Interactions between Pigmy Rattlesnakes (*Sistrurus miliarius*) and a Suite of Prey Species: A Study of Prey Behavior and Variable Venom Toxicity

DISSERTATION

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Abstract

Interactions between predators and prey are widespread in nature but the ecological and evolutionary factors that shape these interactions are poorly understood. In my dissertation, I use pigmy rattlesnakes (*Sistrurus miliarius*) and their prey as a system in which to examine several aspects of this species interaction where different ecological and evolutionary factors may be shaping variation in adaptive traits. In Chapter 1, I review factors affecting predator-prey interactions and explain why the pigmy rattlesnake system is valuable for addressing important research questions. In Chapter 2, I present research on the behavioral component of this interaction, demonstrating that native cotton mice do not change their foraging behavior in the presence of a sit-and-wait rattlesnake predator. In Chapter 3, I explore the toxicity of venom to native prey versus non-native “models” to determine to what extent non-native species are representative of prey in the same broad taxonomic group. I show that native prey have higher resistance to venom than non-natives and encourage the use of native prey in future toxicity work. In Chapter 4, I use native treefrog prey from two different populations in Florida and venom from snakes in the same populations to see if there is a signal of local adaptation present in these populations. I show that detection of a signal of local adaptation depends on the measure of venom function used: evidence for local adaptation was observed in the time to death measure of mortality but not in the 24 hour mortality measure. In Chapter 5, I look at the function of venom at a smaller scale by exploring the amount of functional
variation present across and within populations of snakes using a lizard model prey. I found the individual component of venom toxicity to be larger than the population-level differences that have been the focus of previous research. Overall, this dissertation demonstrates that rattlesnake venom function differs at both the individual and population scale and that toxicity is relative, depending on the specific prey species tested.
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Chapter 1: Introduction

Predator-prey interactions are an important ecological relationship that result in significant evolutionary forces that shape organismal phenotypes. Predation involves several sequential steps including detection, capture, and handling of sympatric prey by a predator (Brodie and Brodie 1999). When predation is viewed as this multi-step process it shows how this sequence of events creates multiple points where natural selection can potentially influence variation in the traits of both predators and prey. For example, under high predation pressure, selection can lead to evasive prey behaviors to which predators may respond with altered hunting tactics (Miller and Surlykke 2001) or prey may evolve distasteful toxins to deter predation leading to co-evolution of toxin resistance in the predator (Hanifin et al. 2008).

Interactions between predatory rattlesnakes and their prey differ from typical predator-prey interactions in two significant ways. First, rattlesnakes predominately use a sit-and-wait style of foraging rather than actively pursuing their prey. This type of predation is expected to have high impacts on prey in the absence of consumption relative to other foraging modes but is also a poorly studied mode of predation (Preisser et al. 2007). Second, rattlesnakes use venom, a complex and protein-rich biological toxin, that allows them to immobilize prey (Zimmerman et al. 1990; Richards et al. 2012; Urdaneta et al.
2004; Torres-Bonilla et al. 2016) while minimizing the risk of physical harm to the predator during the capture process. These features are expected to lead to the evolution of defensive traits in prey and corresponding offensive traits in the predatory snakes, thus resulting in significant adaptations in both snakes and prey. My dissertation explores the presence and functional consequences of adaptive traits in a rattlesnake-prey system in the Southeastern United States using new conceptual perspectives and novel methods of functional analysis.

I studied interactions between pigmy rattlesnakes (*Sistrurus miliarius*) and their vertebrate prey in Florida. The pigmy rattlesnake lives in the southeastern US coastal plain, ranging from eastern Texas and Oklahoma east to southern North Carolina and south to the tip of the Florida mainland (Conant and Collins 1998). The biology of this species is arguably best understood in central Florida where the population has been the subject of long term study (May and Farrell 2012). In these populations, older snakes can reach lengths of 50 cm with body masses exceeding 100 grams (May and Farrell 2012). As a consequence of its small size, this snake has a broad diet of small prey from several taxonomic groups that it consumes. In particular, four broadly defined groups of animals (anurans, lizards, small mammals, and centipedes) each comprise at least 15% of the prey of the pigmy rattlesnake based on museum gut analyses (Gibbs and Mackessy 2009). Although snake diet fluctuates with the seasonal and location-specific abundance of prey, the venom of pigmy rattlesnakes must be able to subdue this diverse array of prey.
species. This broad diet permits study of functional aspects of pigmy rattlesnake venom across a range of naturally-encountered types of prey.

Like all venomous snakes, pigmy rattlesnakes subdue their prey using a protein-rich venom. Snake venom composition is highly variable across multiple taxonomic levels (Chippaux et al. 1991). The variation in venom is thought to be adaptive; venom-coding genes have been shown to be under positive selection relative to neutral markers in snakes (Rokyta et al. 2011) including the PLA₂ component of venom in *Sistrurus* species (Gibbs and Rossiter 2008). Molecular techniques allow venom to be separated into its component parts resulting in the determination of the relative amounts of specific proteins. In pigmy rattlesnakes, significant variation in venom has been documented even at small spatial scales (S.A. Smiley-Walters, unpublished data; D. Rokyta, personal communication). The selection pressures which drive venom variation in snakes like the pigmy rattler are presumably related to snake diet as evidenced by relationships between venom and diet composition (Daltry et al. 1996). However, the direct links between venom variation and whole-venom function in native prey that are needed to support this hypothesis are lacking.

In my dissertation research, I focus on using pigmy rattlesnakes (*Sistrurus miliarius*) and their prey to broaden our knowledge of the functional aspects of molecular and behavioral phenotypes involved in this predator-prey interaction. The overall objective of my research is to determine whether defensive behavioral and molecular traits of prey
and offensive traits of the predator (rattlesnake venom) show functional variation and to use this information to infer the evolutionary history of these traits. I use a multi-faceted approach to study this species interaction. I first use an experimental approach to study the behavior of a common prey species, the cotton mouse (*Peromyscus gossypinus*), in response to the presence of the snake. In Chapter 2, I describe a semi-natural experiment in which environmental complexity and snake presence were manipulated to determine if cotton mice alter their foraging behavior when a rattlesnake is nearby and if this response to the snake is context-dependent. I hypothesize that mice will be able to detect the presence of the rattlesnake predator and alter their foraging behavior in its presence by reducing seed consumption and/or changing the location of their foraging in response to the snake.

In Chapters 3 through 5 I examine different aspects of venom function in the native prey of pigmy rattlesnakes. I use venom lethal toxicity assays to test for venom function in this system by creating dose-response curves for each prey species followed by regression analyses and median lethal dose estimates. In Chapter 3, I look at venom function at a higher taxonomic level in the prey, comparing responses of native and non-native prey species to the venom in terms of toxicity. I test representatives from each of the major vertebrate prey groups (mammals, lizards, anurans) to compare the native and non-native species as well as to look at the influence of evolutionary history on venom susceptibility of the prey. In Chapter 4, I take a closer look at one possible mechanism driving intraspecific venom variation, namely local adaptation of snakes to be able to
better subdue sympatric prey. I evaluate the response of squirrel treefrogs (*Hyla squirella*) from two populations to venom from the same two populations by analyzing the venom-induced mortality at 24 hours and time to frog death responses in a local adaptation framework. The objective of this chapter is to evaluate the specific claim that prey have a major influence on venom evolution by investigating if snake venom has evolved to better kill sympatric prey. In Chapter 5, I look at the functional response of variation in venom both across and within snake populations. The importance of variation at the individual level has been largely ignored in studies of venom toxicity and I address this knowledge gap with this chapter. As with previous chapters, I use lethal toxicity assays to assess venom function, but now focus on the level of individual snake rather than higher-level effects.

Overall, my dissertation represents a comprehensive exploration of the functional significance of offensive and defensive trait variation in a rattlesnake-predator vertebrate-prey system. This research clarifies the role that natural selection plays in the evolution of behavioral and molecular variation that characterizes this predator-prey system and lays a groundwork for future comprehensive studies of the ecological and evolutionary significance of variation in venom and prey resistance in venomous snakes and their prey.
Chapter 2: The Effect of Direct Cues of a Predatory Snake on the Foraging Behavior of Native Cotton Mice (*Peromyscus gossypinus*)

Abstract

Nonconsumptive effects such as altered behavior in the presence of predators can be large in magnitude relative to direct consumptive effects of predation. We studied nonconsumptive effects of an ambush foraging predator, the pigmy rattlesnake (*Sistrurus miliarius*), on the foraging behavior of the cotton mouse (*Peromyscus gossypinus*). We placed mice in semi-natural enclosures that varied in their structural habitat complexity and contained foraging trays at heights close to (low) and distant from (high) a caged snake. During an experimental trial, we examined how much seed mice consumed and where foraging took place under both snake and control treatments. Significant individual variation was observed in foraging behavior of cotton mice. Mice foraged nearly equally at the two heights and were not influenced by experimental treatments. In contrast with prior studies, habitat structural complexity did not affect the amount of seed eaten by cotton mice. There was a significant effect of experimental night on total amount of seed consumed; in novel (unfamiliar) environments, mice reduced their foraging. The direct presence of a snake predator did not significantly influence the amount of seed consumed by cotton mice. Indirect cues of predation risk, especially the
familiarity of the environment, appeared to invoke the largest response in foraging behavior in this study system.

Introduction

Predators can greatly impact prey at both the individual and population level. Direct consumption of prey and the resulting reduction in population abundance is one effect of predators on prey. However, predator presence can induce stress and defenses in the prey that can alter prey behavior (Abbey-Lee et al. 2015), life history (Peckarsky et al. 2002), physiology (Janssens et al. 2015), or morphology (Teplitsky et al. 2004). These risk-induced effects of predation which can occur even in the absence of consumptive mortality (Nelson et al. 2004) are often referred to as nonconsumptive effects (NCEs) (Peckarsky et al. 2008; Pressier et al. 2009; Orrock et al. 2013) but are also called trait-mediated effects (Werner & Peacor 2003; Pressier et al. 2005), trait effects (Luttbeg & Kerby 2005), or risk effects (Creel & Christianson 2008).

Nonconsumptive effects include, but are not limited to, shifts in temporal activity (Kotler et al. 1994), increased refuge use (Downes 2001; Davenport & Chalcraft 2013; Lagrue et al. 2015), display of startle and escape behaviors (Losey & Denno 1998; Brodie & Brodie 1999), shifts in utilization of vertical habitat (Davidson et al. 2015), reduced foraging (Nelson et al. 2004; Reynolds & Sotka 2011), reduced growth rate (Janssens et al. 2015), diminished recruitment (Ellrich et al. 2015), and altered prey stoichiometry (Janssens et al. 2015). For example, prey can display stress-hormone induced reductions in
reproduction and body condition during periods of high predator density (Boonstra et al. 1998). The effect size of NCEs of predators on prey can be comparable or greater in magnitude relative to the effect size of consumptive effects of predation (Preisser et al. 2005). NCEs often trigger cascading impacts on the ecological community, changing relationships between multiple species (Werner & Peacor 2003; Preisser et al. 2005; Trussell et al. 2006; Bestion et al. 2015).

