-subjects factors (mouse population, mouse age). None of the dependent variables met the standard parametric assumptions of normality and homogeneity of variance; duration variables were therefore logarithmically transformed using the equation $\log(X + 1)$, while count variables were square-root transformed using the equation $\sqrt{(X + 3/8)}$ (Zar, 1996). Variables that failed to meet either assumption following transformation were further subjected to the conservative Geisser-Greenhouse correction (Keppel, 1991). Post-hoc comparisons using Tukey's HSD were conducted (JMP, version 3.0) on only those dependent variables exhibiting significant main effects from the ANOVAs.

RESULTS

Recognition Phase:

None of the prey items were rejected by the grasshopper mice; every single cricket and scorpion, from both genera, was attacked within just a few seconds of its introduction into the test chamber. Typically, movement by the cricket or the scorpion attracted the

mouse's attention, which then approached, briefly sniffed the prey, and immediately lunged. The average latency from the moment the prey item was introduced until the mouse's first attempt to grasp or bite the prey was 3.11 sec (SE = 0.57 sec). An average of 1.11 secs of the 3.11 sec latency was spent sniffing the track traversed by the prey, with another 1.00 sec (SE = 0.13) sniffing within 5 mm of the prey, generating a total sniff time of 2.11 sec (SE = 0.39). Of the 114 total prey items presented during the 38 trials, 101 were partially or entirely consumed by the mice, including every cricket, 35 of 38 scorpions in the genus *Vaejovis*, and 28 of 38 scorpions in the genus *Centruroides*. When feeding, the grasshopper mice typically held the prey by its mesosoma and began feeding on the head, as previously described (Horner et al., 1965). The mice consumed all of the prey species from anterior to posterior end, generally leaving the pincers, walking legs, and the final segment of the tail and stinger of the scorpions, and the walking legs and wing covers of the crickets.

Other than fleeting responses to the pain of a scorpion's sting, none of the grasshopper mice showed any behavioral or physiological signs of envenomation by the scorpions of either genus.

Pursuit/Attack Phase:

Grasshopper mice did not appear to be differentiating crickets from scorpions, nor *Vaejovis* from *Centruroides*, during the three seconds the mice conceivably spent deciding not just whether, but how, to attack. None of the three duration variables associated with the pursuit phase of a predatory sequence (total inspection time, total sniffing time, and prey-sniffing time) were significantly different across prey items; e.g., there was no significant effect of type of prey on total inspection time ($F = 0.489$; $df = 2, 64$; $p = 0.615$), total sniffing

time ($F = 1.383$; $df = 2, 64$; $p = 0.258$), nor on prey-sniffing time ($F = 2.676$; $df = 2, 64$; $p = 0.077$).

There were also no significant population effects on any of the three dependent variables; not for total inspection time ($F = 0.376$; $df = 2, 32$; $p = 0.690$), nor for total sniffing time ($F = 0.584$; $df = 2, 32$; $p = 0.564$), nor finally for prey-sniffing time ($F = 1.939$; $df = 2, 32$; $p = 0.160$).

The behavior of juvenile grasshopper mice during the attack phase was not significantly different from that of adults. There was no significant effect of mouse age on total inspection time ($F = 0.627$; $df = 1, 32$; $p = 0.434$), total sniffing time ($F = 0.221$; $df = 1, 32$; $p = 0.642$), or prey-sniffing time ($F = 0.013$; $df = 1, 32$; $p = 0.909$).

None of the two-way interactions (prey type by population, prey type by age, population by age), nor the three-way interaction (among prey type, age, and population) was significantly different for the three duration variables.

Qualitatively, there is limited evidence to suggest that grasshopper mice may orient their initial predatory attacks to different morphological regions of different types of prey. Grasshopper mice primarily directed their initial attacks to the tail region of scorpion species (66.7%), less often to the head (21.3%), and only rarely toward the scorpion's body (12%). In contrast, initial predatory attacks on crickets were oriented most often towards its body (63.2%), frequently to the head (36.8%), but never to the cricket's posterior.

Subjugation/handling phase:

This study measured five behaviors associated with the ability of grasshopper mice to effectively handle prey after the initial attack. These five dependent variables were: total prey-handling time; number of attacks by the mouse before the prey was either incapacitated

or the mouse gave up; number of retreats by the mouse during the encounter; number of stings delivered to the mouse (from scorpions only); and the number of times a mouse dropped its prey (giving the prey an opportunity to escape) during an encounter. In general, crickets were easy for the grasshopper mice to handle, scorpions in the genus *Vaejovis* took a bit more effort (but often not significantly so), while scorpions in the genus *Centruroides* were exceedingly difficult to dispatch, even though in these staged encounters the mouse almost invariably did so. Indeed, the main effect of type of prey was a significant factor in the analyses of each of the five subjugation-related variables.

Effects of Prey Type - The average amount of time it took a grasshopper mouse to subdue a cricket (i.e., elapsed time from initial attack until the prey was incapacitated) was 1.63 secs; for *Vaejovis spinigerus*, it took 6.76 secs; and for scorpions in the genus *Centruroides*, it required an average of 93.72 secs. This difference across prey types in average handling time was highly significant ($F = 71.424$; $df = 1, 32$; $p < 0.0005$; Fig. 1); post-hoc comparisons using Tukey's HSD test indicates that average handling times are significantly different for each of the three different prey items.

The most likely reason that both genera of scorpions took longer than crickets for the mice to subdue is because scorpions sting and crickets do not. Grasshopper mice often responded to being stung by breaking off their attack and briefly grooming the body part that had been stabbed. Interestingly, there appear to be differences in the ability of the two scorpion genera used in this study to deliver effective stings; the average number of stings delivered by *Centruroides* during an encounter was 4.42, for *Vaejovis* it was only 1.16, a difference that was highly significant ($F = 50.392$; $df = 1, 32$; $p < 0.0005$; Fig. 2).

Grooming in rodents involves the use of their feet. So, for a grasshopper mouse to groom a stung hind paw, the mouse by necessity must drop the scorpion. Not surprisingly, the number of times that mice dropped a prey item during an encounter depends on the type of prey it was holding; crickets were dropped on average only 0.26 times during an encounter, *Vaejovis spinigerus* a mean of 0.29 times, and *Centruroides* an average of 2.21 times. These differences also are highly significant ($F = 39.902$; $df = 1.6, 49.9$; $p < 0.0005$; Fig. 3); post-hoc comparisons show that the number of times *Centruroides* was dropped is significantly greater than the number of drops for either crickets or for *Vaejovis*, which are not significantly different from each other.

A truly noxious prey might not only be dropped, it might also, at least temporarily, be abandoned. Grasshopper mice almost never retreated from a cricket after beginning an attack (mean = 0.03 retreats per encounter), rarely retreated from *Vaejovis spinigerus* (mean = 0.05 per encounter), but turned and retreated from *Centruroides* much more frequently (mean = 1.68), differences that once again are highly significant ($F = 8.412$; $df = 1.0, 33.2$; $p = 0.006$; Fig. 4); post-hoc tests show that the number of retreats during trials with *Centruroides* was significantly greater than for each of the other two prey items, which did not differ.

Grasshopper mice, however, are tenacious predators. In the confines of the test terrarium, retreats were temporary, re-attacks the norm. Crickets required an average of only 1.34 attacks before they were incapacitated, *Vaejovis spinigerus* 1.95, while *Centruroides* took 4.61 attacks before the scorpion was either subdued or the mouse broke off its attack. Here again the pattern is the same – highly significant differences across prey types ($F = 37.719$; $df = 1.4, 46.1$; $p < 0.0005$; Fig. 5), with *Centruroides* requiring significantly more attacks than crickets or *Vaejovis*, which did not themselves differ.

Only one of the two-way interactions between prey type and mouse age was significant, involving the variable “number of dropped prey.” Results of this interaction are presented in the section on *Age effects* below.

None of the two-way interactions between prey type and population was significant for any of the 5 subjugation-related variables. There was only one significant three-way interaction of prey type by population by mouse age, this involving the dependent variable “number of attacks.” Results of the three-way interaction are presented in the section on *Population effects* below.

Age effects – the main effect of mouse age (juvenile vs. adult) was not a significant influence on four of the five dependent variables associated with the subjugation/handling phase of a predatory sequence. There were no significant effects of mouse age on handling time ($F = 2.134$; $df = 1, 32$; $p = 0.154$), number of times the mouse was stung ($F = 0.070$; $df = 1, 32$; $p = 0.793$), number of times the mouse retreated ($F = 0.618$; $df = 1, 32$; $p = 0.438$), or the number of attacks by the mouse ($F = 0.595$; $df = 1, 32$; $p = 0.446$).

Adult mice did, however, appear to be more adept at hanging onto a prey item during an encounter; prey were dropped on average only 0.591 times per trial by adults, but 1.375 times per trial by juveniles, a difference that was significant ($F = 6.004$; $df = 1, 32$; $p = 0.020$; Fig. 6). Young mice dropped scorpions in the genus *Centruroides* more often as evidenced by the significant two-way interaction between mouse age and prey type ($F = 3.820$; $df = 1.6, 49.9$; $p = 0.038$; Fig. 7). As with the main effects, the two-way interactions between mouse age and prey type were insignificant for the other four dependent variables.

There was but a single variable, “number of attacks”, in which there was a significant two-way interaction between mouse age and mouse population. “Number of attacks” is also

the only variable resulting in a significant three-way interaction among age, population, and prey type. Both of these results are discussed under *Population effects* below.

Population effects – Significant differences in the manner in which grasshopper mice from the three different populations reacted to their prey appeared in only two of the five post-attack variables. There were no significant differences across populations in handling time ($F = 0.129$; $df = 2, 32$; $p = 0.879$), in the number of times that prey were dropped during handling ($F = 0.965$; $df = 2, 32$; $p = 0.392$), or in the number of retreats by the mouse during the encounter ($F = 0.615$; $df = 2, 32$; $p = 0.547$). The three populations did, however, differ in the number of times they were stung while handling a scorpion; mice from the Santa Rita population were stung an average of 1.86 times during a trial, mice from the Beaver Dam population were stung 3.71 times, while mice from the Organ Mountains were stung 4.33 times. While the main effect of these differences was significant ($F = 35.955$; $df = 2, 32$; $p = 0.042$; Fig. 8), only the Organ Mountain vs. Santa Rita comparison was significant in the post-hoc tests.

The three populations of mice also differed in the number of attacks required to incapacitate, or in only two cases to abandon, the prey; Santa Rita mice required an average of 2.106 attacks per trial, Beaver Dam mice 2.619 attacks, and Organ Mountain mice 3.926 attacks. These differences were significant ($F = 3.666$; $df = 1, 32$; $p = 0.037$; Fig. 9); however, only the comparison between Santa Rita and Organ Mountain mice was significant using Tukey's HSD.

“Number of attacks” was also the only variable exhibiting a significant two-way interaction between mouse age and population ($F = 3.409$; $df = 2, 32$; $p = 0.045$; Fig. 10); while juvenile mice from the Santa Rita and Beaver Dam populations required more attacks

to incapacitate their prey than did adults, mice from the Organ Mountains showed the opposite pattern, with adults requiring more attacks than did juveniles (Fig. 11). Moreover, adult mice from the Organ Mountains directed an exceptionally high number of attacks at *Centruroides*, generating our only significant three-way interaction among age, population, and prey type ($F = 4.605$; $df = 2.9, 46.1$; $p = 0.007$; Fig. 11).

Trial Effects:

Given that the order of presentation of each prey item was systematically counterbalanced among mice, I expected, and found, no significant influences of trial number on any of the measurement variables.

DISCUSSION

Because I staged the interactions between grasshopper mice and their prey, I cannot evaluate whether mice make risk-sensitive decisions regarding how or where to search. There is no doubt, however, that the mice recognized all three genera as potential prey. Every prey item, whether relatively defenseless crickets or neurotoxin-wielding scorpions, were attacked with little hesitation, almost always killed, and usually consumed. Most dramatically, there was no hint that grasshopper mice avoid scorpions in the genus *Centruroides*. Mearns' grasshopper mice unhesitatingly attacked *C. vittatus*, and southern grasshopper mice were equally voracious in attacking *C. exilicauda*, a species whose sting can be lethal to human infants (Russell and Madon, 1984; Simard et al., 1992; LoVecchio and McBride, 2003). These observations suggest that grasshopper mice may routinely encounter *Centruroides* in habitats where they are syntopic, and that mice treat these scorpion species as prey.

Is there any evidence that grasshopper mice make risk-sensitive decisions regarding how to attack prey that differ in their dangerousness? Results from the feeding trials demonstrate that grasshopper mice have the opportunity to engage in such pre-attack assessment. On average, grasshopper mice spent approximately three seconds attending to a prey item before attacking. Approximately two seconds were spent visually and olfactorily tracking the prey, with one second spent sniffing the prey item directly, prior to the mouse's first lunge. However, grasshopper mice do not appear to use the cues they may have acquired during this brief three-second period to discriminate among prey species that differ dramatically, or so I presumed, in their ability to inflict injury on the mouse. There were no significant differences between scorpions and crickets, or between *Vaejovis* and *Centruroides*, in the latency between attending and attacking, in how long the mouse spent tracking the prey, or in the duration spent sniffing the prey from within just a few millimeters. My results add to the numerous studies which suggest a rather simple rule of thumb for grasshopper mice during this phase of a predatory sequence; i.e., if something nearby is moving, and if it is small and smells like food, attack!