The magnitude of NCEs is influenced by traits of the predator, with actively foraging predators having a smaller effect on prey compared to predators with a more sedentary foraging style (Preisser et al. 2007). Whereas the NCEs of sit-and-wait predators on prey are predicted to be high, the impacts of these predators are poorly known, especially in terrestrial environments (Preisser et al. 2007). In a historical analysis of ecological publications that used manipulation, Sih (1985) found that the majority of papers on predator effects involved aquatic ecosystems, with terrestrial research focused on arthropod predators and little work on the effects of vertebrate predators. While recent research on predator NCEs is more diverse than that of the past, it is still biased toward aquatic systems (Pressier et al. 2007; Orrock et al. 2013) and invertebrate predators (Pressier et al. 2007). In a recent meta-analysis of prey response to manipulated predation risk, an overwhelming 91% of experiments took place in aquatic systems (Orrock et al. 2013). In a meta-analysis by Preisser et al. (2007) that focused on studies recording prey growth, fecundity, or density, only one of 17 papers on terrestrial vertebrate predators examined NCEs evoked by a reptilian predator (Downes 2001).
Preisser et al. (2007) found studies involving terrestrial vertebrate predators are dominated by NCEs evoked by mammalian predators. Likewise, Nowak et al. (2008) found that terrestrial ectothermic vertebrates are under-represented in the predator-prey literature. If predator traits influence prey NCEs, then our understanding of NCEs would benefit from broader taxonomic study. Broader taxonomic study is especially important because some prey behaviors which result in avoidance of one predator may make prey more vulnerable to another type of predator (Kotler et al. 1992; Cresswell & Quinn 2013; Embar et al. 2014), making generalization of prey responses to all predators difficult.

Reduction in foraging is a proximate NCE of predators on prey (Nelson et al. 2004; Reynolds & Sotka 2011). In regards to foraging, the marginal value theorem (MVT) predicts net energetic benefits must exceed the long term expected gain from moving for foraging in a patch to continue (Charnov 1976). If costs, including predation costs, are high at a patch, then an animal is expected to leave that patch earlier, all else being equal. Building off the MVT, established methods in foraging theory use the remaining density of food in a patch (the giving-up density, GUD) to evaluate decisions made by organisms faced with energetic harvest costs, predation risk, and missed opportunity costs associated with foraging (Brown et al. 1988). Other factors being equal, differences in perceived predation risk can be measured using GUDs (Brown et al. 1988). This method predicts less food will be eaten (and higher GUDs documented) in environments with higher predation risks (Brown et al. 1988); this change in foraging behavior is a NCE of predators on prey.
Experimental studies of predation risk can manipulate direct cues of predation (such as predator presence, odor, or sound) or indirect cues of predation (often habitat characteristics associated with higher risk). In a review of terrestrial studies using GUDs to evaluate the behavioral responses of prey to indirect or direct cues of predation, Verdolin (2006) found significant decreases in foraging effort associated with higher predation risk. However, the prey response was much greater with respect to habitat characteristics than it was in response to the direct cues of predator odor or predator presence (Verdolin 2006). Rodents, in particular, displayed a widespread reduction in foraging associated with the indirect predation cues of high illumination and open microhabitats (Kotler 1984a; Kotler 1984b; Kotler et al. 1993; Arthur et al. 2004; Bird et al. 2004; Orrock et al. 2004; Pastro & Banks 2006; Strauß et al. 2008; Capers 2010; Hinkelman et al. 2012; but see Meyer & Valone 1999). In contrast to these indirect cues of predation, mammalian prey species generally show mixed responses to the direct cue of predator odor (reviewed by Apfelbach et al. 2005). In small mammals, foraging activity is reduced by the direct presence of predators such as fire ants (Orrock & Danielson 2004) and owls (Kotler et al. 1991; Kotler 1992), but is not reduced in response to the cue of sound (Troxell-Smith et al. 2016).

Studies that investigate rodent NCEs specifically in response to direct cues of snake predators have mixed results, possibly reflecting species or context-dependence. Bouskila (1995) documented higher GUD in the presence of caged snakes in the
kangaroo rat *Dipodomys deserti*, but not in *D. merriami*. Gerbils (*Gerbillus andersoni* and *G. pyramidum*) left resource patches at higher GUDs in response to the presence of a snake (Kotler et al. 1993), but a significant response to only snakes was not observed when predatory owls were also included in the experiment (Kotler et al. 1992). Striped mice (*Rhabdomys pumilio*) decrease activity in response to feces from an elapid snake (Pillay et al. 2003). California ground squirrels (*Otospermophilus beecheyi*) tail-flag and vocalize in the presence of rattlesnakes (Owings & Coss 2008), possibly at a cost of other forgone activities like foraging and reproduction. Therefore, additional information is needed to determine under which circumstances NCEs to predatory snakes would most likely occur.

Habitat characteristics are an extremely important context to consider in predator-prey interactions. For example, the direction and magnitude of NCEs depends on the habitat in which they are measured (Trussell et al. 2006). Structurally complex habitats can lead to differences in predator behavior and change the capture probability of prey (Michel & Adams 2009). Additionally, the indirect cues of predation risk associated with habitat are often more important than direct cues of predation in determining foraging behavior, especially in rodents (Orrock et al. 2004; Verdolin 2006). In rodent-predator interactions, selecting structurally complex habitats may lower the risk of avian predation but increase the risk of predation by snakes (Kotler et al. 1992).
Here, we examine the NCEs of a sit-and-wait predator, the pigmy rattlesnake (Sistrurus miliarius), on the foraging behavior of the cotton mouse (Peromyscus gossypinus). Our study manipulated predator presence and enclosure structural complexity to determine if mice altered their foraging in response to these direct and indirect cues of predation risk. We varied direct cues through the presence or absence of visual and olfactory cues of predator presence. We varied indirect cues of risk through variation in habitat structure. Structurally complex habitats should make it more difficult for a prey to visually detect and avoid a cryptic sit-and-wait predator. However, in very complex habitats, structure may physically interfere with a successful rattlesnake strike. Finally, we varied the riskiness of patches by providing patches at different heights. Many cricetine rodents that inhabit the hardwood forests of the eastern United States, such as P. gossypinus, are semi-arboreal and incorporate above-ground foraging into their behavioral repertoire (Wolfe & Linzey 1977; Packer & Layne 1991). Therefore, if predation risk varies with height, rodents should shift their foraging to microhabitats where predation risk is reduced.

We predicted that cotton mice would exhibit higher GUDs when direct cues of predators were present and in structurally simple environments. We expected patch height to matter most when the predator was present, with mice exhibiting higher GUDs in lower height food patches in the presence of the snake. We also predicted an interaction between direct and indirect cues, with higher GUDs in simple environments when snakes
are absent versus present. In the absence of differences in risk or if foraging is random, mice should consume seeds at nearly equal rates from the two foraging trays.

Methods

Study System

The pigmy rattlesnake (*Sistrurus miliarius*) is a generalist predator with a diet that includes frogs, centipedes, lizards, and small mammals (Gibbs & Mackessy 2009). One dietary constraint is the size of prey that pigmy rattlesnakes can ingest because they are small compared to other pit vipers (adult snout-vent lengths, SVLs, of 35-55 cm, May & Farrell 2012). The cotton mouse has been documented in the gut contents of adult pigmy rattlesnakes (SSW, personal observation) and is one of the most common small mammals in peninsular Florida (Smiley 2010). The cotton mouse likely has various predators, including several snake species, bobcats, and owls. In the mesic hardwood hammocks of Florida, pigmy rattlesnakes can reach exceedingly high densities (up to 50 individuals per ha) and are surface-active and feeding year-round (May et al. 1996; May & Farrell 2012). This same habitat is preferred by the cotton mouse (Wolfe & Linzey 1977), resulting in a high likelihood of frequent encounters between predator and prey.

Experimental Design

We conducted an experiment within semi-natural enclosures to investigate the effects of pigmy rattlesnake presence and environmental complexity on cotton mouse foraging behavior. Four enclosures (121.9 cm x 91.4 cm x 121.9 cm height) were constructed,
each with a plywood base, four sides composed of wire mesh (6.4 mm x 6.4 mm openings) and wood support beams, and a removable plywood ceiling. We placed the enclosures approximately 15 m apart in habitat consisting of mixed hardwood hammock and pine at Lake Woodruff National Wildlife Refuge (DeLeon Springs, FL) (29°6′22″N, 81°22′15″W); the surrounding trees provided partial shade from both sun and moon light. The bottom of each enclosure received 38 L of sandy soil beneath 19 L of mixed oak-pine leaf litter. All enclosures also included a glass water bowl (diameter= 15.0 cm, height= 7.2 cm), a tower (height= 71.1 cm) on which three sections of PVC pipe (diameter= 4.4 cm, length= 20.3 cm) were attached horizontally (tube mounting heights= 0, 20, 41, and 61 cm above the base), a finch bamboo nest (Super Pet, Elk Grove Village, IL), and a foraging station. The aforementioned components provided water, refuge, and food to mice. The foraging station (height= 76 cm) had two wooden platforms (each 45.7 cm x 30.5 cm), one placed at ground (0 cm) height (hereafter, “low level”) and another 48.3 cm above the enclosure leaf litter (hereafter, “high level”). A foraging tray was placed on each level. Foraging trays were clear, shoe-box sized plastic containers (dimensions= 33.0 cm x 19.1 cm x 10.8 cm height, The Container Store, Coppell, TX). Two square rodent access holes (5.0 cm x 5.0 cm) were cut out of each box, one centered on a length and one on a width of the container. A 500 µm sieve was used to remove coarse grains from all purpose sand (Quikrete, Atlanta, GA). Each tray received 500 mL of sieved sand into which 8.0 g white millet seed was mixed; this mixture of sand and seed was replenished after every night of mouse foraging. To investigate the influence of environmental complexity on mouse foraging behavior, we placed a scaffold apparatus
(length= 78.7 cm, width= 66.0 cm, height= 43.2 cm) constructed from four wood post corners, 12 pieces of bamboo at three heights, and eight criss-crossing lengths of twine (contact author for configuration details) into half (n= 2) of the enclosures. The two enclosures that received the scaffold were considered structurally complex enclosures (or briefly “complex”), and the two without the apparatus were defined as “simple” enclosures.

We captured cotton mice at Lake Woodruff National Wildlife Refuge using Sherman live traps (dimensions: 22.9 cm x 8.9 cm x 7.6 cm, H.B. Sherman company, Tallahassee, FL) baited with black oil sunflower seeds. Captured cotton mice were individually marked with ear tags (Monel, size 1005-1; National Band and Tag, Newport, KY, USA). We ran trap lines until four adult cotton mice were captured and then placed one mouse in each enclosure to start a round of data collection (see “trials” as described below). Cotton mouse capture rates were not especially high. For example, in November and December 2013, we caught 20 cotton mice in 1061 trap nights, an uncorrected trap rate of 1.9%. Thus, it was necessary at times to hold mice in captivity for a few nights (average =1.9) before beginning a trial. In captivity mice were held in standard plastic mouse containers (dimensions: 33.0 cm x 21.0 cm x 18.5 cm) with a wire lid, and food and water were provided ad libitum. Our study overlapped with a portion of the cotton mouse breeding season; mice that gave birth during their captive hold time were not used in the experiment. We used 28 mice in our study; 13 (46.4%) of the mice were female. The average weight of mice was 30.39 g (SD= 2.15 g).
Snakes used in our study were also captured at Lake Woodruff National Wildlife Refuge. The snakes were located by walking visual survey. We provided snake stimulus to the mice by placing an adult rattlesnake within a Sterilite brand plastic container (dimensions: 30.5 cm x 35.6 cm x 30.5 cm) which was modified to have screen openings on the side (25 cm x 15 cm) and top (27 cm x 23 cm) of the box. These openings allowed the scent of the snake to readily leave the container while preserving visual snake cues. To maintain snake well-being, the snake cage contained a small amount of leaf litter and a water dish. This set up permitted snakes to take refuge under the leaf litter, which they did a portion of the time. In our daily visits to the enclosures, snakes were recorded completely below the surface 25% of the time (Smiley-Walters, unpublished data). To control for the introduction of a foreign object into the mouse enclosures, we placed an empty Sterilite container into the enclosures that did not receive a snake (control treatment).

We conducted foraging trials with 28 cotton mice. Trials were conducted between the months of November and early January and spanned two cool seasons (2012-2013 and 2013-2014). Each trial consisted of five nights of mouse foraging in which one mouse consumed seed in one of the four enclosures. At the start of a trial, we placed one mouse in each of the four enclosures; two mice were randomly placed in their own structurally complex environments and two were each in simple environments. The mice were allowed to forage for one night (night zero) to acclimate to the enclosures. On the second
day, we randomly added a snake treatment to two of the enclosures during the routine
seed tray replenishment; the other two enclosures received the control treatment. The
mice were allowed to forage for two nights (nights one and two) under these conditions
after which point the control and snake treatments were switched by moving the snake
stimuli into the previous control treatment and vice versa. The mice foraged for two
additional nights (nights three and four) under these new conditions. After five nights
(one acclimation and four experimental), we removed the mice from the enclosures and
returned animals to their capture location. We repeated these methods seven times,
including three times the first cool season and four times the second season. In this way
each mouse (n=28) received both the snake and control treatments over the course of a
trial. However, an individual mouse was only subject to one level of structural
complexity.

Following each day of seed replenishment, mixtures of millet seed and sand in which
mice had foraged were transported from the field to the lab in plastic bags. Because each
mouse (n=28) foraged for five nights in both low and high foraging trays, we collected
and processed 280 bags of seed and sand. In the lab we separated the seeds from sand
using a 1 mm sieve. After sieving, we separated remaining non-seed material (mouse
feces, dirt, etc.) from the millet seeds by hand. We then weighed the remaining amount
of millet seed, including husks. This post-consumption weight was subtracted from the
initial value (8.0 g) to calculate the weight of seed that was consumed in each foraging
A greater mass of consumed seed corresponds to a lower GUD and a lower mass consumed corresponds to higher GUD.

All procedures involving animals were approved by the Stetson University Animal Care and Use Committee (protocol # 2012TF102). This research study was conducted under Florida Fish and Wildlife Conservation Commission Venomous Reptiles License numbers 411-95180 and 411-104419 and Scientific Collecting Permit numbers LSSC-11-00067 and LSSC-11-00067A.

Data Analysis
We used R version 3.3.1 (R Core Team, 2016) and JMP version 10.0 for data analysis. We investigated whether cotton mice changed how much they foraged and/or where they foraged (high versus low tray) in response to manipulated variables, including snake presence (a direct cue of predation) and structural complexity (a potential indirect cue of predation risk). To investigate these questions, we created two response variables. The first variable was the total amount consumed by a mouse in a given night of foraging (hereafter, “total amount eaten”). The total amount eaten was the sum of the weight of seed eaten in the high and low foraging trays. The second response variable was the percentage of seed eaten out of the high tray (“percent high”) calculated by dividing the weight of seed consumed from the high foraging tray by the total amount of seed eaten by that mouse that night and then multiplying by 100. The unit of analysis was the individual mouse.
We first determined how much and where mice were foraging and whether this changed over the course of the experimental trial. Nightly differences in total amount eaten were examined using an ANOVA followed by Tukey HSD pairwise comparisons using a 95% family-wise confidence level. Next, we examined foraging response variables (total amount eaten and percent high) from only the acclimation night (night zero) of the trial using a two-way ANOVA to assess whether foraging patterns differed by sex or environmental complexity. We report Type III marginal sum of squares for this test. After this analysis, in order to focus on snake treatment, we disregarded the acclimation night (night zero) in subsequent analyses. Thereafter, our primary mode of data analysis was mixed linear regression models. Mixed linear regression models were examined for both total amount eaten and percent high as response variables; for each variable, we used the average response of each mouse from two nights of foraging under fixed conditions. Mixed models include both random and fixed effects. All of our models included individual mouse as a random effect. Fixed effects consisted of the variables that were of primary interest in our study (snake presence and environmental complexity) as well as other variables that we deemed potentially relevant (sex and night of the experiment). We also examined the interaction terms between these fixed effects. These models were carried out in R using the lme function in the nlme package (Pinheiro et al. 2011).
Results

The total amount eaten per night was not constant over the five nights of the experimental trials ($F = 24.33, P< 0.0001$; Table 1). Foraging was lowest during the acclimation night, increased, and then leveled off (Fig. 1). Tukey HSD pairwise comparisons found the total amount eaten on night zero to be significantly lower than all other nights (one to four). The weight of seed eaten on night one was significantly lower than the average weight of seed eaten during nights three and four. The total amount eaten on nights two, three, and four did not differ from one another nor did the total amount eaten on nights one and two (Fig. 1). Excluding the acclimation night, an average of 5.17 g of seed was consumed per mouse per night.

Where mice chose to forage did not differ over the nights of the trial ($F = 0.28, P = 0.89$; Table 1). Mice predominately foraged opportunistically by splitting their consumption nearly equally between the high and low foraging trays. The average percent of foraging that occurred from the high tray was 50.2% (range = 0 to 98.3%); the nightly average percent high varied little (from 48.7% to 51.9%) over the five-night period.

During the acclimation night, mouse foraging was not significantly influenced by the main effects of sex or environmental complexity (Table 1). The average amount of seed eaten during the acclimation night was 2.99 g with 51.1% of seed mass consumed from the high tray. The total amount eaten was not significantly affected by sex ($F = 0.26, P =$
0.61; Table 1) or by environmental complexity ($F = 0.22$, $P = 0.64$; Table 1). On average male mice ate slightly more than female mice (mean = 3.13 g and 2.84 g, respectively); however, females were more variable in their total amount eaten (SD: males= 0.80 g, females = 1.89 g). Mice consumed slightly more on average in the structurally simple environment compared to the complex environment (average total amount eaten: simple = 3.13 g, complex = 2.86 g). Additionally, the percent high foraging was not significantly affected by sex ($F = 2.97$, $P = 0.10$; Table 1) or by environmental complexity ($F = 1.40$, $P = 0.25$; Table 1). On average, the percent high foraging was similar between the sexes (mean percent high: male = 55.6%, females = 45.9%) and percent high foraging differed little between the complex and simple environments (mean percent high: complex = 54.3%, simple = 48.0%).

During the experimental period (nights one through four), we found individual variation in foraging behavior to be high in this system, accounting for 60.4% of the variation in the total amount eaten. After accounting for individual random effects, the average total amount eaten over nights one and two was significantly less than the average total amount eaten during nights three and four (mixed linear regression model, $t = 4.39$, $P = 0.0002$; Table 1). We found the fixed effects of snake ($t = -0.72$, $p=0.48$), environmental complexity ($t = -0.08$, $P = 0.93$), and sex ($t = 1.35$, $P = 0.19$) were not significant in explaining variation in the total amount eaten using mixed regression models (Table 1). Two night averages in total amount eaten were similar between snake present (mean= 5.10 g) and control (mean= 5.25 g) treatments as well between simple (mean= 5.16 g)
and complex (mean= 5.19 g) environments. There was no interaction between snake presence and environmental complexity (t = -0.26, P = 0.80; Table 1) and all other interaction terms lacked significance in explaining total amount of seed eaten. Overall, the total amount eaten was most strongly influenced by how long the mice had been in their enclosures and little by habitat characteristics or snake presence.

During the experimental period, individual variation also was significant in explaining where mice foraged, accounting for 63.79% of the variation in percent high foraging. Again, we included individual mouse variation when examining all fixed effects. Mice showed little variation in where they foraged with regard to the variables that we considered, including snake presence (t = -0.28, P = 0.79), environmental complexity (t = 0.53, P = 0.60), sex (t = 0.31, P = 0.76), and night (t = 0.42, P = 0.68) (Table 1). No significant interaction terms were found, including with regard to the fixed effects of snake presence and environmental complexity (t = -0.94, P = 0.36; Table 1), on the percent of foraging that occurred out of the high tray.
A. Total Amount Eaten Response

<table>
<thead>
<tr>
<th>Nights(^w) (#)</th>
<th>Model Type</th>
<th>Effect</th>
<th>DF</th>
<th>Test Statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (0-4)</td>
<td>ANOVA</td>
<td>Night</td>
<td>4</td>
<td>F= 24.33</td>
<td>&lt;0.001*</td>
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<tr>
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<td>F= 0.22</td>
<td>0.64</td>
</tr>
<tr>
<td>Accl. (0)</td>
<td>ANOVA</td>
<td>Sex</td>
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<td>F= 0.26</td>
<td>0.61</td>
</tr>
<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Snake</td>
<td>27</td>
<td>t= 0.72</td>
<td>0.48</td>
</tr>
<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Complexity</td>
<td>26</td>
<td>t= 0.08</td>
<td>0.93</td>
</tr>
<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Sex</td>
<td>26</td>
<td>t= 1.35</td>
<td>0.19</td>
</tr>
<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Nights(^+)</td>
<td>27</td>
<td>t= 4.39</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Snake x Complexity</td>
<td>26</td>
<td>t= 0.26</td>
<td>0.80</td>
</tr>
</tbody>
</table>