Labeling the grasshopper mouse's pre-attack strategy a "rule of thumb" does not mean that these desert carnivores *never* modify their behavior in the pre-contact phase of a predatory sequence. One result, which could only be assessed qualitatively (as it is inappropriate to conduct χ^2 tests on repeated measures), suggests that grasshopper mice orient their initial attacks differently to undefended crickets than to noxious scorpions; crickets were grabbed mainly by the body (63%), while scorpions of both genera were seized mostly by the tail (67%). This might suggest that grasshopper mice recognize the sting of a scorpion as a potent defensive weapon and respond by directing their initial attacks to

immobilize the scorpion's tail. Alternatively, grasshopper mice may be reacting to the first moving object they encounter during an attack, an argument first proposed by Cyr (1972). For example, when a grasshopper mouse is pursuing a fleeing scorpion, the arachnid's tail is the first "anatomical part" the mouse encounters. When, however, a mouse and scorpion meet head-on, the scorpion invariably becomes defensive and thrusts its tail into the face of the mouse. Crickets do not possess long tails, seldom confront mice head-on, and are simply pounced on by the mice as the cricket attempts to escape. Thus, my qualitative observations provide only equivocal support for pre-contact behavioral modification by grasshopper mice. The best evidence that *Onychomys* makes cost-benefit decisions about the dangerousness of a given prey prior to an attack comes from a study using palatable (*Brachystola magna*) and chemically-protected (*Taeniopoda eques*) grasshoppers (Whitman et al. 1986). When these authors presented grasshopper mice (*O. torridus*) with a quick series of prey (*T. eques*, then *B. magna*, followed by a *B. magna* that had been smeared with the defensive secretions from *T. eques*, and finally a second, untreated *B. magna*), the mice frequently rejected both the *T. eques* and the chemically coated *B. magna* in the "sniff at close range" phase leading up to a pounce. Even in Whitman et al.'s (1986) elegant study, however, grasshopper mice appeared to employ their standard rule of thumb, as half of the *T. eques* and 65% of the treated *B. magna* were attacked, often killed, and, for the treated *B. magna* at least, occasionally eaten (killed *T. eques* were never ingested). To quote the authors' conclusion, the grasshopper mouse's evolutionary dictate is to "kill first and ask gustatory questions later".

Whitman et al.'s wonderfully worded conclusion may be just a touch premature, as assessment could still be important in the period separating an attack from a kill. My results suggest that grasshopper mice may engage in prey assessment during the subjugation stage of

the predatory sequence, as they handle the different prey species differently. Indeed, the highly significant prey-type effect observed for every one of the subjugation-related dependent variables is the most robust result of this study. For example, grasshopper mice required more time, and thus exerted more effort, to subdue prey species possessing anti-predator defenses compared to those that did not; i.e., both scorpion genera were more difficult and took longer to incapacitate than crickets (Fig. 1). Prehensile, syringe-tipped tails filled with painful venom appear to work against grasshopper mice, at least temporarily.

Interestingly, the defenses of the two scorpion genera are not equally effective. Grasshopper mice, for instance, required significantly more time to incapacitate *Centruroides* than *Vaejovis* (Fig. 1). Additionally, *Centruroides* appears capable of delivering more, or perhaps just more irritating, stings than does *Vaejovis* (Fig. 2). The less effective defenses of *Vaejovis* relative to *Centruroides* is even more apparent in the remaining three subjugation-related variables, since in none of these did grasshopper mice experience any greater difficulty in handling *Vaejovis spinigerus* than they did in handling crickets. For example, grasshopper mice mounted repeated attacks against *Centruroides* in order to immobilize these scorpions, significantly more attacks than required to subdue either *Vaejovis* or crickets, which were indistinguishable (Fig. 5). Similarly, grasshopper mice retreated more often from *Centruroides* than they did from *Vaejovis* or crickets, which again did not differ (Fig. 4). Each additional retreat requires an additional attack if the mouse is to prevail and, with two exceptions (both with *Centruroides*), the mouse always did. Lastly, and not surprisingly, grasshopper mice dropped *Centruroides* significantly more often during handling than they dropped *Vaejovis* which, even though the latter can and did sting, was dropped no more frequently than were crickets (Fig. 3). Collectively, these results

demonstrate that *Centruroides* is extremely difficult to handle, while *Vaejovis spinigerus* is not. The biological significance of these generic differences will be discussed later, but here I return to the topic of assessment.

Does the fact that grasshopper mice handle different types of prey differently indicate that the mice are using sophisticated cost-benefit analyses to negate their prey's defenses? An argument based on Occam's Razor would say "no." The fact that it is extremely difficult for grasshopper mice to subdue *Centruroides*, requiring more time, more retreats, and more attacks than required by crickets or *Vaejovis*, may involve very little cognitive processing at all -- at least no more cognitive machinery than is involved when a human being unknowingly picks up a hot cast-iron skillet and immediately drops it. A grasshopper mouse hears or sees something move, approaches it quickly, sniffs it briefly, and then attacks. If the prey is *Centruroides*, and it is successful in landing its apparently irritating sting, the mouse drops the scorpion, retreats, grooms the site of the sting, and then re-attacks, with no apparent modification of its attack behavior. The mouse does not do what we would do when unwittingly picking up a hot skillet; i.e., look around for a hot-pad holder before our second attempt to grab the skillet. To state, for example, that "grasshopper mice attack crickets, stink beetles, and scorpions differently" (Langley 1981, pg. 115) when all that was demonstrated was that mice handled the three prey species differently not only conflates the attack phase with the subjugation phase of a predatory sequence, it implies a level of assessment that may not characterize the mice. To reinforce my point that failing to distinguish different phases of a predatory sequence can prevent our understanding of predator assessment strategies, I turn to another investigation (O'Connell & Formanowicz 1998) that used scorpions in the genus *Centruroides* as their exemplar of a dangerous prey.

O’Connell and Formanowicz (1998) clearly demonstrated that whiptail lizards (*Cnemidophorus gularis*) handle *Centruroides vittatus* differently than they handle lab crickets (*Acheta domesticus*). Indeed, their results strongly parallel those I presented here; i.e., whiptail lizards required more time and more attacks to subdue scorpions than crickets. Additionally, the lizards shook and threw scorpions violently after biting onto them, a behavior not observed when lizards bit crickets. For the authors to conclude that their “experiments indicate that *C. gularis* may distinguish between different levels of risk associated with foraging on different types of prey” is, however, premature. The “shake and throw” response of lizards may be the equivalent of the “drop, retreat, and groom” response of grasshopper mice to having just been stung by *Centruroides* – assessment, other than the immediate sensation of and response to pain, need not be involved.

I do not mean to imply that grasshopper mice are predatory automatons, incapable of adjusting their behavior to the exigencies of different situations. My results, for example, are consistent with other studies (Cyr 1972; Langley 1986) showing prey handling behavior in grasshopper mice improves with experience; adults dropped their prey items less often than did juvenile mice, significantly so when feeding on *Centruroides*. This suggests that adults are either more adept than juveniles at avoiding being stung, or have habituated to the pain of the stings. Furthermore, the results of this study tentatively suggest that prey handling behavior may be influenced by population level factors. Grasshopper mice from the Organ Mountains required significantly more attacks to subdue their prey and sustained more stings from scorpions than did mice from the Santa Rita Mountains, with mice from the Beaver Dam Mountains exhibiting intermediate values not significantly different from the other two populations. One possible explanation for these differences is that the stings of *Centruroides*

vittatus (sympatric with the *O. arenicola* from the Organ Mountains) are more effective than the stings of *C. exilicauda* (sympatric with *O. torridus* from the Santa Rita and Beaver Dam Mountains). Weak support for this hypothesis is provided by the significant two-way interaction between number of attacks and mouse population (Fig. 10) and the significant three-way interaction among population, age, and attacks (Fig. 11). While juvenile mice from the Santa Rita and Beaver Dam populations required more attacks to subdue their prey than did adults, especially when attacking *Centruroides exilicauda*, adult rather than juvenile mice from the Organ Mountains required the most attacks, especially when subduing *C. vittatus*. I caution, however, against making strong inferences regarding population level differences in handling behavior until a larger sample of mice from both the Santa Rita and Organ Mountain populations can be studied.

The reason that grasshopper mice neither avoid *Centruroides* nor attack them more cautiously than they attack *Vaejovis* or crickets could be because they are resistant to the neurotoxins produced by scorpions in the genus *Centruroides*. This is perhaps the most interesting of my findings; indeed, the dogma among arachnologists and mammalogists, at least those I polled informally, was that grasshopper mice would not attack *Centruroides*, especially *Centruroides exilicauda*. But the mice do attack, they are stung, yet they do not demonstrate any of the systemic physiological effects typically associated with *Centruroides*' venom. Neurotoxins produced by bark and striped scorpions that selectively bind the sodium (Na^+) and potassium (K^+) ion-channels of vertebrates typically produce a cascade of physiological malfunctions ranging from hyper-excitability and seizures, to loss of motor control and paralysis, and finally to cardiac and respiratory failure; i.e., death (Simard et al., 1992). The "cocktail" of neurotoxins produced by *Centruroides* species could exert strong

selection on grasshopper mice, especially on young and inexperienced mice that require multiple attacks, and thus receive multiple stings, when battling a scorpion. For a predator that initiates attacks in response to movement and olfactory cues, and for whom discrimination of scorpions wielding potentially lethal neurotoxins could be difficult, selection would favor individuals possessing biochemical or physiological mechanisms that neutralize, degrade, or prevent such toxins from binding. Thus, grasshopper mice may have developed resistance to neurotoxins as an evolutionary response to selection by *Centruroides*. In turn, the extreme potency of the venom in certain groups of scorpions, including *Centruroides*, may be the result of selection by tenacious and resistant mammalian predators including *Onychomys*.

Comparative differences in the potency of scorpion venoms across species may provide insight into the strategies scorpions employ to evade their predators. Here again, it is useful to divide a predatory encounter into the three phases of detection/recognition, attack/pursuit, and handling/subjugation, as Vermeij (1982) has argued convincingly that selection on any given prey species will be most intense during a single phase. The results of this study strongly suggest, for example, that predator-induced selection has operated most intensely on *Centruroides* during the subjugation phase, as scorpions in this genus are extremely difficult to handle. By comparison, the anti-predator adaptations of *Vaejovis spinigerus* are most likely associated with either avoiding detection or escaping pursuit, as these scorpions are barely more difficult to subdue than crickets. Support for these predictions awaits more detailed ecological studies than are now available for either genus of scorpions. I can, however, suggest several proximate explanations for why *Centruroides* is more difficult than *Vaejovis* for *Onychomys* to handle. Perhaps the tails of *Centruroides* are

more agile than the tails of *Vaejovis*, or their stingers more penetrating, or their venom more debilitating. Experimental evidence (Rowe & Rowe, unpubl. data) shows that *Centruroides* does indeed have more potent venom than *Vaejovis*, but not in the manner I originally suspected. Of relevance here is a recent study demonstrating that scorpion venoms, although a complex cocktail of chemicals, can coarsely be divided into those causing immediate pain and those responsible for toxicity and death (Inceoglu et al. 2003). I originally predicted that grasshopper mice would be especially cautious when interacting with *Centruroides* given their potentially lethal neurotoxins. Obviously, I was incorrect – *Onychomys* can afford to attack *Centruroides* with the same voracity as they attack *V. spinigerus* or crickets because the mice have evolved resistance to *Centruroides*' neurotoxins. What makes both species of *Centruroides* so difficult for grasshopper mice to handle are not their neurotoxins, but rather the pain component, of their venom (Rowe & Rowe unpubl. data). Thus, in the arms race between *Centruroides* and *Onychomys*, evolution of more toxic venoms in the scorpions appears to have been effectively countered by evolution of physiological resistance to those toxins by the mouse. A painful sting, however, causes the mouse to drop a *Centruroides* and, in a habitat more complex than the experimental terrarium, may allow it to live to sting another day.

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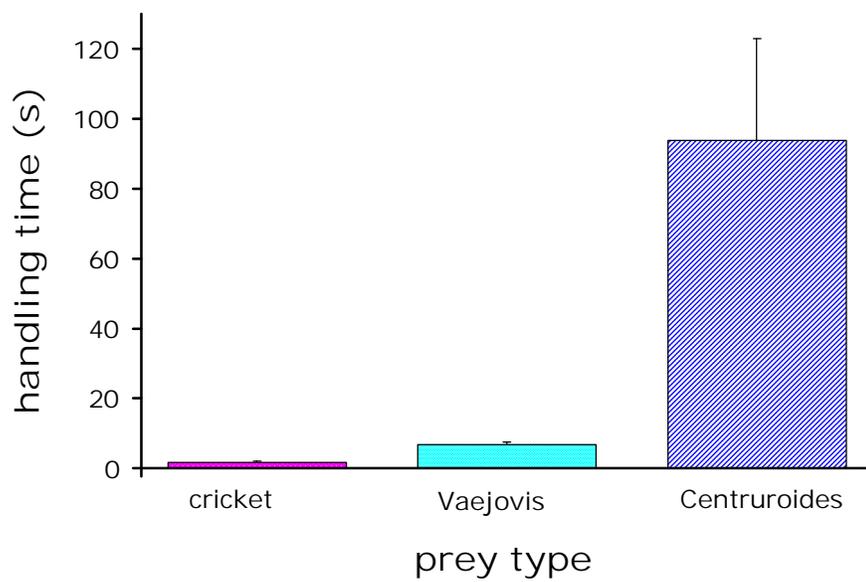


Figure 1. Mean and SE of latency, for each prey type, between a mouse's first attack and the moment the prey is incapacitated. Post hoc comparisons using Tukey's HSD show that the handling times were significantly different across each of the three prey types.

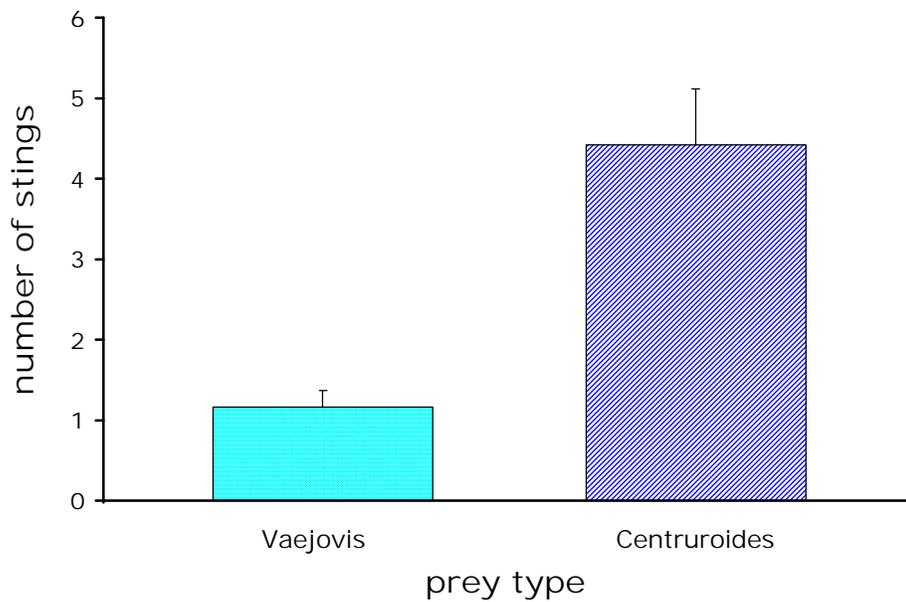


Figure 2. Mean and SE of the number of successful stings landed by either *Vaejovis spinigerus* or *Centruroides* spp. during an encounter with *Onychomys*. The average number of stings delivered by the two species was significantly different.

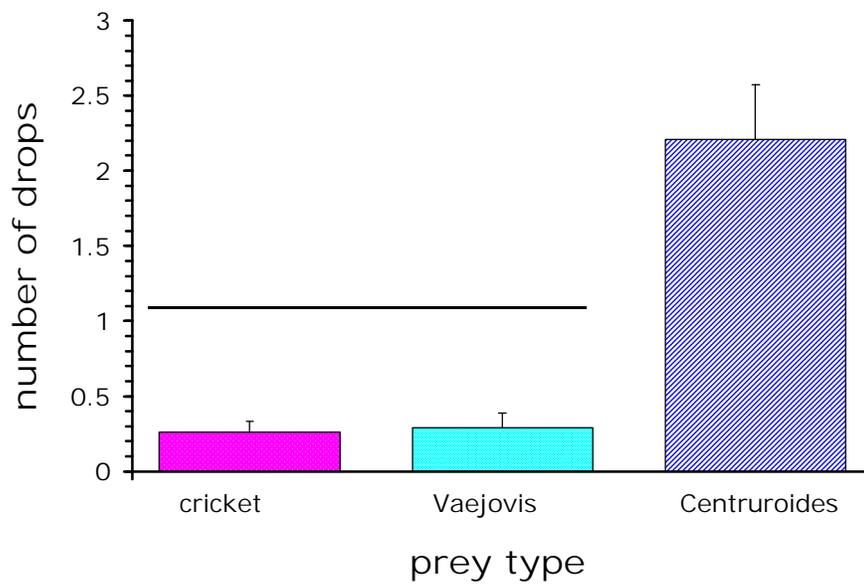


Figure 3. Mean and SE of the number of times each prey type was dropped during the handling phase of an encounter with *Onychomys*. Histograms overscored by the same line failed to differ at the $p < 0.05$ level in post-hoc comparisons using Tukey's HSD.

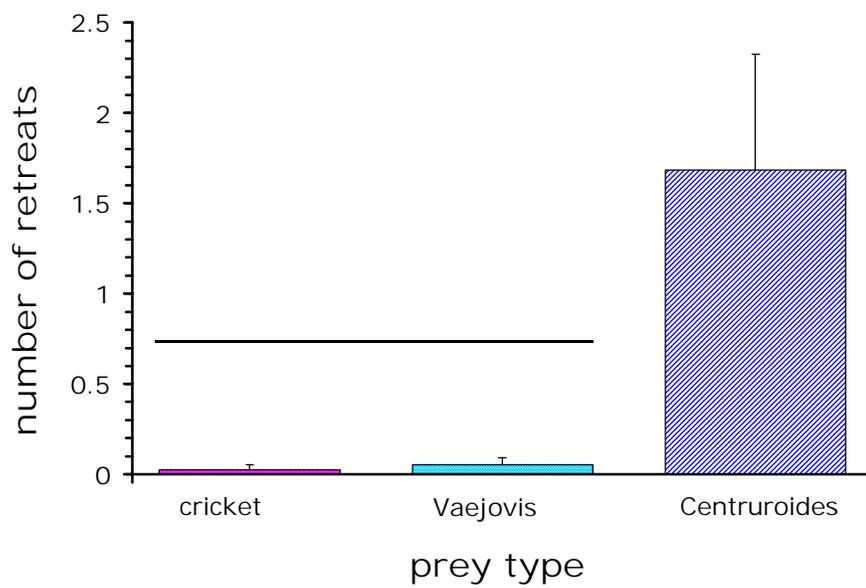


Figure 4. Mean and SE of the number of retreats from each prey type exhibited by *Onychomys* during the handling phase of an encounter. Histograms overscored by the same line failed to differ at the $p < 0.05$ level in post-hoc comparisons using Tukey's HSD.

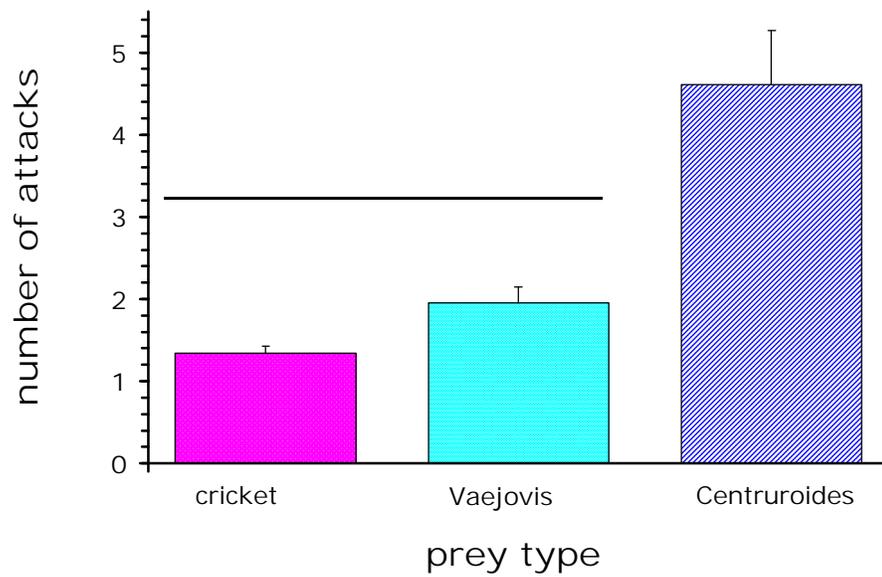


Figure 5. Mean and SE of the number of attacks required for *Onychomys* to subdue each prey type during the handling phase of an encounter. Histograms overscored by the same line failed to differ at the $p < 0.05$ level in post-hoc comparisons using Tukey's HSD.

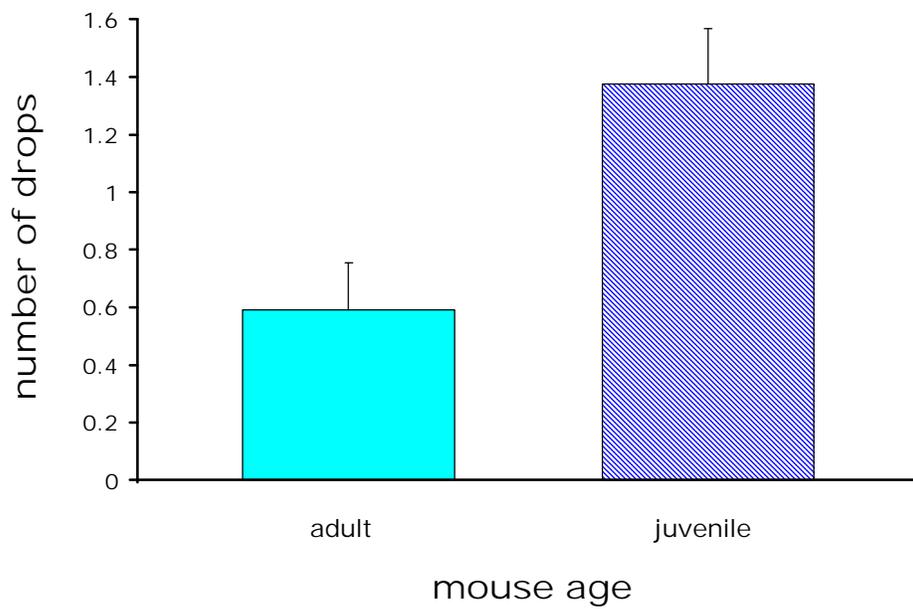


Figure 6. Mean and SE of the number of times prey were dropped by juvenile vs. adult *Onychomys* during the handling phase of an encounter. Prey were dropped significantly more often by juvenile than by adult grasshopper mice.

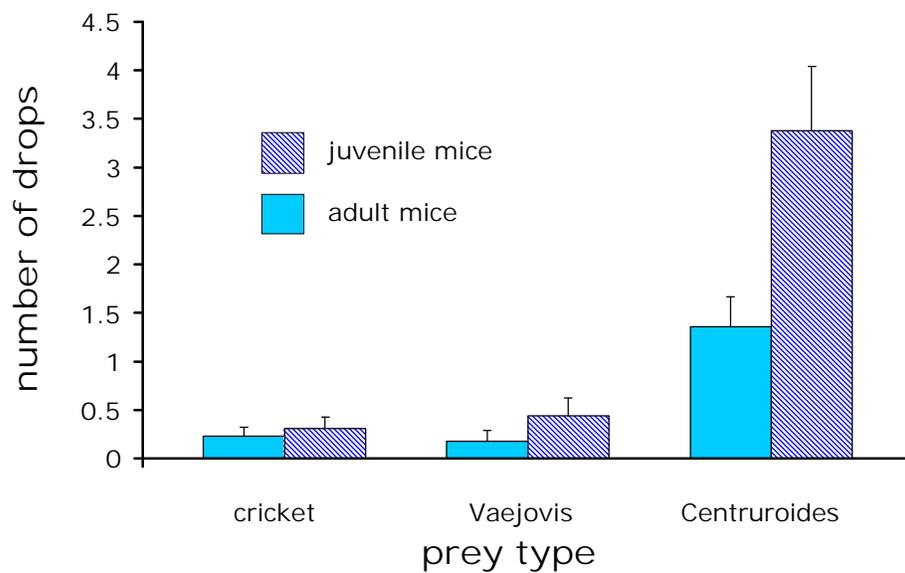


Figure 7. Mean and SE of the number of times prey were dropped during handling as a function of type of prey and the age of the grasshopper mouse. Although juvenile mice dropped prey more often than did adults across all prey types, the juveniles had an especially difficult time with *Centruroides*.

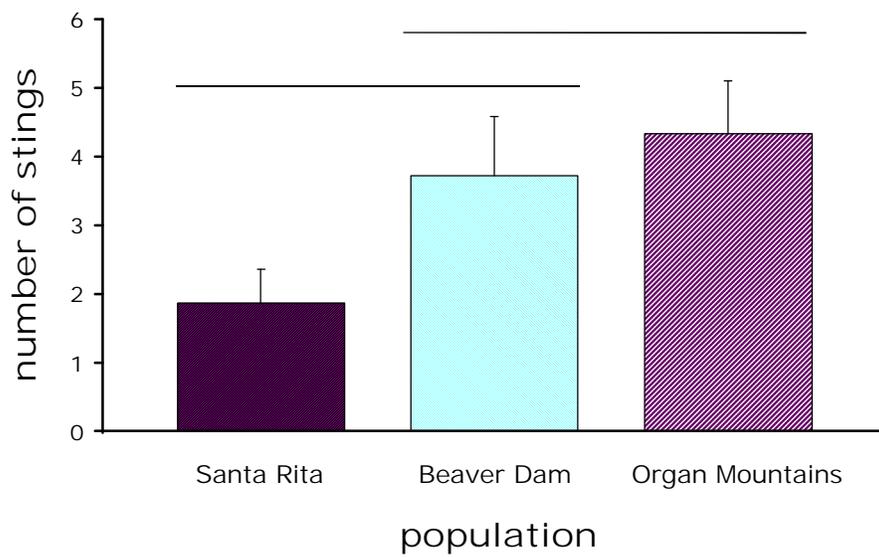


Figure 8. Mean and SE of the number of successful stings delivered by scorpions to *Onychomys* from the three different study populations. Histograms overscored by the same line failed to differ at the $p < 0.05$ level in post-hoc comparisons using Tukey's HSD.

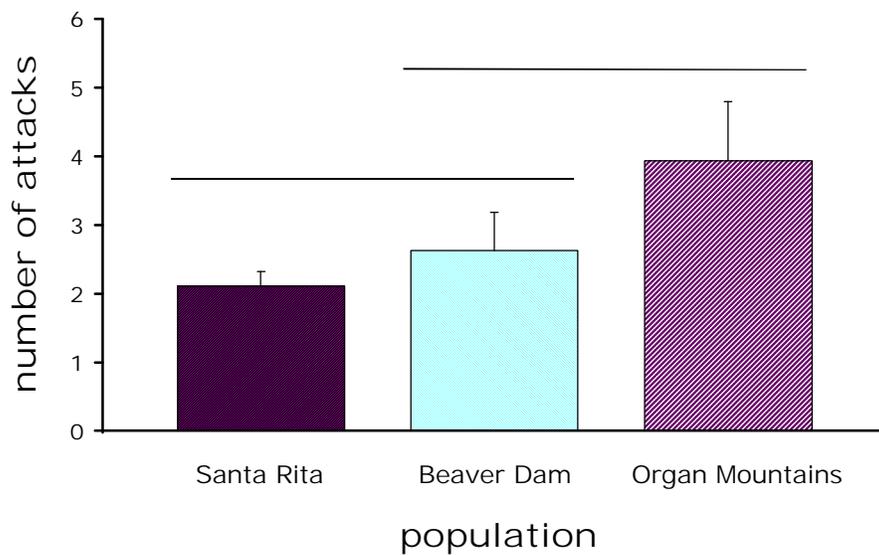


Figure 9. Mean and SE of the number of attacks required by *Onychomys* from the three different study populations to subdue their prey during the handling phase of a predatory encounter. Histograms overscored by the same line failed to differ at the $p < 0.05$ level in post-hoc comparisons using Tukey's HSD.