B. Percent High Response

<table>
<thead>
<tr>
<th>Nights(^w) (#)</th>
<th>Model Type</th>
<th>Effect</th>
<th>DF</th>
<th>Test Statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (0-4)</td>
<td>ANOVA</td>
<td>Night</td>
<td>4</td>
<td>F= 0.28</td>
<td>0.89</td>
</tr>
<tr>
<td>Accl. (0)</td>
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<td>F= 1.40</td>
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<td>F= 2.97</td>
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<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Snake</td>
<td>27</td>
<td>t= 0.28</td>
<td>0.79</td>
</tr>
<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Complexity</td>
<td>26</td>
<td>t= 0.53</td>
<td>0.60</td>
</tr>
<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Sex</td>
<td>26</td>
<td>t= 0.31</td>
<td>0.76</td>
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<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Nights(^+)</td>
<td>27</td>
<td>t= 0.42</td>
<td>0.68</td>
</tr>
<tr>
<td>Exp. (1-4)(^\beta)</td>
<td>MRM(^^)</td>
<td>Snake x Complexity</td>
<td>26</td>
<td>t= 0.94</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Table 1. Results of statistical analyses run on the mouse foraging response variables: (A) total amount eaten and (B) percent high. The ANOVA tests included the acclimation (accl.) night (night 0), while mixed regression model (MRM) results focused on the experimental (exp.) period (nights 1-4). \(^w\) The nights of foraging data that were included in the analysis. \(^\beta\) Two-night treatment averages were used for regression analyses. \(^^\) All mixed regression models included individual mouse as a random effect. \(^+\) Regression examined blocks of two nights (ie. 1 and 2 versus 3 and 4) to correspond with the interval of control or snake treatment. * Significant at \(\alpha=0.05\).
Figure 1. The amount of millet seed eaten each night (average +/- SEM) by cotton mice (n=28) during the trial. The amount of seed consumed on night zero was lower than all other nights. Night one was lower than nights three and four. Significant pairwise differences between nightly averages are indicated by letters A, B, and C.
Discussion

Our results show individual variation and the novelty of environment (an indirect cue of predation risk) are important in governing the foraging behavior of cotton mice, whereas snake presence (a direct cue of predation risk) and structural complexity of the environment (a probable indirect cue of predation risk) did not significantly influence the mouse foraging behavior. Generally, our findings agree with previous studies that have found responses of rodents to indirect cues of predation are larger than their responses to direct cues of predation (Orrock et al. 2004; Verdolin 2006).

Cotton mice showed the biggest changes in the total amount eaten in response to how familiar they were with the surrounding environment. Unfamiliar environments are likely risky habitats because escape routes or potential predator ambush sites are largely unknown. For example, fish in a familiar environment were able to escape threatening stimuli quicker than fish in an unfamiliar environment (Brown 2001). During our experiment, many of the mice constructed retreat tunnels within the enclosure over the trial period. In addition, changes such as the arrival of a predator, are more difficult to detect in novel environments. A cryptic stationary predator, such as a rattlesnake, is likely difficult to visually separate from the background environment. Predator odor cues also may be important in some predator-prey systems (Apfelbach et al. 2005), although the closely related *Peromyscus leucopus* does not reduce foraging in the presence of predator scent (Orrock & Danielson 2009).
It is possible that cotton mice could have reduced foraging at the start of each trial in response to other factors besides predation risk that are associated with novel environments, including potential conspecific aggression or increased exploratory behavior. However, we find these alternative explanations unlikely based on the natural history of this species and personal observations of mice during field visits. For example, if mice were wary of infringing on conspecific territories, then male and female mice likely would have responded differently to the novel environment as male mice have high overlap in home ranges (Wolfe & Linzey 1977) and are passive toward cage mates (SSW, personal observation), while females are more exclusive and aggressive.

We did not detect any significant effect of snake presence on foraging behavior. A similar result was found by Wasko et al. (2013) who failed to detect a reduction in foraging in *Peromyscus mexicanus* and *Melanomys caliginosus* in response to snake feces or actual snake presence. It is possible that rodents are not wary of venomous snakes when foraging. However, it is also likely that mice may not need to forgo foraging opportunities if they are able to visually detect a venomous snake, track the movement of the snake, and remain out of striking distance of the ambush foraging predator. It is unknown what percentage of mice were aware of snake presence. However, we know from the distribution of seed husks that at least some mice consumed seeds in the center of the enclosure floor where they would be able to watch the snake but remain outside the snake’s strike distance.
We may have failed to detect a response of mice to snakes because of the season in which our study was conducted; response of mice to snakes may be dependent on season. Herman and Valone (2000) showed the response of Dipodomys merriami to mammalian predator scent was seasonally dependent. If the behavioral response is seasonal, then the response of mice to snakes would be expected to be largest in warmer months when snakes are more active. Unfortunately, rodents are difficult to trap during the summer in Florida. Therefore, while behavioral foraging of mice may change with season, a similar experiment would be quite challenging to conduct during the warm months of the year. Additionally, pigmy rattlesnake feed throughout the year in Florida (May & Farrell 2012) so our experimental design is not unnatural.

We did not detect a significant effect of structural complexity within an enclosure on the total amount of seed consumed. This result was surprising because prior research shows an overwhelming amount of evidence of habitat features being important as indirect cues of predation risk and dictating foraging decisions in rodents (Arthur et al. 2004; Bird et al. 2004; Orrock et al. 2004; Pastro & Banks 2006; Verdolin 2006; Strauß et al. 2008). For our prey species, Packer and Layne (1991) documented cotton mice visiting covered foraging stations more often than open microhabitats in the Florida scrub. We suspect during the acclimation night, foraging by mice was already greatly reduced and the novelty of the environment superseded the importance of structural complexity. During the four-night experimental period, it is possible differences in structural complexity between the two treatments may not have been perceived as different enough by mice to
affect foraging decisions. Hinkelman et al. (2012) found GUDs of cotton mice did not differ between microhabitats with downed woody debris and those that were either open or shrub-dominated, but they did find differences in GUDs between shrub and open microhabitats. Doherty et al. (2015) showed foraging microhabitat preference is context-dependent for a suite of three rodents found in Australia. In this system, rodents demonstrated significant preference for sheltered microhabitats over open ones only in recently burned habitat patches; no difference was found in long unburned areas. Additionally, in our study, large individual variation may have made the signal of habitat structural complexity difficult to detect because individual mice only received one structural complexity treatment. In any case, we believe that the interaction between predator type (pursuit versus ambush) and microhabitat features warrants further investigation to determine if the trade-offs and predator facilitation described by Kotler et al. (1992) exist in other systems. Open microhabitats are likely safer for rodents only when snake predation is greater than the combined effects of mammalian and avian predation.

Our results demonstrate that cotton mice behave optimally with respect to foraging height in terms of maximizing net energy returns. Cotton mice in our study foraged from above-ground platforms nearly half of the time. An equal distribution of foraging between the high and low trays would allow mice to have the largest return of seed per unit of search effort (sand digging) and is consistent with the predictions of the marginal value theorem, maximizing energetic returns when travel time between patches is small. The proportion
of arboreal foraging displayed by cotton mice in our study is greater than that described previously by Packer and Layne (1991) in the Florida scrub. However, our foraging platforms (0.48 m) were lower than the bait stations used by Packer and Layne (1991) (heights of 1 to 1.5 m). Therefore, cotton mice displayed optimal foraging when given choices corresponding to the heights of ground and shrub layers of habitat and may reduce foraging as height increases further, approaching the tree layer. Mice in our study also used several heights for their daytime refuges, with half of individuals constructing ground-level retreats and half utilizing above-ground (finch nest) retreats.

We show there is a high degree of individual variation in foraging decisions made by cotton mice in our study system. Individual variation is an important aspect of ecology and evolution research, although the importance of individual variation in determining behavioral syndromes has only in the past decade become a subject of intense research focus (Sih et al. 2004). The proximate causes of individual variation in our mice are unknown but could include small differences in nutritional needs influenced by body weight, metabolic rate, and reproductive status, as well as differences in foraging efficiency and stress response. Because enclosures were not completely closed systems, it is also possible that some mice supplemented their foraging with invertebrates. Other factors, such as variation in environmental temperature, which we did not specifically include in our mixed models are also likely contributing to the individual variation component.
In summary, we failed to detect altered foraging behavior of cotton mice in response to the direct presence of pigmy rattlesnakes. Our research differs from much previous work in failing to detect an effect of environmental complexity, a likely indirect cue of predation risk, on foraging behavior. The indirect cue of predation which affected cotton mouse foraging behavior the most was the novelty of the environment; adequate acclimation periods would be a critical component of any future enclosure-based research using a rodent system. Our study demonstrates the importance of including individual variation in behavioral analyses as it was very high in this study system.

Acknowledgments

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Chapter 3: Species matters: comparison of pigmy rattlesnake venom toxicity to non-native and native prey

Abstract

Venom toxicity assessments are widely based upon non-native organisms that are not consumed in the wild by the venomous predator. This practice raises questions about the relevance of toxicity results on these “model” prey in addressing ecological or evolutionary questions regarding venom effects on native prey. Here, we explore this issue by comparing the toxicity of venom from pigmy rattlesnakes (*Sistrurus miliarius*) on taxonomically-diverse sets of model and native vertebrate prey. Specifically, we compared rattlesnake venom toxicity using regression analyses of mortality data and LD$_{50}$ estimates for nine species from three broad taxonomic groups of prey (reptiles, mammals, and amphibians) to determine whether the non-native model organism of each group was representative of other group members. In all three groups, model species (*Anolis sagrei*, *Mus musculus*, and *Lithobates pipiens*) had a significantly different mortality response from one or more of the native prey species that the models were meant to represent. Two features of our results suggest an importance of evolutionary history in understanding these differences. First, there was a phylogenetic component to prey responses to venom. Within vertebrate groups, models and congeneric prey showed more similar responses than non-congeneric prey suggesting that venom may act on
common prey targets that result from common ancestry. Second, there was evidence of a possible adaptive response by native prey to the toxic effects of venom. Native prey often showed higher LD\textsubscript{50} values than their model counterparts, suggesting greater resistance to venom. We recommend that, to generate measures of venom toxicity that are ecologically and evolutionarily relevant, researchers use native prey when possible. If native prey are not available, choosing “model” species that are close taxonomic relatives to natural prey may be the best alternative.