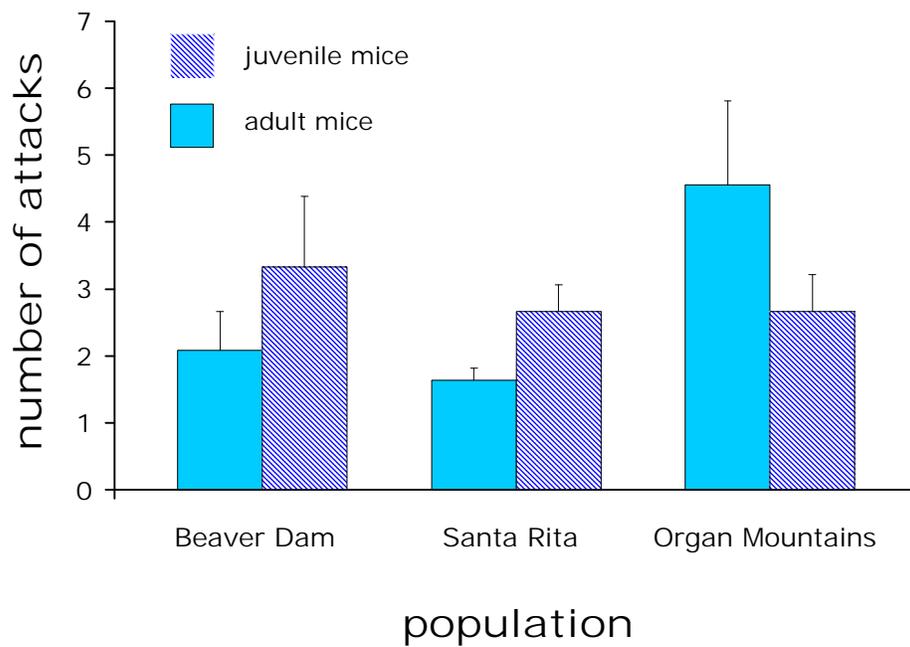


Figure 10. Mean and SE of the number of times juvenile and adult mice from each study population had to attack their prey before the prey was either incapacitated or abandoned. While juvenile mice from the Beaver Dam and Santa Rita populations had to attack more often than the adults, adult mice from the Organ Mountains exhibited more attacks than the juveniles.

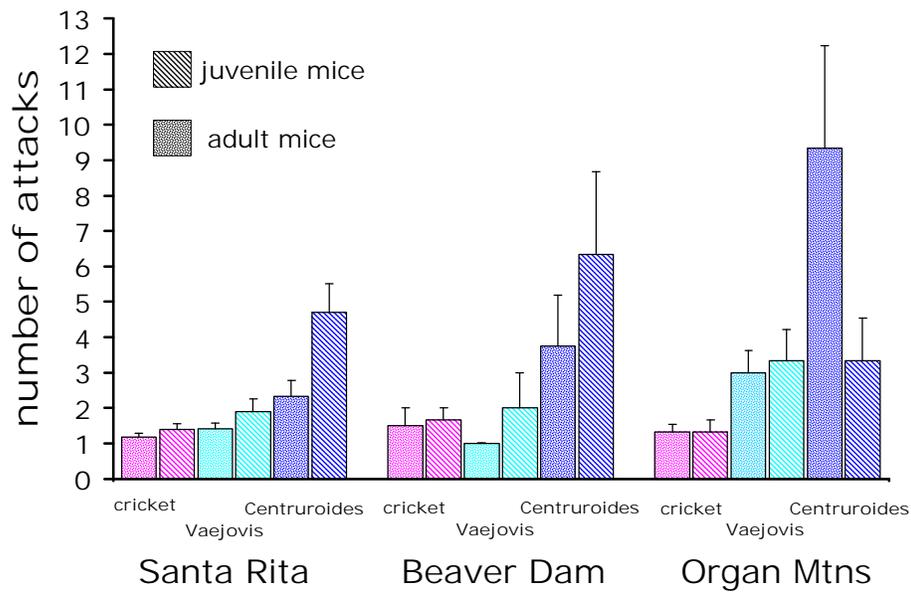


Figure 11. Mean and SE of the number of attacks directed by juvenile and adult mice from each population at each of the three prey types. Note, in particular, the high number of attacks adult mice from the Organ Mountain population directed against *Centruroides vittatus* compared to the number directed by adult mice from the Santa Rita and Beaver Dam populations against *C. exilicauda*.

CHAPTER THREE

**CONFIRMATION OF THE RESISTANCE OF GRASSHOPPER MICE
(*ONYCHOMYS* SPP.) TO THE VENOM OF BARK SCORPIONS (*CENTRUROIDES
EXILICAUDA*)**

INTRODUCTION

Results from my behavioral study of the predator-prey relationship between grasshopper mice (*Onychomys* spp.) and bark and striped scorpions (*Centruroides* spp.) demonstrated that grasshopper mice are likely predators on bark and striped scorpions in regions where these species co-occur (Chapter Two). Bark scorpions (*C. exilicauda*) and striped scorpions (*C. vittatus*) are members of the genus *Centruroides*, a group of scorpion species best known for their production of neurotoxins that selectively bind the sodium (Na^+) and potassium (K^+) ion-channels of vertebrates (Possani et al., 2000; Corona et al., 2001; Corona et al., 2002). While K^+ ion-channel toxins are associated with pain and paralysis, Na^+ ion-channel toxins are capable of producing death in mammals, especially small mammals (Simard et al., 1992; Possani et al., 2000). My behavioral analyses of the interactions between grasshopper mice and *Centruroides* spp. established that mice attack, incapacitate, and consume both bark and striped scorpions. During these interactions, mice were usually stung at least once, and some mice were stung several times. However, none of the envenomated mice showed any physiological effects of the neurotoxins, and all the mice survived. This behavioral study suggested that grasshopper mice have developed physiological resistance to bark and striped scorpion K^+ and Na^+ ion-channel neurotoxins.

The purpose of this chapter was to confirm or refute the apparent resistance of grasshopper mice to bark and striped scorpion neurotoxins. If populations of grasshopper mice that are sympatric with bark or striped scorpions have developed resistance to K^+ and Na^+ ion-channel neurotoxins, then a lethal dose of venom (as determined from tests using standard laboratory mice, *Mus* sp.) should result in a reduction of the physiological effects in grasshopper mice. Here, an initial toxicity test is used to determine the effects of bark

scorpion (*C.exilicauda*) venom on grasshopper mice. Grasshopper mice were injected with a single dose of bark scorpion venom. The dose administered to grasshopper mice was equivalent to the dose previously determined to be lethal to at least 50% of a non-resistant *Mus* sp. population (Simard et al., 1992). I chose to use bark scorpion venom for this assay because it is more toxic than striped scorpion (*C.vittatus*) venom, and because the median lethal dose is known for a non-resistant mammal model. The median lethal dose (LD₅₀) for bark scorpion venom (*C.exilicauda*) in *Mus* sp. is equivalent to 1.0-1.5 µg/g (Simard et al., 1992).

METHODS

Study Sites and Animals:

Southern grasshopper mice (*Onychomys torridus*) were collected from two study sites in Arizona (AZ). The first site represents a population of mice that occur syntopically with bark scorpions in the Santa Rita Mountains (SR) of Pima County, AZ; 16 mice were collected from the Santa Rita Mountains in July 2000. The second location, near the Chiricahua Mountains (CR) of Cochise County, AZ, represents a site where the mice are parapatric with a nearby population of bark scorpions (or occur in the nonoverlapping zone of a partially sympatric distribution, *sensu* Fig. 4B in Key 1981). The nearest bark scorpion population is located approximately fifteen to twenty miles to the east of the Chiricahuas in the Peloncillo Mountains (PL); nine mice were collected from the Chiricahua Mountains in early August of 2000. Mearns' grasshopper mice (*Onychomys arenicola*) were collected from a third study site in the Organ Mountains (OR) of Doña Ana County in New Mexico (NM). In this region, Mearns' grasshopper mice occur syntopically with striped scorpions; eight mice were collected from the Organ Mountains in mid-August 2000.

All grasshopper mice were returned to North Carolina to establish a breeding colony. The grasshopper mice were housed in a USDA approved animal facility and cared for according to USDA guidelines for wild mice. Several of the female grasshopper mice were pregnant when they were collected. All of these mice gave birth to pups, increasing the colony size before I began matching breeding pairs. Grasshopper mice were paired by population, and sister-brother pairs were avoided.

The bark scorpions that provided the venom sample for this study were collected from Pinal County in AZ. The venom was extracted using electrical stimulation and the insoluble components were removed using a combination of water extraction and centrifugation. The remaining supernatant (neurotoxins dissolved in water) was lyophilized (freeze dried) and stored at -20°C.

Procedure:

A sample of lyophilized bark scorpion venom was weighed and placed in a sterile vial. The sample of freeze-dried venom was re-hydrated in physiological saline (0.9% NaCl) and maintained at 4°C for approximately 30 minutes prior to administration to grasshopper mice. Three experimental groups of eight grasshopper mice each, representing the three populations (SR, n = 8; CR, n = 8; OR, n = 8), were injected with a dose of bark scorpion venom equivalent to the higher end of the median lethal dose range of venom reported for non-resistant *Mus* sp. (1.56µg/g). A control group of three grasshopper mice representing each population (SR, n = 3; CR, n = 3; OR, n = 3) was injected with an equivalent volume of physiological saline. This control group was included to demonstrate that the injection had no effect on the mice. A control group consisting of two non-resistant *Mus* sp. were each injected with a dose of bark scorpion venom equivalent to the higher end of the median lethal

dose range reported for bark scorpion venom in non-resistant *Mus* sp. (1.56µg/g). This control group was included to demonstrate that the batch of venom used in this test was biologically active. All mice were weighed and venom doses were adjusted to each individual mouse's weight. The volume of venom injected ranged from approximately 0.2-0.4 mls. The venom samples were injected into the intraperitoneal (i.p.) cavity of the mice. The mice were observed for several hours and the effects of the venom were recorded.

RESULTS

All grasshopper mice (100%) in the experimental groups that received bark scorpion venom survived, and none of the mice (0%) demonstrated any of the physiological effects typically associated with bark scorpion neurotoxins (i.e., excessive salivation, muscle spasms, hyperactivity, seizures, loss of motor control, paralysis, respiratory distress, and cardiac and respiratory failure). Control mice that received a dose of saline exhibited no physiological effects of either the saline or the injection. Both mice in the non-resistant *Mus* sp. control group exhibited all the physiological effects typically associated with bark scorpion neurotoxins (see above) and both expired, thus demonstrating that the batch of venom used in this assay was biologically active.

CONCLUSION

This initial toxicity test demonstrates that southern and Mearns' grasshopper mice are not affected by the K⁺ and Na⁺ ion-channel neurotoxins produced by bark scorpions, at least up to a dose of 1.56 • g/g of venom protein per gram of mouse. These results suggest that grasshopper mice, in populations where they co-occur with bark and striped scorpions, exhibit physiological resistance to the neurotoxins produced by bark scorpions.

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CHAPTER FOUR

THE EVOLUTIONARY SIGNIFICANCE OF GEOGRAPHIC VARIABILITY IN VENOM TOXICITY AND VENOM RESISTANCE: COEVOLUTION BETWEEN BARK AND STRIPED SCORPIONS (*CENTRUROIDES* SPP.) AND GRASSHOPPER MICE (*ONYCHOMYS* SPP.)

INTRODUCTION

Empirical studies of predator-prey coevolution typically evaluate systems with respect to three questions. The first question is concerned with whether both predator and prey exert reciprocal selection on each other during their interaction. The second question focuses on whether selection produces an evolutionary response in both populations, and the third addresses whether the evolutionary response generates novel adaptations or modifies existing ones (Dawkins and Krebs, 1979; Futuyma and Slatkin, 1983; Abrams, 1986a, 1986b). In order to address these questions, researchers often employ an “arms race” analogy as a kind of simple model to explain how predator-prey coevolution proceeds (Dawkins and Krebs, 1979; Abrams, 1986a, 1986b; Brodie and Brodie, 1990, 1999a). The arms race model describes a process in which individual predator and prey exert reciprocal selection on each other during their interaction, which in turn increases the rate of evolutionary response in both populations, resulting in the production of anti-predator adaptations in prey and counter adaptations in predators (Edmunds, 1974; Dawkins and Krebs, 1979; Futuyma and Slatkin, 1983; Abrams, 1986a, 1986b; Thompson, 1994; Vermeij, 1994; Brodie and Brodie, 1999a). The arms race model implies that the adaptations one observes in predators and their prey that mediate their interaction are the product of coevolution due to the reciprocal selection pressure exerted on each other during their contact; i.e., when a prey increases its anti-predator defenses, the predator responds by increasing its counter defenses, which induces new defenses in the prey resulting in an “arms race” (Edmunds, 1974; Dawkins and Krebs, 1979; Ridley, 1993; Vermeij, 1994).

If the arms race model realistically describes antagonistic interactions between predator and prey and reliably predicts the outcome of these interactions, numerous examples

of coevolved predator-prey systems should exist that clearly demonstrate the traits observed in both species are the product of reciprocal selection (Vermeij, 1994; Brodie and Brodie, 1999a). The literature is indeed replete with examples of predator-prey associations frequently referred to as “arms races”, with the behavioral, morphological, and chemical adaptations mediating the interactions referred to as “arms” or “armaments” attributed to the outcome of the “race” (Cott, 1940; Edmunds, 1974; Dawkins and Krebs, 1979; Abrams, 1986b; Brodie and Brodie, 1999a). However, the majority of these studies demonstrate *only* that the prey have developed anti-predator defenses in response to interaction with and selection imposed by their predators. Arthropods, for example, possess a myriad of chemical defenses which they use to deter their enemies. Some arthropods secrete noxious substances to discourage predators from attacking: the European caterpillar *Papilio machaon* secretes a mixture of isobutyric and 2-methylbutyric acid; the Florida millipede *Narceus gordanus* secretes benzoquinones; the carabid beetle *Galerita janus* sprays formic acid at its predators; and the whip scorpion *Mastigoproctus giganteus* discharges a mixture of acetic and caprylic acid (Eisner, 1970). Instead of chemical defenses, some invertebrates respond to their predators with modifications of their morphology; e.g., populations of the marine snail *Littorina obtusata* that coexist with the predatory crab *Carcinus maenas* exhibit thicker shells than snails that do not coexist with this crab (Trussel and Smith, 2000). For some species of prey, the adaptations to predation have been behavioral. For instance, damselfly larvae (*Enallagma* spp.) have evolutionarily responded to their recent colonization of lakes inhabited by fast-swimming nymphal dragonfly predators by an increase in their own swimming speed (McPeck, 1999). Other species of prey have countered with physiological adaptations to their predators. Five species of fish (pomacentrids and blennies), for example,

that are potential prey for the sea snake *Aipysurus laevis* exhibit varying degrees of resistance to the venom produced by this predator (Zimmerman et al., 1992). Two species of muraenid eel also demonstrate resistance to the venom of their sea snake predators, *A. laevis* and *Laticauda colubrina* (Heatwole and Poran, 1995; see also Heatwole and Powell, 1998). And finally, populations of California ground squirrel (*Spermophilus beecheyi*) that coexist with northern Pacific rattlesnakes (*Crotalus viridis oreganus*) exhibit resistance to this snake's potent venom, while populations of ground squirrels allopatric with the rattlesnakes do not (Poran et al., 1987).