Introduction

Venoms of predatory snakes play an essential role in successful foraging by contributing to prey immobilization (Zimmerman et al. 1990; Richards et al. 2012; Urdaneta et al. 2004; Torres-Bonilla et al. 2016), prey digestion (Thomas and Pough 1979, but see Chu et al. 2009), and chemosensory location of prey (Chiszar et al. 1999) following envenomation. These activities are the functional result of venom enzymatic and non-enzymatic proteins, small organic molecules, and nonorganic components (Mackessy 2008; Mackessy and Saviola 2016), the composition of which varies across multiple taxonomic levels (Chippaux et al. 1991; Nawarak et al. 2003; Serrano et al. 2005; Gibbs and Mackessy 2009; Barlow et al. 2009; Gibbs and Chiucchi 2011; Margres et al. 2015a). This variation in composition is widely thought to represent adaptive variation that has evolved via natural selection and enhances snake foraging success on preferred prey (Barlow et al. 2009; Gibbs and Mackessy 2009). Lines of evidence that support this claim include research demonstrating the prey-specific toxic effects of snake venom.
(Mackessy et al. 2008; Barlow et al. 2009; Gibbs and Mackessy 2009) and a match between snake diet and venom performance on representative prey (Barlow et al. 2009; Gibbs and Mackessy 2009). However, most of this work has used model laboratory species that are not envenomated by snakes in the wild (e.g. Mackessy 2008). This reliance on model organisms raises questions about how representative these results are for making inferences about venom toxicity and function in relation to native prey which have a shared ecological and evolutionary history with a given venomous snake predator.

The most widely used method for assessing prey-specific effects of venom involves constructing a dose-response curve by exposing prey to a range of venom doses, including those that result in zero and 100 percent prey mortality. The S-shape of a typical dose-response curve can be approximated with a probit or logistic regression model by assuming a tolerance distribution of responses (Agresti 2007); this property is useful because model predicted values can estimate mortality responses for doses intermediate to those tested. The median lethal dose (LD$_{50}$) is a summary statistic of the dose-response curve that enables comparison of toxicity of a single snake venom across different prey species (Jorge da Silva and Aird 2001; Gibbs and Mackessy 2009) or comparison of different snake venoms when a single prey species such as $Mus$ is used (Jorge da Silva and Aird 2001; Mackessy 2008; Barlow et al. 2009; Gibbs and Mackessy 2009). While specific enzymatic assays of venom function are available (e.g. Jorge da Silva and Aird 2001; Debono et al. 2016; Modahl et al. 2016), a lethal toxicity assay and associated LD$_{50}$ provide a whole-organism response to snake venom inclusive of all
proteins and any synergistic interactions (Borkow et al. 1993) between them. Thus, LD$_{50}$ estimates allow venom toxicity to be assessed in a way that is most realistic in terms of the interaction between predator and prey in the wild.

Previous research has used LD$_{50}$ estimates to demonstrate differential toxic effects of venom across broad taxonomic groups of prey, often using non-native, commercially available animals. For example, domestic chicks (Gallus domesticus) ranked as the most susceptible of several prey species tested with venom from the brown treesnake (Boiga irregularis), followed by introduced lizards (Hemidactylus geckos and Carlia skinks), and house mice (Mus musculus) (Mackessy et al. 2006). Venom from coral snakes (Micrurus sp.) had different LD$_{50}$ estimates in house mice (Mus), native fish, and reptiles (Jorge da Silva and Aird 2001). The results of both of these studies were based on LD$_{50}$ point estimates without confidence intervals. Gibbs and Mackessy (2009) was important in documenting significant differences in LD$_{50}$ estimates which incorporated error estimates across broad taxonomic groups of prey when tested with venoms from four species of Sistrurus rattlesnakes. Their results showed that wild caught leopard frogs (Rana pipiens) were, in general, more resistant to Sistrurus venom when compared to invasive brown anoles (Anolis sagrei) and house mice (Mus musculus).

Although laboratory or commercially available organisms like Mus or Gallus are readily accessible for toxicity tests, they typically have no direct evolutionary history with the snake species whose venom is being tested. Thus, the relevance of toxicity measures on
these species for ecological and evolutionary inferences about venom effects on prey is unclear. Prey species that naturally co-occur with specific venomous snakes are subject to these ecological interactions and the evolutionary consequences that results. As such, native prey may offer more relevant measures of venom toxicity, yet few studies have assessed whether this is the case (but see Mackessy 1988; Jorge da Silva and Aird 2001).

The complications associated with drawing toxicity inferences using commercial species has been previously recognized (Jorge da Silva and Aird 2001; Richards et al. 2012). For example, Mackessy et al. (2006) suggested that inbred house mice may have limited utility for comprehending the natural roles of venoms in snakes that consume primarily non-mammalian prey. Additionally, data from saw-scaled vipers (*Echis* sp.) show that the easily available desert locust (*Schistocerca gregaria*) was a poor proxy for venom toxicity and performance on natural scorpion prey (Richards et al. 2012). It remains to be seen whether the results from this work are generalizable to other taxonomic groups of snake prey. New studies that rigorously compare venom toxicity on commercially available model organisms to native prey in the same taxonomic groups would be valuable in addressing this uncertainty. This information would allow an assessment of the utility of toxicity measures collected on commercially available organisms relative to taxonomically similar native prey as well as permit an evaluation of the effect of shared evolutionary history between predator and prey on venom toxicity measures.
Our objective was to examine whether non-native “model” organisms are representative of other species in terms of their susceptibility to rattlesnake venom. We were specifically interested in 1) whether the toxicity of venom to non-native model species reflected venom toxicity to native prey species in the same broad taxonomic group and 2) whether evolutionary relatedness between species pairs influenced the similarity of their mortality response to venom. To accomplish these objectives, we conducted lethal toxicity assays with venom from the pigmy rattlesnake (*Sistrurus miliarius*) on different prey species representative of three broad taxonomic groups (reptiles, mammals, and amphibians) preyed upon by this generalist predator. Within each taxonomic group, we made comparisons using prey LD$_{50}$ estimates and conducted probit regression analyses to examine the effects of species and, where possible, genus on the mortality response data. Our work builds on studies by Gibbs and Mackessy (2009) and Richards et al. (2012) by examining pigmy rattlesnake venom toxicity of different broad taxonomic groups and comparing model organisms to natural prey, respectively. Our research focuses on venom toxicity to vertebrate species because these taxa (especially *Mus*) are widely used in classic lethal toxicity tests and were not the focus of previous research comparing model organisms to naturalistic prey (see Richards et al. 2012).

**Methods**

*Collection and processing of rattlesnake venom*

We located pigmy rattlesnakes (*Sistrurus miliarius*) by visual survey at Lake Woodruff National Wildlife Refuge (DeLeon Springs, FL, USA). We transported these wild-caught
snakes to Stetson University (Deland, FL, USA) and collected venom by inducing rattlesnakes to bite a parafilm-covered beaker. We weighed each snake and recorded its snout-vent-length (SVL) before returning the snake to its site of capture in the wild. We defined adult snakes as those at least 45 g mass or 38 cm SVL, corresponding to a minimum of two years of age in this population (May and Farrell 2012). We combined venoms from adult snakes to form a common solution (hereafter, “pooled venom”) which we used in lethal toxicity assays. We stored snake venoms at -80°C when not in use. We diluted pooled venoms with physiological saline (Scholar Chemical) and then quantified their protein content in replicate using the Bio-Rad Protein Assay (Bio-Rad Laboratories) and the bovine gamma globulin standard.

Prey acquisition and lethal toxicity assays

We obtained LD<sub>50</sub> estimates for pigmy rattlesnake venom on nine different prey species from both the laboratory and the literature. We conducted laboratory toxicity tests with seven different species. Five species were native prey field captured in the vicinity of Lake Woodruff National Wildlife Refuge including the green anole (*Anolis carolinensis*), the cotton mouse (*Peromyscus gossypinus*), the southern leopard frog (*Lithobates sphenoecephalus*), the green treefrog (*Hyla cinerea*), and the squirrel treefrog (*Hyla squirella*). We field captured non-native brown anoles (*Anolis sagrei*) from Stetson University (DeLand, FL, USA). We purchased house mice (*Mus musculus*) from a local pet store. The laboratory methods conducted for these seven species are detailed below. We supplemented our laboratory data with raw data available from a previously
published study (Gibbs and Mackessy 2009). We reanalyzed the dose-response data from Gibbs and Mackessy (2009) using the same statistical methods as our data. This re-analysis resulted in additional information on the house mouse and brown anole as well as on a species not tested with our laboratory methods, the northern leopard frog (*Lithobates pipiens*). We also used an LD$_{50}$ estimate for pigmy rattlesnake venom reported on the Norway rat (*Rattus norvegicus*) (Assi and Nasser 1999) for comparison to our mammal data. Thus, our study compares LD$_{50}$ estimates from two lizard species (reptiles), three rodent species (mammals), and four frog species (amphibians) to examine toxicity of pigmy rattlesnake venom to all major taxonomic groups of vertebrate prey eaten by this snake (Gibbs and Mackessy 2009). Following Gibbs and Mackessy (2009), we defined our model species as *A. sagrei* for reptiles, *M. musculus* for mammals, and *L. pipiens* for amphibians.

In all species for which laboratory data were collected, we weighed each individual and assigned it to a venom-dose treatment using a mass-stratified random design to ensure that the smallest and largest animals of each species were not all in the same dose-treatment group. We delivered a mass-adjusted dose of venom diluted in saline intraperitoneally to the ventral side of each test animal using an insulin syringe. We monitored prey in the hours following injection and report results based on 24-hour mortality status (alive or dead). Our goal was to construct a dose-response curve inclusive of both zero, 100 percent, and intermediate mortality values for each prey species.
Statistical analyses

We used R version 3.3.1 (R Core Team 2016) to conduct analyses on our lethal toxicity assay results. We fit the dose-response data, comprised of venom dose and associated end-point mortality status, with a probit logistic regression from which we 1) estimated the LD$_{50}$ for each prey species and 2) tested for significant differences between data sets, species pairs, and frog genera. To estimate the LD$_{50}$ for a given prey species, we used the glm function followed by the dose.p function (MASS package) on the dose-response data available for that species. The output from these commands provided a species-specific LD$_{50}$ point estimate and its associated standard error. As described in Smiley-Walters et al. (submitted), we calculated a 95% confidence interval for each LD$_{50}$ by multiplying the standard error (output given by dose.p) by the sample-size dependent 97.5 percent quantile of the student’s t-distribution (function qt). These procedures allowed us to compare lethal toxicity estimates on all species.

We tested for statistical differences between species within each broad taxonomic group (reptiles, mammals, and amphibians) using dose-response data that were available for species in that group. In the two species (*M. musculus* and *A. sagrei*) for which data were available from both Gibbs and Mackessy (2009) and this study, the first step of our analysis was to test for an effect of study. If no study effect was found, we combined the datasets for greater sample size in subsequent analyses; detection of an effect resulted in us treating the data separately. All significance testing was conducted using probit
regression models (glm function) with dose included as a model parameter. In the reptiles, we compared the data for *Anolis sagrei* (n=47) and *A. carolinensis* (n=21) by testing for an effect of species on the mortality response. We repeated this process to look at species differences within the mammals, comparing *M. musculus* (n=18, data only from this study) versus *P. gossypinus* (n=42). In the frogs, we also included a genera term in the regression model. We found this model term to be significant and so we performed separate regressions for the *Lithobates* data (n=39) and the *Hyla* data (n=68) in which we tested for species-level effects on mortality. In these last models, we examined *H. cinerea* (n=40) versus *H. squirella* (n=28) and *L. pipiens* (n=18) versus *L. sphenocephalus* (n=21). We did not collect data on *L. pipiens* in this study, thus we made the assumption that the two *Lithobates* datasets were comparable.

**Results**

Overall, our results demonstrate significant species-specific toxicity of pigmy rattlesnake venom on prey. In the reptiles, we found no statistically significant effect of study on mortality (*z*=0.966, *p*=0.334, df=44). Thus, we combined data from both this research with that of Gibbs and Mackessy (2009) for our species-level analysis. In the *Anolis* mortality data, we found that the species term was significant in our probit regression analysis (*z*=2.17, *p*=0.0298, df=65). The non-native model species, *A. sagrei*, was approximately four times more susceptible to pigmy rattlesnake venom in our analysis than the native species, *A. carolinensis* (Figure 2).
In the mammals, a study effect was found for the *M. musculus* mortality data (*z*=1.97, *p*=0.049, df=30). The Non-Swiss Albino (NSA) mice used by Gibbs and Mackessy (2009) had a lower LD$_{50}$ estimate (7.19, n=15) than our non-albino mice purchased commercially which had an estimated LD$_{50}$ of (15.18, n=18). Because of the significant study effect, we took a conservative approach and only used data from our non-albino mice in the species significance test. Our probit regression analysis found species to be a significant predictor of rodent mortality at 24 hours (*z*=2.35, *p*=0.019, df=57). The overall LD$_{50}$ estimate for *M. musculus* was lower than the LD$_{50}$ estimated for *P. gossypinus* and comparable to that of *Rattus norvegicus* (Figure 3). Thus, our data on mammals suggests that *M. musculus* is not a good model for the native rodent (*P. gossypinus*) in our rattlesnake-prey system.

In amphibians, we found large differences in the LD$_{50}$ estimates for the four frog species tested (Figure 4). *L. sphenocephalus* had the highest LD$_{50}$, followed by *L. pipiens*, *H. cinerea*, and *H. squirella*. Our probit regression analysis found genus to be a significant predictor of frog mortality at 24 hours (*z*=4.14, *p*<0.001, df=104) with *Hyla* being more susceptible to pigmy rattlesnake venom than *Lithobates*. Within the *Lithobates*, we did not find a statistically significant difference between the two species included in our probit regression (*z*=1.25, *p*=0.211, df=36). Within the *Hyla*, we found species to be a significant predictor of frog mortality (*z*=2.48, *p*=0.013, df=65). *H. squirella* were more susceptible than *H. cinerea* to pigmy rattlesnake venom (Figure 4). Our data on
amphibians suggest that *L. pipiens* may be a reasonable model species for the congeneric
*L. sphenoecephalus* but not for all frogs as it is a poor proxy for treefrogs (*Hyla* sp.).

Our results show significant species-specific toxicity of pigmy rattlesnake venom on
many prey. Because prey species within a taxonomic group differed in their
susceptibility to venom, it is difficult to rank broad taxonomic groups with respect to one
another in a straightforward manner. However, if we consider the frogs as two separate
groups, a crude ranking of taxa from the most susceptible to the least susceptible to
pigmy rattlesnake venom based on their LD$_{50}$ estimates would be as follows: *Anolis*
lizards, similar levels of susceptibility between *Hyla* treefrogs and rodents, followed by
*Lithobates* frogs.
Figure 2. Estimated median lethal dose (LD$_{50}$) for two congeneric lizard species. Error bars represent the 95% confidence interval. The LD50 of the model *A. sagrei* is a poor approximation of the native *A. carolinensis*.
Figure 3. Estimated median lethal dose (LD$_{50}$) for three rodent species. Error bars represent the 95% confidence interval. The estimate for *R. norvegicus* was taken from Assi and Nasser (1999). The estimate for *M. musculus* illustrated here is based on combined data from both Gibbs and Mackessy (2009) and this study. The LD$_{50}$ for the model *M. musculus* is similar to the taxonomically related and non-native *R. norvegicus*, but lower than the estimated LD$_{50}$ of the native *P. gossypinus*. 
Figure 4. Estimated median lethal dose (LD$_{50}$) for four frog species. Error bars represent the 95% confidence interval. The estimate for *L. pipiens* is based on data from Gibbs and Mackessy (2009). The LD$_{50}$ for the model *L. pipiens* is similar to the congeneric *L. sphenocephalus*, but both leopard frogs have considerably high LD$_{50}$ estimates than the *Hyla* treefrogs. *H. cinerea* and *H. squirella* are more similar to each other than the *Lithobates* but the two display species-specific LD$_{50}$ estimates.
Discussion

Measure of Venom Toxicity

Our study indicates that many easily available model species may be poor proxies for the toxicity responses of native prey species. In each of the taxonomic groups of prey that we tested, the non-native model species was statistically different from one or more native species. In some instances, however, the models did provide broad-scale information about the effect of venom on the native prey. For example, the response of model species to venom had greater similarity to the response of congeneric native species than to the response of native prey from a different genus. Specifically, in our frog toxicity data the LD$_{50}$ estimates from the two Lithobates species did not statistically differ, but their toxicity response data were very different from both H. squirella and H. cinerea. Additionally, although the two Anolis lizard species differed from with regards to their toxicity responses, the responses of these two species had greater similarity to each other than to any other species tested. Thus, a model species that is chosen carefully with respect to evolutionary proximity to native prey species may offer an accurate assessment of the toxic effects of a specific venom in a broad sense. Whether or not the toxicity information provided by a model species is a satisfactory substitute for that of a native prey species will ultimately depend on the particular research question being investigated.

We found a significant effect of study in M. musculus when results from this study are compared to that of Gibbs and Mackessy (2009). Several explanations might be
considered for this difference. First, population-level differences in venom function have been documented within the pigmy rattlesnake (Smiley-Walters et al. submitted) and the different studies used venom of slightly different geographic origin and composition. However, these differences would fail to explain why this study effect was limited to the mammals; *Anolis* and *Lithobates* data did not differ between studies. It is possible that there was a venom difference that differentially impacted venom function in mammals or that we failed to detect a difference in venom source effect in the other species. In either case, if venom composition differences caused the study effect here, then the combined data provide a more generalized result of the toxic effects of pigmy rattlesnake venom on *M. musculus*. Second, it is possible that laboratory procedures (site and depth of injection) differed enough between labs to influence the LD$_{50}$ estimates and that these had a greater impact on endothermic animals like mice. Observations suggest that venom injection factors can affect rodent survival post-envenomation (S. A. Smiley-Walters, unpublished data). However, we feel that the most likely explanation for the significant effect of study on mammal mortality is that each study used different strains of *M. musculus* to carry out toxicity testing. These strains of mice may differ in their degree of outbreeding or basic physiology. Several studies have found behavioral (Augustsson and Meyerson 2004), physiological (Barnabei et al. 2010; Moreth et al. 2014), and other differences (Brosnan-Watters et al. 2000) between strains of *M. musculus*, making this explanation plausible. The mice that we used in our toxicity assays in this study showed multiple pelage colors and were not albino like those used by Gibbs and Mackessy (2009) suggesting they were a different strain.
**Effects of Prey Evolutionary History on Venom Toxicity**

Our results also provide evidence of the effects of prey evolutionary history on venom toxicity across different evolutionary timescales. First, more closely-related prey had more similar venom responses than more distantly related prey suggesting an effect of a shared evolutionary history on venom resistance. For example, among the amphibians tested, although there were congeneric differences between species in response to venom, the largest differences in LD$_{50}$ estimates was between the two frog genera. While *L. sphenocephalus* was only 1.38 times more resistant to pigmy rattlesnake venom than *L. pipiens*, *L. sphenocephalus* was 15.5 times more resistant compared to *H. squirella*. Within the treefrogs, *H. cinerea* displayed 4.28 times the resistance to pigmy rattlesnake venom of *H. squirella*. One reason that *Hyla* treefrogs may be so much more susceptible to pigmy rattlesnake venom than leopard frogs is that they prefer above-ground refugia (Boughton et al. 2000), spending substantial time at 2–4 m vegetation heights than at ground-level where pigmy rattlesnakes forage. Although most treefrogs will need to come to ground level to breed and potentially forage efficiently, this behavioral preference for arboreality in *Hyla* compared to *Lithobates* frogs (which spend large amounts of time directly on the ground) may reduce selection pressures that favor the evolution of resistance to *Sistrurus* venoms in *Hyla* species. These differences in species’ venom resistance within the amphibian group could have consequences in conclusions made by researchers who only use only a single representative frog species in their study to make conclusions about all frogs or amphibians. For example, the rank order of
taxonomic group’s (reptile, mammal, amphibian) susceptibility to rattlesnake venom made by Gibbs and Mackessy (2009) would have changed if they had included a *Hyla* species instead of a *Lithobates* in their study. Species within a taxonomic group are not equally resistant to venom; one frog is not necessarily equivalent to any frog or all frogs with respect to their estimated LD$_{50}$. Because frogs are not equivalent, choosing a single appropriate model to represent all frogs is impossible.

Second, comparisons of toxicity estimates for native prey and related non-natives suggest evidence for the evolution of venom resistance in native prey. Specifically, our results show several examples of species pairs where a native species has a greater resistance (higher LD$_{50}$ estimate) to co-occurring rattlesnake venom than a naïve model species. Although these repeated outcomes could be caused by chance, the repeated pattern suggests the occurrence of selection for increased resistance to pigmy rattlesnake venom in native prey. Recent venom research offers evidence of local adaptation of snake venom to prey at intraspecific scales (Holding et al. 2016; Smiley-Walters et al. submitted). These traits and processes should also be considered in selecting species with which to conduct toxicology research. Native prey should be used in place of easily available model species whenever possible to collect the most relevant toxicity data for the study objectives, especially when venom studies have a focus on ecological or evolutionary interactions between venomous predators and their prey.
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Chapter 4: Evaluating local adaptation of a complex phenotype: Reciprocal tests of pigmy rattlesnake venoms on native treefrog prey

Abstract

Predator-prey interactions can create reciprocal selection pressures on populations of species pairs which can result in local adaptation yet the presence and pattern of local adaptation is poorly studied in vertebrate predator-prey systems. Here we used a reciprocal common garden (laboratory) experimental design involving comparisons between local and foreign populations to determine if local adaptation was present between a generalist predator, the pigmy rattlesnake (*Sistrurus miliarius*), and a co-occurring prey, the squirrel treefrog (*Hyla squirella*). We conducted toxicity trials using snake venom from two populations separated by 340 km tested on prey from sympatric and allopatric populations, resulting in data from four venom origin-frog origin combinations. We assessed venom effectiveness using two measures (frog mortality at 24 hours and time to frog death) and then used regression analyses to look for a signal of local adaptation with either measure. We found evidence for local adaptation for one measure (time to death) but not the other (frog mortality). We argue that in this system, the time to death of a prey item is a more ecologically relevant measure of venom effectiveness than is frog mortality at 24 hours. Our results document an example of local adaptation between two interacting vertebrates using a whole-organism assay and a
local versus foreign criteria and provide evidence that population-level variation in snake venom is adaptive.