While these studies overwhelmingly demonstrate that prey species have responded evolutionarily to pressures imposed by their predators, relatively few studies have shown that predators respond in kind to selection by their prey (Brodie, 1990; Dietl, 2003). Even fewer studies have successfully demonstrated that the adaptations of the predators are in *specific* response to the predator-induced adaptations in the prey; i.e., that the evolutionary responses for both parties in the ecological relationship are linked (Futuyma and Slatkin, 1983; Abrams, 1986b; Brodie and Brodie, 1990, 1999a).

The lack of valid examples of coevolved predator-prey systems that fit the arms race model is often attributed to the inability of the model to accurately describe predator-prey interactions in natural populations and, thus, reliably predict their outcome (Sih, 1984; Abrams, 1986a; Vermeij, 1982, 1994). While it appears to be widely accepted that predators are responsible for the evolution of anti-predator defenses in their prey, counter adaptations on the part of the predator are thought instead to be a more generalized response to diffuse groups of prey species. Predators may improve their visual, auditory, or olfactory ability in order to better locate and detect multiple types of prey, or predators may increase their

running speed in order to better capture a variety prey, but specific counter-adaptations to the evolved defenses by one species of prey appear rare (Edmunds, 1974; Curio, 1976).

Dawkins and Krebs (1979) proposed the “Life-Dinner Principle” as a potential explanation for the lack of congruence between the arms race analogy and what one observes in natural populations. The explanation focuses on the role of selection during antagonistic interactions between predators and their prey. The Life-Dinner Principle suggests that predators exert strong selection on prey because individuals are at risk of losing their lives during an interaction. Strong selection on heritable variation for defensive traits increases the prey’s evolutionary rate of evolving anti-predator adaptations or modifying existing ones.

Conversely, prey species are thought to exert only weak selection on their predators because predators are rarely at risk of losing their lives during an interaction with their prey. Weak selection on predators would have little impact on heritable variation in their offensive traits, explaining the dearth of examples of prey-induced modifications of a predator’s morphology, behavior, or physiology (Dawkins and Krebs, 1979).

There is, however, an exception to the asymmetric selection inherent in the Life-Dinner principle – predators occasionally must deal with dangerous prey. Prey that impose “severe and predictable consequences” on their predators during an interaction may level the “evolutionary” playing field by increasing selection pressure on the predator, which could then lead to reciprocal selection (Brodie and Brodie, 1999a). The possibility that prey can impose selection on their predators is suggested, for example, in the interaction between the bivalve *Mercenaria mercenaria* and the predatory whelk *Sinistrofulgur sinistrum* (Dietl 2003). The whelk uses the edges of its shell to pry open the bivalve, but can suffer damage to its own shell while doing so, especially when attacking larger-sized bivalves; the cost of

having a chipped shell to the whelk is speculative, but may include increased mortality from its own predators as the whelk's operculum would no longer protect that part of its body lying exposed beneath its broken shell (Dietl 2003).

Vermeij (1982) proposed that the type of predator-prey system most likely to demonstrate reciprocal selection and linked evolutionary responses would be one in which the predator interacts with a prey species that itself relies upon potentially lethal chemical defenses. Chemical anti-predator defenses that have the potential to produce mortality and strongly affect predator fitness could certainly be considered as imposing severe and predictable consequences. Predators subjected to intense selection by chemically mediated defenses may respond in turn by evolving counter mechanisms to neutralize or degrade such chemicals. The mongoose (*Herpestes* spp.), for example, a known snake predator, possesses mutations in its nicotinic acetylcholine receptor (AChR) that decrease binding of the alpha-bungarotoxin (alpha-BTX) found in the venoms of many cobras (*Naja* spp.) and kraits (*Bungarus* spp.), rendering the mongoose resistant to the defensive strikes it elicits when attacking these serpents (Barchan et al., 1995; Asher et al., 1998; Fuchs, 1997). While the mongoose-cobra system is an excellent example of a predator responding to chemically mediated selection by its prey, this specific predator-prey relationship has never been evaluated from a coevolutionary or arms race perspective.

An excellent example of a chemically mediated predator-prey system that has been evaluated from a coevolutionary perspective involves a series of investigations into the relationship between the tetrodotoxin producing newt, *Taricha granulosa*, and its resistant predator the garter snake, *Thamnophis sirtalis* (Brodie and Brodie, 1990, 1991, 1999a, 1999b; Hanifin et al., 1999; Motychak et al., 1999; Brodie et al., 2002; Geffeney et al.,

2002). The Brodies and their colleagues have demonstrated geographic patterns of variability in tetrodotoxin production by the newt and toxin resistance by the garter snake, which indicate that the newt and snake have increased their defensive and counter-defensive strategies, respectively, in direct response to their opponent's escalation (Brodie and Brodie, 1990; Brodie and Brodie, 1991; Hanifin et al., 1999; Motychak et al., 1999; Brodie et al., 2002).

The newt-garter snake system is, to date, the sole well-documented example of reciprocal coevolution between a predator and its prey. The Brodies (1990, 1999) have challenged other investigators using the "arms race" analogy when studying predation to focus on traits at the "interface" of the interaction and evaluate whether predators "can and do" respond evolutionarily to increased anti-predator defenses in their prey. The purpose of this investigation is to accept that challenge. In this study, I investigate the predator-prey relationships between populations of grasshopper mice (*Onychomys* spp.) with their highly toxic prey, bark and striped scorpions (*Centruroides* spp.), in order to test the hypothesis that interaction between a predator and a chemically defended prey will result in reciprocal selection and linked evolutionary responses. Specifically, I evaluate whether bark and striped scorpions and grasshopper mice have increased their defenses (toxicity) and counter defenses (resistance) in response to each other by examining geographic patterns of expression in venom toxicity and venom resistance in both sympatric and allopatric populations.

STUDY SYSTEM

The relationship between grasshopper mice and the bark/striped scorpion group provides an excellent opportunity to test hypotheses on predator-prey coevolution using an

arms race perspective. This system involves chemically mediated interaction between the predator and prey, with the potential for strong reciprocal selection. The genus *Centruroides* belongs to the family Buthidae, comprising scorpion species well known for producing neurotoxins (Fet and Lowe, 2000; Loret and Hammock, 2001). *Centruroides* is a New World genus composed of 46 species and 22 subspecies distributed throughout the Americas and the Caribbean; *Centruroides*' center of distribution lies in Mexico and Central America (Fet and Lowe, 2000). Members of the genus *Centruroides* produce venom that contains neurotoxins specific to the ion channels of vertebrates, as well as some that are specific to invertebrates (Possani et al., 2000). Vertebrate-specific neurotoxins pose a threat to humans and have been the impetus for much of the research into the biochemical nature of scorpion toxins (Loret and Hammock, 2001). The majority of neurotoxins identified in *Centruroides* that are specific to vertebrates target the sodium (Na^+) ion-channel (Possani et al., 2000). Additional neurotoxins have been identified that target the (K^+) ion-channel (Corona et al., 2002). Neurotoxins that target the Na^+ ion-channel produce lethal effects in mammals, while K^+ ion-channel blockers are associated with paralysis and severe pain in mammals (Simard et al., 1992). Approximately seven species of *Centruroides* produce neurotoxins that selectively target the Na^+ ion-channels of vertebrates and have the potential to produce lethal effects in humans and other mammals (Possani et al., 1981; Martin et al., 1987; Becerril et al., 1993; Dehesa-Davila et al., 1996; Pintar et al., 1999; Padilla et al., 2003). These seven species occur, for the most part, as contiguous populations that are distributed from southern Mexico northward to southern Utah.

This study examines two species of *Centruroides* that differ in venom toxicity and geographic distribution, but are similar in that each has a close ecological association with a

distinct species of grasshopper mouse. The bark scorpion (*Centruroides 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 (including several islands), and eastward into the westernmost region of New Mexico (Fet and Lowe, 2000). The distribution of bark scorpions is broadly coincident with the distribution of southern grasshopper mice (*Onychomys torridus*) throughout much of their range in the Sonoran, Sinaloan, and Mojave Deserts of the U.S. and Mexico (Riddle and Honeycut, 1990; Riddle, 1995). Approximately 22 neurotoxins (a combination of 11 structurally unique toxins and their isoforms) have been identified in the venom of bark scorpions that target the Na⁺ channels of vertebrates (Simard et al., 1992; Corona et al., 2001; SCORPION, molecular database of toxins). Bark scorpions are included in the group of *Centruroides* species capable of producing lethal effects in mammals (Stahnke, 1971; Simard et al., 1992). Curiously, bark scorpions exhibit variability in venom components and toxicity throughout their range in the U.S. and Mexico (Simard et al., 1992; Josselyn, 1997; Dean Watt, personal communication; Lourival Possani, personal communication). For example, the median lethal dose (LD₅₀) reported for various populations of bark scorpion in Arizona and Sonora, Mexico ranges from 0.67 – 1.5 µg/g venom protein in a non-resistant mammal model (*Mus* sp.) (Stahnke, 1971; Simard et al., 1992; Alejandro Alagon, unpublished data; Lourival Possani, unpublished data). While few published studies exist concerning the natural history of bark scorpions, unpublished data suggest this species feeds on other invertebrates and interacts with vertebrate species only as prey (Bigelow, unpublished data; Rowe, unpublished data). Thus, the production of vertebrate-specific neurotoxins as components of bark scorpion venom is most often attributed to evolution by natural selection

due to vertebrate predators, and the geographic variability in venom toxicity is attributed to differences in predator composition and density among populations of bark scorpion (Simard et al., 1992; Possani et al., 2000).

The common striped scorpion (*Centruroides vittatus*) is distributed from Mexico (Chihuahua, Coahuila, Nuevo Leon, Tamaulipas, Zacatecas) northward through Texas and Oklahoma, westward through New Mexico to the Rio Grande, and eastward through Louisiana, Arkansas, and Mississippi (Fet and Lowe, 2000). The distribution of striped scorpions overlaps with the distribution of Mearns' grasshopper mice (*Onychomys arenicola*) throughout most of their range in the Chihuahuan Desert of the U.S. and Mexico (Riddle and Honeycutt, 1990; Riddle, 1995). The venom of striped scorpions has not been well characterized at the biochemical or physiological level because this species does not pose a health risk to humans (Stahnke, 1971). However, high performance liquid chromatography (HPLC) and toxicity tests reveal that striped scorpions produce both Na⁺ and K⁺ ion-channel neurotoxins that target vertebrate species. While striped scorpions do not pose a health risk to humans, they are potentially lethal to small mammals (Lourival Possani, personal communication). As with bark scorpions, few published studies exist documenting the natural history of striped scorpions with respect to their predators or their prey. However, what is known about the diet of striped scorpions indicates they consume only invertebrates, using their venom against vertebrate species as an anti-predator defense strategy (O'Connell and Formanowicz, 1998; Rowe and Rowe, unpublished data).

The genus *Onychomys* comprises three species of carnivorous mice; *O. torridus*, *O. arenicola*, and *O. leucogaster*. Grasshopper mice 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, the southern grasshopper mouse (*O. torridus*) routinely eats scorpions (Horner et al., 1965; Cyr, 1972; Langley, 1981), and was historically referred to as the scorpion mouse (Bailey and Sperry, 1929). Indeed, results from my 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 lethal bark and striped scorpions as effectively as they attack and subdue crickets and non-lethal species of scorpions (Chapter Two). The manner in which *O. torridus* and *O. arenicola* disabled and then consumed bark and striped scorpions suggest these mice may routinely encounter and prey on *Centruroides* in communities where they coexist. During feeding trials, the mice were usually unable to evade stings by bark and striped 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 physiological effects associated with *Centruroides*' venom. Moreover, initial toxicity tests reveal that populations of grasshopper mice, which coexist with bark and striped scorpions, are physiologically resistant to the venom of bark scorpions (Rowe, unpublished data).

Finally, both grasshopper mice and bark and striped scorpions are arid adapted species, which not only share similar distributions, but also share similar biogeographical histories in the Sonoran and Chihuahuan Deserts of the southwestern U.S. and Mexico

(Bigelow, unpublished data; Rowe and Rowe, unpublished data; Hinesley, 1979; Williams, 1980; Sullivan et al., 1986; Riddle and Honeycutt, 1990; Riddle, 1995; Shelley and Sissom, 1995; Fet and Lowe, 2000). Fossil records of both *Centruroides* and *Onychomys* indicate these groups may have had a close ecological association on an evolutionary timescale (approximately 6-3.6 MYA) sufficient to generate reciprocal adaptations (Baskin, 1979; Carleton and Eshelman, 1979; Sullivan et al., 1995; Fet and Lowe, 2000; Gantenbein et al., 2001).

METHODS

The Institutional Animal Care and Use Committee approved the vertebrate animal care and use protocols for the procedures described below. Vertebrate species were housed in USDA regulated animal facilities and cared for according to USDA guidelines. The appropriate permits were obtained for the collection and transport of grasshopper mice.

Description of Biological Assay:

I used a biological assay to measure venom toxicity in populations of bark and striped scorpion, and to measure venom resistance in populations of grasshopper mice. The Up-and-Down Procedure (UDP) was used to estimate acute toxicity levels associated with venom toxicity and venom resistance (NIEHS, 2001; USEPA, 2002). The UDP provides an estimate of the median lethal dose (LD₅₀) for the test substance (i.e., toxin) in an animal model. I chose the UDP to minimize the number of animals required to estimate median lethal doses. Briefly, an initial dose is selected based on information about the LD₅₀ for the particular toxin being investigated. Single animals are tested in a sequential order. The first test animal is administered a dose of toxin just below the estimated LD₅₀. If the first test animal

survives, the next subject receives an increased dose of toxin. If, however, the first animal dies, the following animal receives a decreased dose of toxin. The dose progression factor is determined by the antilog of the reciprocal of the estimated slope of the dose-response curve for the toxin being tested. The pattern of dose decision is continued for the remaining test animals. Each test animal is injected with one dose of venom and animals are not tested more than once. The time interval between tests depends on the duration of toxic effects in test animals and the outcome of the test with respect to survival or mortality. A test is not considered complete until the test animal either dies or recovers from the effects of the toxin. Testing is continued until one of the following criteria are met: 1) three consecutive animals survive at a pre-determined dose designating an upper bound limit; 2) five reversals occur in survival and mortality in a minimum of six animals tested. The outcome of each test is recorded as either a survival or a death. The dose for each test animal in a group, the outcome for each test, and the testing order are used to estimate the LD₅₀ and confidence intervals for each group of test animals (i.e., an LD₅₀ is a population level estimate). Median lethal doses are estimated using the maximum likelihood method, and confidence intervals are estimated using the profile-likelihood-based procedure. The calculations are performed using the software program AOT₄₂₅StatPgm provided by the USEPA (2002).

Venom Resistance Analyses: grasshopper mice

Intraspecific patterns in venom resistance - To evaluate patterns of neurotoxin resistance in southern grasshopper mice (*O. torridus*), I estimated resistance in populations of mice both sympatric with and allopatric to bark scorpions (*C. exilicauda*). Southern grasshopper mice were collected from two study sites in Arizona (AZ) and three study sites in Nevada (NV) (Fig. 1). The first study site was located at the Santa Rita Experimental

Range, in the Santa Rita Mountains of Pima County, in south-central AZ. Southern grasshopper mice are syntopic (i.e., biotic sympatry) with dense populations of bark scorpion in the Santa Rita Mountains. The second study site was located in the Chiricahua Mountains of Cochise County, in southeastern AZ. Southern grasshopper mice do not exist syntopically with bark scorpions in the Chiricahua Mountains. Instead, mice coexist with several species of non-toxic (i.e., non-lethal to vertebrate species) scorpions belonging to the genus *Vaejovis*. However, dense populations of bark scorpion are located approximately 15 miles to the east in the Peloncillo Mountains of southwestern New Mexico (NM). Thus, southern grasshopper mice from the Chiricahua Mountains might be considered parapatric with populations of bark scorpion to the east (or to occur in the nonoverlapping zone of a partially sympatric distribution, *sensu* Fig. 4B in Key 1981). Study sites in Nevada were located near the Carson River in Lyon County, and in the mountains surrounding the towns of Tonopah and Beatty, both in Nye County. The distribution of bark scorpions does not extend into Nevada beyond this state's border with the Colorado River. Thus, populations of southern grasshopper mice from the northwestern Nevada counties of Lyon and Nye are allopatric to *Centruroides*. Southern grasshopper mice from Lyon and Nye Counties are, however, sympatric with several species of non-toxic scorpion, including members of the genus *Vaejovis*.

Sixteen southern grasshopper mice were collected from the Santa Rita Mountains (SR) in July 2000, and nine specimens were collected from the Chiricahua Mountains (CR) in early August of that same year. From late July through early August 2001, six southern grasshopper mice were collected from the study site in Lyon County and a total of seven specimens were collected from the two study sites in Nye County. Grasshopper mice from the three study sites in Lyon and Nye Counties were pooled into one population (NV) to

increase sample size. I felt this was justified because all three sites represent populations of mice allopatric to *Centruroides*. 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. Grasshopper mice were maintained in standard mouse cages with bedding and water bottles, and provided commercial mouse chow as a food source. Grasshopper mice were transported back to NC to establish a breeding colony, in order to produce enough mice for resistance assays.

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*. Although the initial sample sizes were relatively small for establishing a breeding colony, several of the females from all three populations (SR, CR, NV) were already pregnant when they were collected. These pregnant females gave birth en route to NC, immediately increasing the sample sizes. I chose males and females from within populations to form breeding pairs and sister-brother pairs were avoided.

Interspecific patterns in venom resistance - I compared patterns of neurotoxin resistance in southern grasshopper mice with the two other species of *Onychomys*. I estimated resistance in a population of Mearns' grasshopper mice (*O. arenicola*), which are broadly sympatric with the common striped scorpion (*C. vittatus*), and in a population of northern grasshopper mice (*O. leucogaster*), which are broadly allopatric to all *Centruroides* species in the majority of its range. Mearns' grasshopper mice were collected from a study site located in the Organ Mountains of Doña Ana County, in southern NM (Fig. 1). Mearns' grasshopper mice are syntopic with dense populations of the striped scorpion in the Organ Mountains. Northern grasshopper mice were collected from a sand-sage prairie community

located in Finney County, in southwestern Kansas, where mice are not only allopatric to *Centruroides*, but also to all other species of scorpion (Fig. 1).

Eight Mearns' grasshopper mice were collected from the Organ Mountains (OR) in June 2000. These grasshopper mice were collected and transported to NC in the same manner as previously described. Mice were paired and bred to increase the sample size of the OR population. Thirteen northern grasshopper mice were collected from the sand-sage prairie in Kansas (KS) in July 2003. Rather than returning these grasshopper mice to NC, resistance analyses were conducted at the study site. Northern grasshopper mice that survived the resistance analyses were released at their trap site.

Procedure - I used the UDP to evaluate the resistance level (i.e., LD₅₀) of each population of grasshopper mice to the venom of bark scorpions (*C. exilicauda*). I chose bark scorpion venom for these analyses because it is more potent than striped scorpion venom and because the median lethal dose has been determined and reported for a non-resistant mammal model. The median lethal dose range for bark scorpion venom from central Arizona is reported as 1.0-1.5µg of venom protein per gram of *Mus* sp. (Simard et al., 1992). The sample of venom used for these analyses was obtained by electrical stimulation of bark scorpions that were collected in Mesa, AZ. The lyophilized sample of venom was re-hydrated in physiological saline (0.9% NaCl) and maintained at 4° C for approximately 30 minutes prior to administration to grasshopper mice. Each dose of venom was adjusted to the test animal's weight, and the samples were injected into the intraperitoneal cavity. Grasshopper mice were observed for signs of the physiological effects of the venom including increased salivation, repetitive head movements, seizures, and paralysis. Visible

effects of the venom and the outcome of the test in terms of survival or mortality were recorded.

Results of an initial toxicity test previously conducted (Rowe, unpublished data) indicated that grasshopper mice from the SR, CR, and OR populations were resistant to a 3.43 μ g/g dose of bark scorpion venom. I determined the starting dose for the acute toxicity tests in this study by multiplying the last known test dose (3.43 μ g/g) by a dose interval progression factor of 1.3. Thus, the starting dose was 4.46 μ g/g, and the dose progression factor of 1.3 was maintained throughout the entire assay. The dose progression factor of 1.3 was selected based on information about the dose-response curve for bark scorpion venom (Lourival Possani, personal communication). Because the extent to which grasshopper mice would exhibit resistance to bark scorpion venom was unknown, the LD₅₀ was estimated first for a single population (NV), before testing the remaining populations. Once the LD₅₀ was estimated for the NV population, the starting dose for the remaining populations was adjusted upwards, thus saving venom and mice. The median lethal dose for each population was estimated using the following sample sizes, gender, and average weights of grasshopper mice: 1) NV, n = 11, mixed gender, avg.wt. = 16.76g; 2) SR, n = 11, all female, avg.wt. = 28.3g; 3) OR, n = 13, mixed gender, avg.wt. = 25.3g; 4) CR, n = 11, mixed gender, avg.wt. = 29.6g; 5) KS, n = 9, mixed gender, avg.wt. = 39.9g. The LD₅₀ for the SR, OR, and CR populations was estimated using grasshopper mice born in captivity. The LD₅₀ for the NV population was estimated using the grasshopper mice originally collected from study sites in Lyon and Nye Counties, and the LD₅₀ for the KS population was estimated from grasshopper mice originally collected from Finney County.

Venom Toxicity Analyses: bark and striped scorpions

Intraspecific patterns in venom toxicity - To evaluate patterns of venom toxicity in bark scorpions (*C. exilicauda*), I estimated toxicity in populations of scorpion both sympatric with and allopatric to southern grasshopper mice (*O. torridus*). Bark scorpions were collected from three study sites in Arizona and one study site in New Mexico (Fig. 1). The first study site was located at the Santa Rita Experimental Range (SR) in south-central AZ, where bark scorpions are syntopic with southern grasshopper mice. The second syntopic study site was located in northern AZ, near the Virgin River Gorge in the Beaver Dam Mountains Wilderness Area (BD). Although the SR and BD study sites are located at the extreme ends of AZ, the habitats are similar, and both regions support dense populations of bark scorpions that co-occur with southern grasshopper mice. The third study site in AZ was located in the north-central region, near Glen Canyon (GC). Dense populations of bark scorpion exist in the rocky hillsides surrounding the canyon. While southern grasshopper mice occur in the very northwestern corner of AZ (i.e., the BD population), their distribution does not extend into the north-central or northeastern portion of AZ. Thus, bark scorpions in Glen Canyon represent a population allopatric to southern grasshopper mice. The fourth study site was located in the Peloncillo Mountains (PL) of southwestern NM, where bark scorpions and southern grasshopper mice are syntopic. The Peloncillo Mountains represent the eastern boundary delineating the distributions of both bark scorpions and southern grasshopper mice.

A total of 1,200 adult bark scorpions (approximately 400 scorpions per site) were collected from the SR (avg.wt. = 0.60g), BD (avg.wt. = 0.77g), and GC (avg.wt. = 0.76g) study sites during June and July 2002. Approximately 150 adult bark scorpions were

collected from the PL (avg.wt. = 0.38g) study site in early August 2003. Bark scorpions were usually collected between the hours of 9:00 pm and 12:00 am, using UV illumination. The scorpions were transported to NC in large freezer bags filled with paper towels for substrate; water was lightly sprinkled over several of the towels to provide a source of moisture for the scorpions during transit. Upon arrival in NC, scorpions were transferred to large, ventilated plastic containers. The plastic containers were filled with a layer of aquarium gravel, and egg crates were added to provide refuge. Water was provided *ad libitum*, and crickets were offered to the scorpions twice a month. Scorpions were maintained in a laboratory with large windows, exposing them to a natural light cycle. The temperature in the laboratory was maintained between 22-23• C.

Interspecific patterns in venom toxicity - I compared patterns of venom toxicity in bark scorpions with one additional species of *Centruroides*, a population of the common striped scorpion (*C. vittatus*) syntopic with Mearns' grasshopper mouse (*O. arenicola*). As previously noted, venom produced by the striped scorpion has not been well characterized in terms of venom components and toxicity level because it does not pose a health risk to humans. Striped scorpions were collected from the Organ Mountains (OR) in NM, where they coexist with Mearns' grasshopper mice (Fig. 1). Approximately 150 striped scorpions were collected from the OR site (avg.wt. = 0.51g) in early August 2003. Scorpions were transported to NC and housed as previously described.

Procedure - Individual scorpions were rinsed in distilled water to clean their telsons (the last tail segment, containing the venom gland). Venom was extracted from the venom gland of each scorpion using electrical stimulation. Venom samples were pooled by population and deposited into sterile petri dishes. Venom was extracted twice from each

population, and the two samples were combined. Venom samples were purified using water-extraction and high-speed centrifugation. The supernatant containing the venom was aliquoted into sterile microcentrifuge tubes and lyophilized. Freezed-dried venom samples were stored at -20° C.

The UDP was used to estimate the LD₅₀ (degree of toxicity) for a sample of venom from each population of bark (*C. exilicauda*) and striped scorpion (*C. vittatus*). The median lethal dose range for bark scorpion venom from central Arizona is reported as 1.0-1.5 µg of venom protein per gram of *Mus* sp. (Simard et al., 1992). The median lethal dose has not been previously reported for striped scorpion venom from New Mexico. However, the estimated LD₅₀ for striped scorpions from Chihuahua, Mexico, is reported as 7.0 µg/g (Lourival Possani, personal communication). A strain of *Mus* sp. (CD-1, female, 20-22 grams) was used as the non-resistant animal model to estimate the LD₅₀ for each population of scorpion. I selected a starting dose below the reported LD₅₀ value for each species of scorpion. In order to detect small differences in the expression of toxicity among populations of scorpion, I chose a dose progression interval of 0.10 µg/g, which increased the sensitivity of the test. Lyophilized venom samples were re-hydrated in physiological saline (0.9% NaCl) and maintained at 4° C for approximately 30 minutes prior to administering the samples to test animals. Groups of approximately ten mice were required to estimate the LD₅₀ for each sample of venom. Mice were weighed prior to the assay, and venom dose concentrations were adjusted to individual mouse weights. A constant volume of venom (0.4 mls) was injected into the intraperitoneal cavity of each mouse in the assay. Visible signs of the physiological effects of the venom were recorded. The outcome of each test was reported as either a survival or a death, and the time to recovery or death was noted. The LD₅₀ and

confidence intervals for each population of scorpion was calculated using the software package AOT₄₂₅Stat.Pgm. (USEPA, 2002).