Introduction

Antagonistic interactions between species, such as those between predator and prey, can create strong selection pressures that are important for shaping evolutionary trajectories. However especially for species with broad geographic ranges, the nature of these interactions may not be static across all populations because of spatial variation in abiotic and biotic factors. This selection landscape creates divergent pressures, which can result in local adaptation as populations evolve traits that enable them to have higher fitness in local compared to foreign environments (reviewed by Kawecki and Ebert 2004). Understanding local adaptation is important because it has broad implications in ecology and evolution including a role in the process of speciation (Schemske 2010; Lenormand 2012), maintenance of species range boundaries (Kirkpatrick and Barton 1997; Gandon 2009; Fedorka et al. 2012), individual fitness consequences related to dispersal (Kawecki and Holt 2002; Peterson et al. 2014), and the ability of species to adapt to changing climates (St Clair and Howe 2007; Schiffer et al. 2012; Golbuu et al. 2016; Mathiasen and Premoli 2016; Mosca et al. 2016). The process of local adaptation applied to species interactions means that one species effectively becomes a dynamic “environment” for the other. Local adaptation in species interactions has been most widely studied in host-parasite systems (reviewed by Greischar and Koskella 2007; Hoeksema and Forde 2008;
Keogh et al. 2016) and can ultimately influence coevolutionary dynamics between interacting species.

In practice, several definitions have been used to identify the pattern of local adaptation including home versus away, local versus foreign, and the sympatric-allopatric contrast (Kawecki and Ebert 2004; Blanquart et al. 2013). When applied to populations within a species, the home versus away criterion focuses on comparing the fitness of a population in two environments; local adaptation is present when a population has higher fitness in its home environment than in geographically distinct ‘away’ environments (Kawecki and Ebert 2004). The local versus foreign definition compares two populations within an environment and is based on the concept that a population must have higher fitness in its’ local environment than any foreign population for local adaptation to exist (Kawecki and Ebert 2004). Lastly the sympatric-allopatric contrast is a broader definition of local adaptation that focuses on the difference between the average fitness in all sympatric population-environment combinations and the average fitness in allopatric population-environment combinations; populations must have higher fitness in sympatric combinations for local adaptation to be present (Kawecki and Ebert 2004; Blanquart et al. 2013).

Debate remains concerning which of these theoretical definitions is the best to use in practice—the local versus foreign criterion is the more rigorous definition advocated by Kawecki and Ebert (2004), while the sympatric-allopatric contrast is suggested by
Blanquart et al. (2013) because of its greater power in detecting local adaptation. Note that these criteria for defining local adaptation are not independent of one another (Kawecki and Ebert 2004). A classic population-by-environment interaction pattern results on graphical fitness plots when the strict local versus foreign definition is used for local adaptation (Kawecki and Ebert 2004; Brennan et al. 2016). Finally, while testing for local adaptation by any criteria is ideally done with multiple populations, the significance of the sympatric-allopatric contrast cannot be evaluated with only two populations (Kawecki and Ebert 2004; Blanquart et al. 2013).

Any definition of local adaptation requires measurement of a trait in which variation has clear functional consequences. Snake venom is one such trait because it has a direct effect on the ability of an individual snake to immobilize (Zimmerman et al. 1990; Richards et al. 2012; Torres-Bonilla et al. 2016) and digest (Thomas and Pough 1979) its prey. In this study we examine interactions between a venomous snake and its prey. Venom is a trait with clear fitness implications for the snake that produces it. Snake venom is a complex phenotypic trait with a strong genetic basis (Wooldridge et al. 2001; Li et al. 2005; Casewell et al. 2013; Dagda et al. 2013). Venom is comprised of a mixture of enzymes, non-enzymatic proteins, and other compounds that cause physical damage and disruption of homeostasis in the envenomated prey (reviewed by Mackessy 2008), allowing prey to be immobilized prior to ingestion. Pit viper venoms may also facilitate prey digestion (Thomas and Pough 1979, but see Chu et al. 2009) as well as enhance chemosensory location of envenomated prey following snake strike and release.
behaviors (Chiszar et al. 1999). In addition to predation, venom may also play a critical role in snake defense (Hayes et al. 2002; Jansa and Voss 2011; Voss and Jansa 2012).

Snake venom is variable across multiple biological scales (individual to family-level taxonomy) (reviewed by Chippaux et al. 1991) yet the evolutionary significance of this variation is unclear. Two competing hypotheses exist for the processes that generate and maintain variation in snake venom — the first is that variation evolves via neutral processes such as genetic drift (Williams et al. 1988) while the second is that variation in venom is adaptive, driven by selection acting through diet variation that enables snakes to better subdue specific prey (Daltry et al. 1996). Recent evidence for a role of natural selection in shaping venom variation has come from the demonstration of prey-specific effects of venom (Barlow et al. 2009; Gibbs and Mackessy 2009) and trait-matching between venom function and broad patterns of snake diet (Barlow et al. 2009; Gibbs and Mackessy 2009). However, studies that assess the functional significance of venom variation at the population level are rare (but see Holding et al. 2016) yet are needed to evaluate whether widespread intraspecific variation in venom has functional significance, supporting the idea that variation has evolved because of selection rather than neutral evolutionary processes. Additional studies that use more rigorous hypothesis testing are needed to clarify this matter. By testing for local adaptation in this rattlesnake-prey system, we hope to contribute hypothesis-driven data that informs the venom community’s lingering debate over whether venom variation in snakes has functional significance in the prey, supporting the adaptive hypothesis of venom variation.
Here we use data on the toxicity of pigmy rattlesnake (*Sistrurus miliarius*) venom on squirrel treefrog (*Hyla squirella*) prey from different populations to assess whether a local versus foreign pattern of local adaptation is present when specific populations of this system are compared. Venom variation is high at the population-level for this snake (Smiley-Walters, unpublished data) and our goal is to determine if this variation has functional significance in terms of effects on a co-occurring prey species. We use reciprocal experiments in a common garden (laboratory) setting to examine the toxicity of pigmy rattlesnake venom from two study sites in Florida to squirrel treefrogs from the same two locations. We use regression techniques to test for interactions between frog origin and venom origin on frog mortality-related data. A significant frog origin by venom origin interaction term would match the local versus foreign definition of local adaptation as described by Kawecki and Ebert (2004). Based on the prevalence of venom variation in pit viper systems (Chippaux et al. 1991), the presence of strong positive selection on venom genes of *Sistrurus* rattlesnakes (Gibbs and Rossiter 2008), and the documentation of snake advantage in other rattlesnake-prey systems (Holding et al. 2016), we predict that a pattern of local adaptation, if documented, would favor the pigmy rattlesnake predator.
Methods

Study System

Pigmy rattlesnakes are generalist predators whose diet includes mice, lizards, frogs, and centipedes (Gibbs and Mackessy 2009). We chose to work with frogs as a model prey item because anurans comprise greater than 25% of the dietary items found in the gut of pigmy rattlesnakes, a percentage that is comparable only to that of lizards (Gibbs and Mackessy 2009). Chemical cues from frogs have been shown to influence the foraging site selection of *S. miliarius* (Roth et al. 1999, Bevelander et al. 2006) suggesting that they are an important prey item. By contrast, snakes do not respond to cues from mice and lizards in selecting microhabitats (Bevelander et al. 2006). We choose to work with squirrel treefrogs because of the ease of collecting this species from our two focal study sites. Additionally, *Hyla sp.* have been documented in the diet of the pigmy rattlesnake (Ernst and Ernst 2011). The pigmy rattlesnake and the squirrel treefrog have large areas of co-occurrence in the southeastern United States (Conant and Collins 1998) implying a long shared evolutionary history.

Collection of Study Animals and Venoms

We used field-collected animals from two locations separated by a Euclidean distance of approximately 340 km: Lake Woodruff National Wildlife Refuge (WOOD) in central Florida (DeLeon Springs, FL) (29.10°, -81.37°) and the Apalachicola National Forest (ANF) in the Florida panhandle (west of Crawfordville, FL) (30.17°, -84.65°). During 2014 and 2015, we collected squirrel treefrogs (n=151) at both study sites by hand and
using passive PVC pipe traps hung on trees (Boughton et al. 2000). Frogs were transported back to Stetson University (DeLand, FL) where they were held in individual plastic containers and maintained on a diet of crickets until used in toxicity assays. We located pigmy rattlesnakes at both study locations by visual survey. Walking searches were conducted at WOOD, while surveys were primarily driving at ANF, resulting in an increased distance between capture sites in the ANF. We transported snakes back to the lab where they were processed and subsequently returned to their site of capture. We recorded each animal’s weight and snout-vent-length (SVL) and collected venom from each snake by encouraging it to bite a parafilm-covered beaker. Venoms were collected between 2011 and 2013 from WOOD snakes and between 2012 and 2014 from ANF snakes. Venoms were stored at -80° C until use.

For each study site, we combined individual venom samples collected from that location into a single, pooled stock solution. The WOOD pooled venom was obtained from 49 snakes and the ANF pooled venom from 25 individuals. WOOD snakes used in this study were slightly larger (snout vent length, SVL, mean= 42.3 cm, range= 32.55-54.05 cm) than ANF snakes (SVL mean= 34.3 cm, range 26.25-46.05 cm), reflecting the paucity of large snakes observed in the ANF population. All snakes used were greater than 28 cm SVL or 20 g body weight; these measurements corresponded to pigmy rattlesnakes greater than one year in age in a long-term study at Lake Woodruff National Wildlife Refuge (May and Farrell 2012).
Laboratory Toxicity Assays

Rattlesnake venom contains high concentrations of proteins (~225-250 mg/mL) (Mackessy 2008). We diluted pooled venom from both ANF and WOOD using physiological saline (Scholar Chemistry). Next we estimated the protein concentration of diluted venoms from absorbance readings at 595 nm using the Bio-Rad Protein Assay Kit (Bio-Rad) and the bovine gamma globulin standard.