RESULTS

Venom Resistance Analyses: grasshopper mice

Intraspecific patterns - The results of the toxicity tests indicate that while all three test populations (SR, CR, NV) of southern grasshopper mice demonstrate physiological resistance to bark scorpion venom, differences exist among populations in the degree of resistance expressed. Venom resistance for each grasshopper mouse population is expressed as the dose of bark scorpion venom that produced mortality in 50% of the grasshopper mice tested. Differences among median lethal dose values are considered significant if the confidence intervals for different populations do not overlap. The SR population of grasshopper mice, which is syntopic with an abundant population of bark scorpions (*C. exilicauda*, the more toxic of the two *Centruroides* species used in this study), expressed the highest level of resistance in the assays (SR: LD₅₀ = 18.38µg/g; 95% C.I. = 15.2-19.8; Table 1; Fig. 2). The CR population of grasshopper mice, parapatric to a population of bark scorpions in the Peloncillo Mountains, expressed a level of resistance less than the syntopic SR population (CR: LD₅₀ = 12.49µg/g; 95% C.I. = NA; Table 1; Fig. 2). Unfortunately, it was not possible to assess the significance of the difference between the SR and CR LD₅₀'s, as individual measures of toxicity within the CR population were too variable to generate a confidence interval using AOT₄₂₅Stat.Pgm. (USEPA, 2002). The NV population of southern grasshopper mice, allopatric to all *Centruroides*, expressed a level of resistance significantly lower than the syntopic SR population, and less than the parapatric CR population (NV: LD₅₀ = 10.27µg/g; 95% C.I. = 9.01-11.7; Table 1; Fig. 2).

Interspecific patterns - Toxicity tests reveal that all three species of grasshopper mice demonstrate a level of physiological resistance to bark scorpion neurotoxins greater than that reported for non-resistant *Mus* sp. However, differences exist in the level of resistance expressed among the three species. Populations of the southern grasshopper mice (*O. torridus*) both sympatric with and allopatric to the highly toxic bark scorpion demonstrated the highest levels of resistance (Table 1; Fig. 2). The population of Mearns' grasshopper mice (*O. arenicola*), which are syntopic with striped scorpions (*C. vittatus*, the less toxic of the two *Centruroides* species employed in this study), expressed a level of resistance less than that of all three populations of southern grasshopper mice I tested, and significantly so when compared to the LD₅₀ for SR mice (OR: LD₅₀ = 8.60µg/g; 95% C.I. = 7.54-9.80; Table 1; Fig. 2). The population of northern grasshopper mice (*O. leucogaster*), allopatric to all *Centruroides* species, exhibited a level of resistance significantly less than either the SR or NV populations of southern grasshopper mice, as well as being significantly less than the resistance shown by my one population (OR) of Mearns' grasshopper mice (KS: LD₅₀ = 3.91µg/g; 95% C.I. = 3.43-4.46; Table 1; Fig. 2).

Venom Toxicity Analyses: bark and striped scorpions

Intraspecific patterns - Venom assays reveal that toxicity levels among populations of bark scorpion (*C. exilicauda*) sympatric with southern grasshopper mice (*O. torridus*) are all relatively similar; however, a significant difference exists in venom toxicity levels when comparing populations of *C. exilicauda* sympatric with vs. allopatric to southern grasshopper mice (Table 2; Fig. 3). Regardless of geographic location, for example, the toxicity levels for all three populations of bark scorpions syntopic with southern grasshopper mice were highly similar (SR: LD₅₀ = 0.8µg/g; 95% C.I. = 0.73-0.85; BD: LD₅₀ = 0.8µg/g;

95% C.I. = 0.75-0.87; PL: LD₅₀ = 0.75µg/g; 95% C.I. = 0.70-0.80; Table 2; Fig. 3). The lone population of bark scorpion I tested that is allopatric to *O. torridus*, in contrast, was significantly less toxic than each of these three syntopic populations (GC: LD₅₀ = 1.0µg/g; 95% C.I. = 0.97-1.10; Table 2; Fig. 3).

Interspecific patterns - Results of the venom assays demonstrate that both bark and striped scorpions are potentially lethal to small mammals with, however, *C. exilicauda* being significantly more toxic than its congener *C. vittatus*. As reported above, the median lethal doses estimated for populations of bark scorpion in this study ranged from 0.75-1.00µg/g, significantly lower than the median LD₅₀'s for striped scorpions syntopic with Mearns' grasshopper mouse (OR: LD₅₀ = 7.7µg/g; 95% C.I. = 7.0-8.0; Table 2; Fig. 3).

DISCUSSION

Venom Resistance in *Onychomys*:

Interspecific patterns - Is there any evidence that grasshopper mice have responded evolutionarily to the potentially lethal neurotoxins produced by bark and striped scorpions? Results from the toxicity assays suggest that grasshopper mice have evolved specific counter defenses in response to the potent venoms of these scorpions. Strong support for this conclusion is provided by the geographic patterns of expression in venom resistance among species of grasshopper mice from regions broadly sympatric with or allopatric to *Centruroides*. The pattern demonstrates that species of grasshopper mice whose distribution overlaps with that of either bark or striped scorpions are more resistant than a species of mouse that does not co-occur with these toxic scorpions. Populations of southern and Mearns' grasshopper mice, two species whose geographical distributions closely coincide with the distributions of *C. exilicauda* and *C. vittatus*, respectively, exhibited levels of venom

resistance significantly greater than that of a population of northern grasshopper mice, a species whose extensive range in the north-central U.S. is broadly, though not exclusively, allopatric to *Centruroides*. Specifically, the LD₅₀'s for southern grasshopper mice ranged from ~10.3 µg to 18.4 µg of scorpion venom per gram of mouse for the three populations of *O. torridus* I tested; for Mearns' grasshopper mouse, *O. arenicola*, the dose was ~8.6 µg/g; and for northern grasshopper mice, *O. leucogaster*, the concentration was only 3.9 µg/g. Thus, species of grasshopper mice that routinely interact with neurotoxic *Centruroides* appear to have evolved physiological defenses against these toxins, a result consistent with predictions from the arms race analogy.

Can the arms race analogy account for the fact that northern grasshopper mice, at least those from the KS population I tested, maintain some resistance to *Centruroides* venom? After all, the median LD₅₀ for *O. leucogaster* to *Centruroides* venom was 3.9 µg/g, approximately three times the LD₅₀ reported for domestic mice, *Mus* sp., at 1.0-1.5 µg/g. 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 antislake behavior in California ground squirrels (*Spermophilus beecheyi*) no longer experiencing predation from rattlesnakes (*Crotalus* spp.) has been shown to require hundreds of thousands to millions of years (Poran et al., 1987; Towers and Coss, 1990; Coss, 1999). Molecular evidence suggests that all three species of *Onychomys* diverged from a common ancestor inhabiting the desert regions of the southwestern U.S. sometime between 2.5-3.5 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). I hope to explore this "signature" of vestigial venom resistance in *O.*

leucogaster by expanding, in future investigations, resistance analyses to include additional populations of the northern grasshopper mouse.

Comparisons of venom resistance in the two populations of *Onychomys* I tested that were syntopic with *Centruroides*, one each for *O. torridus* (SR) with *C. exilicauda* and *O. arenicola* (OR) with *C. vittatus*, provide additional support for the arms race model.

Reciprocal selection requires that the traits one observes in predators to 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. That is exactly what is seen when the venom resistance levels in SR mice (18.4 $\mu\text{g/g}$) and OR mice (8.6 $\mu\text{g/g}$) are compared with the corresponding toxicities of the venoms they must deal with; I will return to this point below.

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

where they are not. This is precisely what the arms race model predicts. In regions where 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 may 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 $\mu\text{g/g}$) than SR mice (18.4 $\mu\text{g/g}$) who probably interact nightly with *C. exilicauda*. Mice from the CR population, which do not currently overlap with bark scorpions, show intermediate levels of venom resistance (12.5 $\mu\text{g/g}$) coupled with extreme levels of inter-individual variability in resistance; these results suggest there may be gene flow between CR mice and grasshopper mice who, less than 15 miles east, *are* interacting with *C. exilicauda*.

Venom Toxicity in *Centruroides*:

If predators develop counter defenses in response to selection by prey, it follows from the arms race analogy that prey should reciprocate by strengthening or increasing their anti-predator defenses. My results, presented above, indeed suggest that grasshopper mice have developed venom resistance as a counter defense to potentially lethal neurotoxins produced by bark and striped scorpions. Is there any evidence to suggest the reciprocal; i.e., that bark and striped scorpions have increased their anti-predator defenses in response to the evolution of venom resistance in the mice? Here again, results are consistent with predictions generated from an arms race perspective. I will present the intraspecific patterns first.

Intraspecific patterns – If the toxicity of *Centruroides* venom is the product of selection imposed by resistant mice, it follows that bark scorpions from regions sympatric with grasshopper mice (and especially those from regions where the mice are most resistant) should possess more toxic venom than scorpions from regions lacking the mice (or where the level of resistance in the mice is negligible). This is consistent with geographic patterns of venom toxicity among different populations of *C. exilicauda*. Regardless of geographic location, populations of bark scorpion that coexist with southern grasshopper mice exhibit increased toxicity levels (as measured in lab mice) compared with a population of bark scorpion that does not coexist with *O. torridus*. The Santa Rita (SR), Beaver Dam (BD), and Peloncillo (PL) populations of bark scorpion, all of which are syntopic with southern grasshopper mice, demonstrated relatively similar venom toxicity values (SR = 0.8 µg/g; BD = 0.8 µg/g; PL = 0.75 µg/g). Moreover, each of these median LD₅₀'s is significantly lower (and, therefore, more potent) than the LD₅₀ of *C. exilicauda* from Glen Canyon (GC; 1.0µg/g), a location devoid of *O. torridus*.

Interspecific patterns - Analyses of venom toxicity levels between *C. vittatus* and *C. exilicauda* reveal that striped scorpions are significantly less toxic (median LD₅₀ = 7.7 µg/g) than bark scorpions (range of median LD₅₀'s for four populations = 0.75-1.00 µg/g). Here again, however, the interspecific patterns support reciprocal selection. A parsimonious explanation for why the OR population of *C. vittatus* is less toxic than the four populations of *C. exilicauda* is that OR scorpions are interacting with OR mice (*O. arenicola*), who themselves are only moderately resistant (8.6 µg/g) to *Centruroides* toxins. In regions where bark scorpions must deal with highly resistant *O. torridus* (as, for example, in the Santa Rita Mountains, where the SR mice's venom resistance is 18.4µg/g), a more potent venom (0.75-

1.00 µg/g) would be required. Thus, in zones of sympatry, venom toxicity in the scorpion and venom resistance in the mice covary and this is consistent with the arms race model.

Alternative strategies for scorpions? – Arms races, by their very nature, are dynamic. Bark scorpions evolve more potent venoms, grasshopper mice counter with increased resistance, leading to more toxic venoms, which selects for increased resistance..... The molecular machinery employed by scorpions in making their venoms appears pre-adapted, if not adapted, for generating the venom variability that might “keep them in the race”. Numerous studies have demonstrated that the plethora of toxins present in a typical scorpion’s venomous cocktail are the product of gene duplication events followed by the accumulation of mutations in protein coding regions (Froy et al., 1999; Possani et al., 1999; Gurevitz et al., 2001; Zhu and Li, 2002; Gordon and Gurevitz, 2003). To date, approximately eleven structural toxins and several of their isoforms have been isolated from bark scorpion (*C. exilicauda*) venom (Corona et al., 2001), suggesting that they too possess the genetic machinery to counter further increases in venom resistance by *Onychomys*. But is toxicity enough? Results from the bioassays presented here suggest that the grasshopper mice are equally competent at running the race. Indeed, coupled with results from my behavioral study (Chapter Two), one could suggest the mice are actually winning. In staged feeding trials with southern and Mearns’ grasshopper mice, bark and striped scorpions were almost invariably killed, while the mice showed no visible effects from the neurotoxins. Preliminary analyses (Rowe, unpublished data) point to modifications in the voltage-gated sodium channels of *Onychomys* neurons as a potential mechanism for their resistance to *Centruroides* neurotoxins. But the scorpions may have alternative tactics for dealing with resistant grasshopper mice. A recent study has shown that scorpion venoms contain

chemicals that cause, not only toxicosis, paralysis, or death, as is the case for *Centruroides*' neurotoxins, but also cause acute, temporary pain (Inceoglu et al. 2003). Evolutionarily, what options might there be for a scorpion whose predator appears capable of staying one step ahead of its toxins? One possibility is to increase the painfulness of a sting, which could encourage the predator to drop the scorpion, allowing it to scurry for cover as the predator temporarily grooms the site of the sting. This appears to be part of *Centruroides* strategy for dealing with *Onychomys*. Results from my feeding study show that grasshopper mice have a very difficult time handling *Centruroides*, frequently dropping these scorpions when stung and grooming the site of the sting. Future investigations will explore whether grasshopper mice nociceptors, like their sodium channels, have been under reciprocal selection from *Centruroides*.

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Table 1. Physiological resistance of grasshopper mouse populations to bark scorpion venom. Values are reported as the dose of bark scorpion venom that produces mortality in 50% of the grasshopper mouse population; i.e., median lethal dose (LD₅₀ µg/g). The median lethal dose previously reported for bark scorpion venom in a non-resistant mammal model (*Mus* sp.) is equivalent to 1.0-1.5 • g/g (Simard et al., 1992).

Study Site	Grasshopper Mouse Species	Overlap With Scorpions	Toxicity LD₅₀ (mg/g) (95% C.I.)
SR	<i>O. torridus</i> (southern grasshopper mouse)	Biotic Sympatry <i>C. exilicauda</i> (bark scorpion)	18.38 µg/g (15.2-19.8)
CR	<i>O. torridus</i> (southern grasshopper mouse)	Parapatric* <i>C. exilicauda</i> (bark scorpion)	12.49 µg/g (not assessed)
NV	<i>O. torridus</i> (southern grasshopper mouse)	Allopatric <i>Centruroides</i> (bark and striped)	10.27 µg/g (9.0-11.7)
OR	<i>O. arenicola</i> (Mearns' grasshopper mouse)	Biotic Sympatry <i>C. vittatus</i> (striped scorpion)	8.60 µg/g (7.5-9.8)
KS	<i>O. leucogaster</i> (northern grasshopper mouse)	Allopatric <i>Centruroides</i> (bark and striped)	3.91 µg/g (3.4-4.5)

*Or trapped in a region of allopatry from a population of *O. torridus* that, 15 miles away, is sympatric with *C. exilicauda*.

Table 2. Venom toxicity levels of bark and striped scorpion populations. Values are reported as the dose of bark or striped scorpion venom that produces mortality in 50% of a non-resistant *Mus* sp. population; i.e., median lethal dose (LD₅₀ µg/g). The median lethal dose previously reported for bark scorpion venom from central Arizona, as tested in a non-resistant mammal model (*Mus* sp.) is equivalent to 1.0-1.5 • g/g (Simard et al., 1992).

Study Site	Scorpion Species	Overlap With Grasshopper Mice	Toxicity LD₅₀ (mg/g) (95% C.I.)
SR	<i>C. exilicauda</i> (bark scorpion)	Biotic Sympatry <i>O. torridus</i> (southern grasshopper mouse)	0.80 µg/g (0.73-0.85)
BD	<i>C. exilicauda</i> (bark scorpion)	Biotic Sympatry <i>O. torridus</i> (southern grasshopper mouse)	0.80 µg/g (0.75-0.87)
PL	<i>C. exilicauda</i> (bark scorpion)	Biotic Sympatry <i>O. torridus</i> (southern grasshopper mouse)	0.75 µg/g (0.70-0.80)
GC	<i>C. exilicauda</i> (bark scorpion)	Allopatric <i>O. torridus</i> (southern grasshopper mouse)	1.0 µg/g (0.97-1.1)
OR	<i>C. vittatus</i> (striped scorpion)	Biotic Sympatry <i>O. arenicola</i> (Mearns' grasshopper mouse)	7.7 µg/g (7.0-8.0)

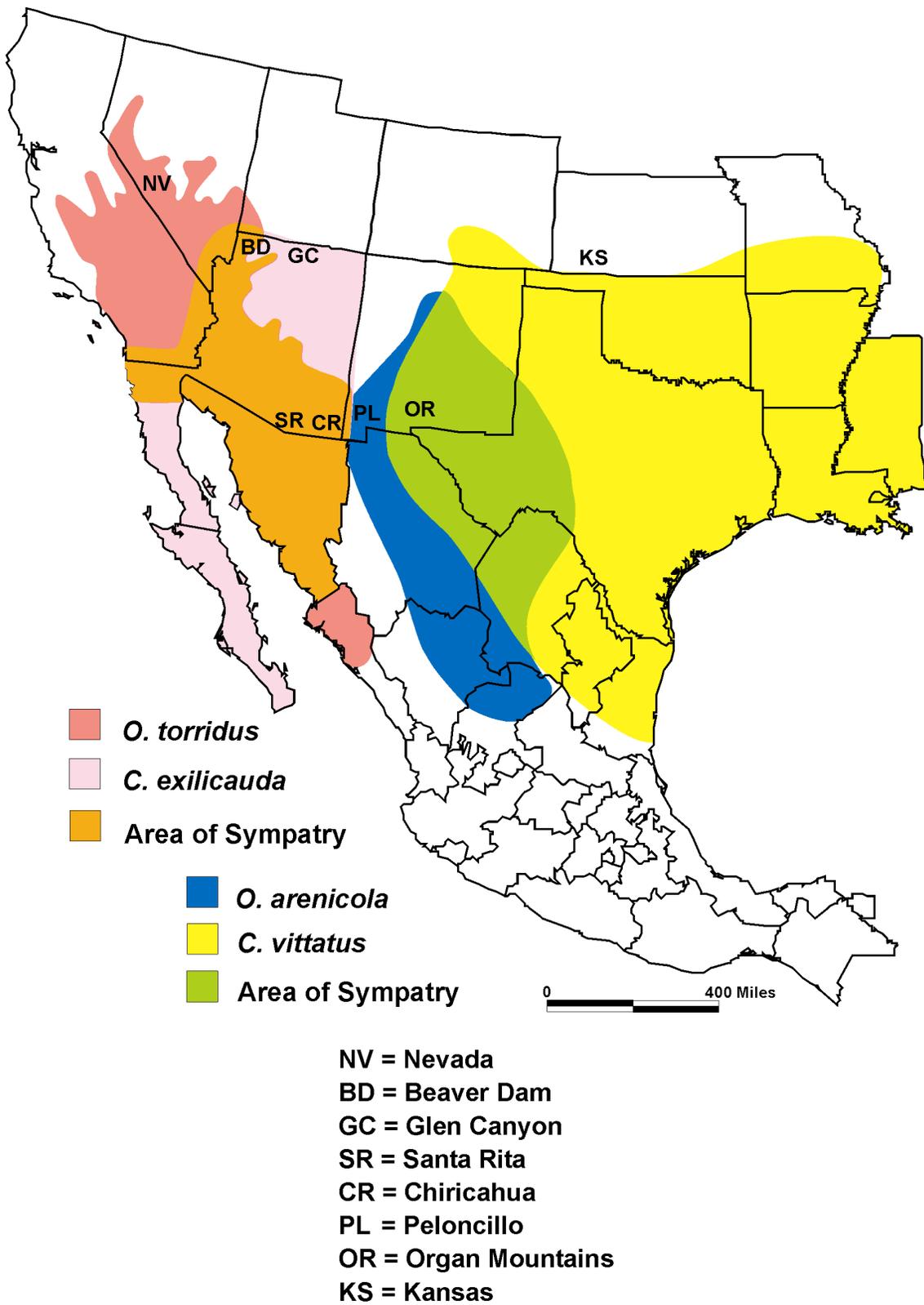
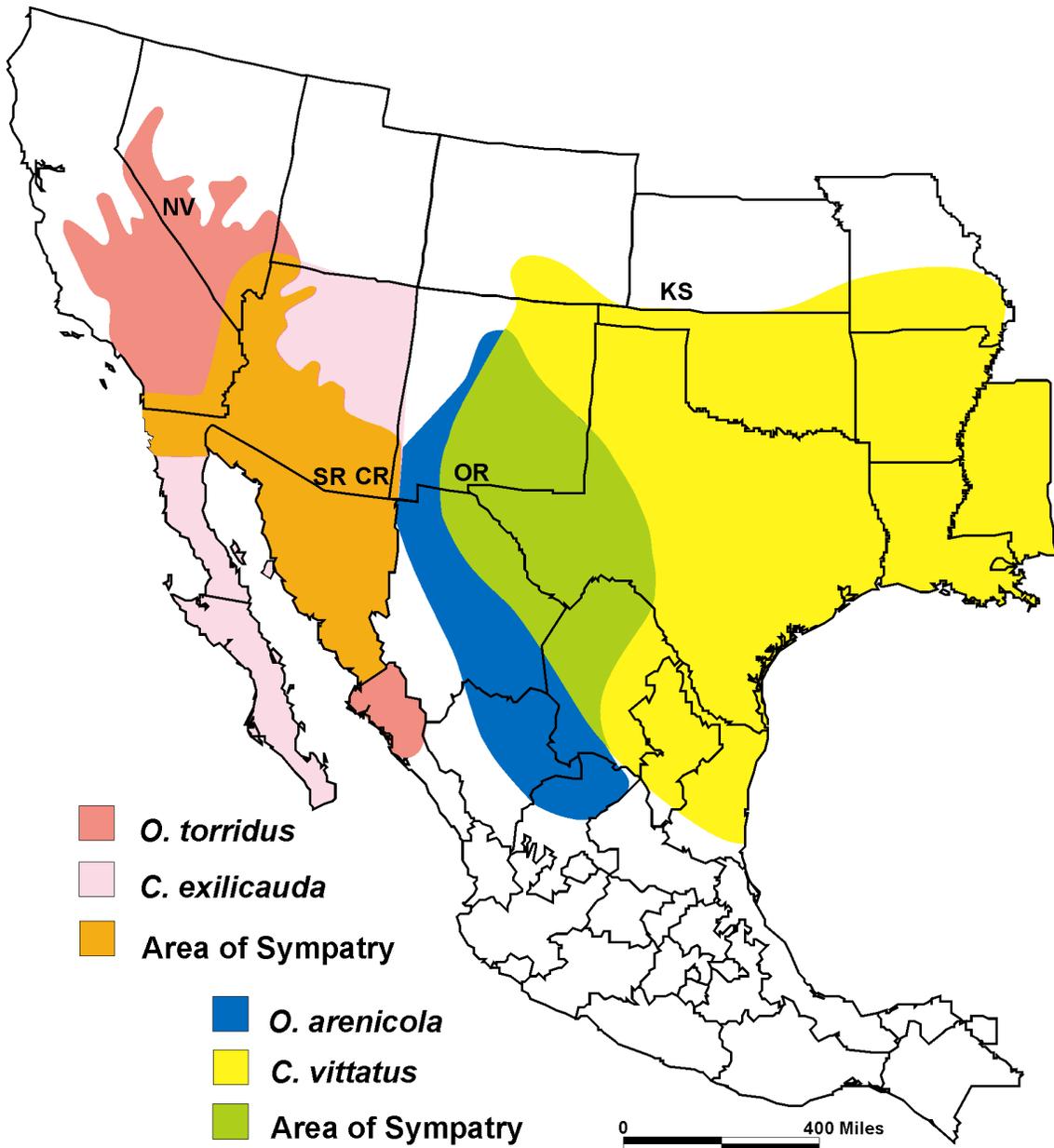


Figure 1. Species distribution and study sites.



SR: *O. torridus* LD₅₀ = 18.38 μg/g

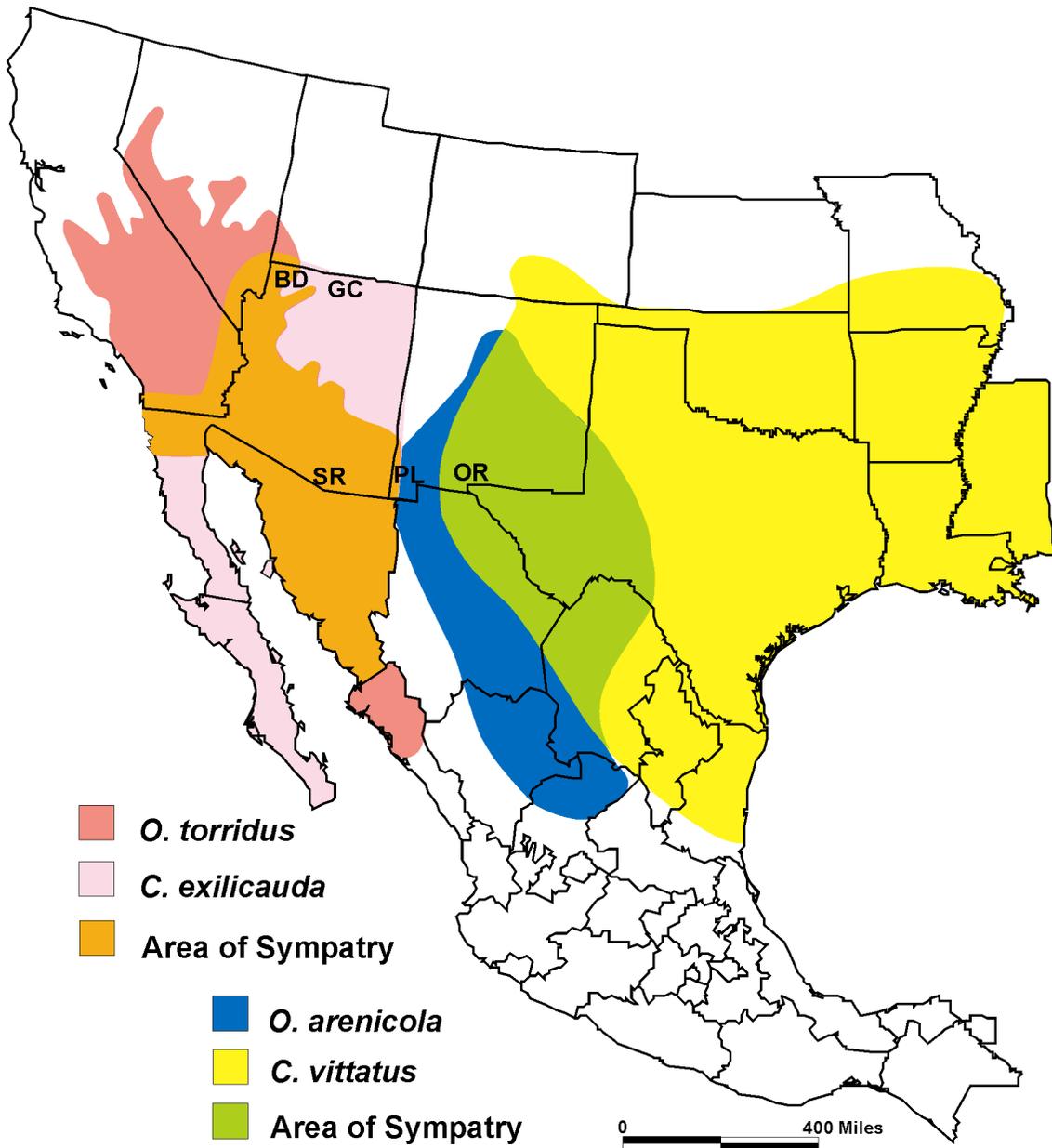
CR: *O. torridus* LD₅₀ = 12.49 μg/g

NV: *O. torridus* LD₅₀ = 10.27 μg/g

OR: *O. arenicola* LD₅₀ = 8.60 μg/g

KS: *O. leucogaster* LD₅₀ = 3.91 μg/g

Figure 2. Species distribution, study sites, and venom resistance of *Onychomys* spp.



SR: *C. exilicauda* LD₅₀ = 0.8 μg/g

BD: *C. exilicauda* LD₅₀ = 0.8 μg/g

PL: *C. exilicauda* LD₅₀ = 0.75 μg/g

GC: *C. exilicauda* LD₅₀ = 1.0 μg/g

OR: *C. exilicauda* LD₅₀ = 7.7 μg/g

Figure 3. Species distribution, study sites, and venom toxicity of *Centruroides* spp.