Following protein quantification, we weighed squirrel treefrogs which were to be used in an upcoming round of venom injections. Frogs were injected over several months, but each round of injections was paired with respect to venom treatment. Most rounds consisted of six or 12 frogs (range: 4-19) injected on one day. We assigned venom treatments (ANF or WOOD) randomly after stratifying the frogs by weight so that each venom treatment included both large and small animals. Using the known protein concentrations of each solution, we calculated the appropriate volume of diluted venom to deliver to each treefrog to reach a desired body-weight adjusted dose. Additional physiological saline was added to bring the injection volume to 20 µl for each frog. Injections were administered intraperitoneally into the frog’s inferior ventral side. Nine different concentrations (sample size indicated) were used for ANF frogs: 4 (6), 6 (6), 7 (12), 8 (12), 9 (12), 10 (18), 12 (12), 18 (12), and 30 (6) mg/kg. Seven concentrations were used for WOOD frogs: 4 (6), 6 (6), 7 (6), 8 (12), 9 (12), 10 (12), and 18 (1) mg/kg. The concentrations used encompassed the entire dose-response curve (0 to 100% mortality). Fewer venom doses were used for WOOD frogs because we reached full
mortality at concentrations less than 30 mg/kg and did not have as many frogs from this location. We monitored frogs for mortality at 1, 2, 3, 4, 5, 6, 8, 10, 24, and 48 hours following injection. In total, 48 ANF frogs received ANF venom, 48 ANF frogs received WOOD venom, 27 WOOD frogs received ANF venom, and 28 WOOD frogs received WOOD venom.

Data Analysis
We analyzed data using R version 3.3.1 (R Core Team 2016). First we fit the entire dataset with a probit regression model which included dose as an explanatory variable and the mortality status at 24 hours (dead or alive) as a response variable. Fitting a regression model allowed us to determine the median lethal dose (LD$_{50}$) for the entire dataset. We used the probit model here because of its long history in toxicity analyses (Finney 1952). We then repeated this process with subsets of data defined by the four unique combinations of frog origin and venom origin. In R, we used the glm function for our probit models. We then used the dose.p function available in the MASS package to estimate the LD$_{50}$ and its associated standard error. Next, we calculated a 95% confidence interval for each of the median lethal dose estimates using the estimated standard error (provided by dose.p) multiplied by the sample-size dependent 97.5 percent quantile of the student’s t-distribution (using the inverse cumulative probability distribution function).
After computing LD$_{50}$ values, we analyzed the entire data set using a logistic regression model to determine which variables (dose, frog origin, venom origin, and their 2° interactions) were significant contributors to observed frog mortality. We used a logistic regression (glm function in R) for this analysis because of a slightly better fit of this model (lower AIC) compared to the probit model as well as the greater use of logistic regression for significance testing. This model allowed us to test for local adaptation explicitly by examining whether the frog origin by venom origin interaction was significant in explaining frog mortality at 24 hours.

Lastly we examined whether the variables in our study (dose, frog origin, venom origin, and their 2° interactions) significantly contributed to the time to death response in squirrel treefrogs. Frog mortality was monitored from 1 to 48 hours as described previously. Because frogs could have died anytime between subsequent checks, we defined time to death as the midpoint number of hours between the last check the frog was recorded as alive and the first check it was recorded as dead. We increased the normality of our response variable with a square-root transformation. We then performed a multiple linear regression on the square-root transformed time to death, using the glm function in R. Similarly, to the mortality data, this model allowed us to test for local adaptation explicitly by examining whether the frog origin by venom origin interaction was significant in explaining time to death in squirrel treefrogs.
Results

In the toxicity trials, 82 of the 151 squirrel treefrogs (54.3%) died during the 24 hour interval following venom injection. We estimated the overall LD$_{50}$ for *Sistrurus miliarius* venom on *Hyla squirella* to be 8.68 mg/kg (95% CI: 7.56 - 9.81 mg/kg) using a probit regression model (Figure 5). The probit-based LD$_{50}$ estimates for each of the four frog origin-venom origin combinations were similar and 95% confidence intervals overlapped substantially (Figure 6). ANF venom injected into ANF frogs had an LD$_{50}$ of 8.80 mg/kg and ANF venom in WOOD frogs resulted in an LD$_{50}$ of 10.07 mg/kg. WOOD venom injected into WOOD frogs had an LD$_{50}$ of 8.42 mg/kg while WOOD venom in ANF frogs resulted in an LD$_{50}$ of 7.07 mg/kg. The relationship between dosage and mortality in most of our datasets were monotonic increasing, the exception being WOOD venom on ANF frogs. However, all displayed a constant percent mortality between at least two treatment doses which contributed to larger confidence intervals.

We first analyzed the treefrog mortality response using a logistic regression. As expected, venom dose (p<0.001) was a significant contributor to treefrog mortality at 24 hours after injection. Venom origin (p=0.107) and frog origin (p=0.107) were not statistically significant predictors of frog mortality in the logistic regression model. There was a strong trend toward venoms from the two populations behaving differently across the range of doses tested; the interaction between dose and venom origin was just above the level of statistical significance (p=0.062) in our logistic regression. This interaction effect can be visualized by differential shapes of the dose-response curves of
the two venoms (Figure 7); frogs that received the ANF venom treatment resulted in a fitted logistic regression model with a steeper slope than those which received the WOOD venom treatment.

The venom origin by frog origin interaction term was not significant (p=0.392) in explaining frog mortality at 24 hours in our logistic regression. Additional secondary interaction terms also were not significant. The lack of a significant interaction between venom origin and frog origin in the treefrog mortality data agrees with the pattern displayed by the LD$_{50}$ estimates (Figure 6). In general, ANF venom tended to be more toxic to treefrogs (ANF venoms displayed lower LD$_{50}$ values) compared to WOOD venom and frogs from ANF were slightly less vulnerable to snake venom (ANF frogs displayed higher LD$_{50}$ values) than frogs from WOOD (Figure 6). The parallel-line pattern displayed by our LD$_{50}$ estimates (Figure 6) and the lack of a significant venom origin by frog origin interaction term in our logistic regression analysis do not fit with the patterns expected under the prediction of local adaptation.

We analyzed the time to death response of treefrogs (n=84) that died within 48 hours after venom injection. The square-root transformation of time to death resulted in a better fitting model (AIC=232) when compared to a model performed on non-transformed data (AIC=550); the same variables remained significant in both models. Using transformed data, we found that venom dose (p<0.001), venom origin (p=0.029), and the interaction between frog origin and venom origin (p=0.033) were all significant
predictors of the time to death for squirrel treefrogs. Venom origin was not significant (p=0.405) when an interaction term between dose and venom origin was included in the model; the interaction between dose and venom origin also was not significant (p=0.834). Frog origin (p=0.442) and the interaction between frog origin and dose (p=0.428) were not significant predictors of a frog’s time to death.

The interaction between frog origin and venom origin represents a direct test for the presence of local adaptation. This interaction term is significant, displaying a classical crossing pattern when mortality for sympatric versus allopatric populations are compared (Figure 8). The average time to death was shorter for sympatric pairs: ANF frogs injected with ANF venom had a mean time to death of 6.32 hours (n=28) and WOOD frogs injected with WOOD venom had a mean time to death of 6.65 hours (n=13). In contrast the average time to death was longer for mismatched combinations: ANF frogs injected with WOOD venom had a mean time to death of 10.04 hours (n=27) and WOOD frogs injected with ANF venom had a mean time to death of 9.50 hours (n=16). The fitness trade-off pattern displayed by our time to death data (Figure 8) and a significant venom origin by frog origin interaction term for this response variable in our multiple regression analysis support the presence of local adaptation in venom function.
Figure 5. A probit regression model generated dose-response curve for pigmy rattlesnake venom on squirrel treefrogs. This graph shows the probit regression fitted values (solid line) and the summarized mortality data (solid points) for all treefrogs.

\[ \text{LD}_{50} = 8.68\text{mg/kg} \]
Figure 6. Median lethal dose (LD$_{50}$) estimates generated from probit regression models for the four combinations of frog origin (ANF or WOOD) and venom origin (ANF or WOOD). Lower LD$_{50}$ estimates correspond to more toxic venom. Error bars represent 95% confidence.
Figure 7. Fitted values from two logistic regression models of frog mortality over the range of venom doses used in our study. The regression shown by the solid line represents frogs injected with ANF venom while the regression depicted by the dashed line represents frogs injected with WOOD venom.
Figure 8. The average time to death (+/- 1.0 SEM) is shown for the four combinations of frog origin (ANF or WOOD) and venom origin (ANF or WOOD). Shorter times to death favor the snake predator; both venoms caused shorter times to death in sympatric frogs and longer times to death in allopatric frogs.
Discussion

Measures of prey mortality

A strength of this study is that it differs from other recent work on local adaptation in venom (Holding et al. 2016) in that it uses a direct measure of prey mortality instead of an indirect measure of venom effectiveness (inhibition of venom enzymatic activity) and therefore is based on a measure of venom function that is more closely tied to snake fitness. However, our ability to detect local adaptation in the S. miliarius and H. squirella predator-prey system differed depending on the measure of prey mortality that was used. Specifically, we found evidence of local adaptation using the response of time to death of the squirrel treefrog prey but not using a measure based on a more traditional measure, frog mortality at 24 hours (LD$_{50}$). This raises the issue of which measure of venom toxicity is more ecologically and evolutionarily relevant for assessing local adaptation in natural populations.

In terms of biological relevance, the LD$_{50}$ and the time from envenomation to death likely differ in how well they measure whether an envenomated prey represents a potential meal for a snake predator. As others have noted, the LD$_{50}$ of venom has limited ecological relevance because prey that die near the commonly used cut-off times of 24 or 48 hours are unlikely to result in a meal for a snake that engages in strike and release predation (Chiszar et al. 1999). This is because if the prey takes a prolonged time to die then the snake may be unable to re-locate the prey post-strike during a longer envenomation-to-
death time frame. In contrast prey that die or are immobilized quickly represent prey that are more likely to be consumed.

Another issue that is pertinent to the ecological relevance of our results is that we conducted toxicity tests with diluted venom. As venom concentration increases towards volumes realistically delivered by the snake to the prey, all prey that receive an accurate strike will likely die from the encounter, while the time to death data may scale in a more direct manner with dosage. In other words, venom that causes more rapid mortality at lower doses may also kill comparatively faster at higher doses, but venom that fails to kill at low doses may be as effective as more toxic venom once the concentration is increased. As dose increases time to death may occur at shorter time-scales. Mackessy (1988) saw a decrease in the time to incapacitation of lizards with an increase in dose. In our study we saw decreased times to death at higher doses, but the specific relationship of this scaling beyond doses tested is unknown. Overall we believe that time to death is a more ecologically relevant measure of venom function to the snake than the binomial mortality response associated with LD$_{50}$ and so it is a more appropriate measure of venom effectiveness when assessing local adaptation.

Despite the above issues, median lethal dose (LD$_{50}$) is a commonly reported measure of function in studies of snake venom toxicity (D'Império Lima et al. 1991; da Silva & Aird 2001; Mebs 2001; Mackessy et al. 2006; Mackessy 2008; Barlow et al. 2009; Gibbs & Mackessy 2009; Mackessy 2010; Richards et al. 2012; Bénard-Valle et al. 2014;
Lomonte et al. 2014; Laustsen et al. 2015) whereas studies that collect both whole-organism response data and the measure of LD$_{50}$ are rare (but see Mackessy 1988; Barlow et al. 2009; Richards et al. 2012). As discussed above the qualities that make the median lethal dose good for studies of comparative toxicity do not necessarily make it the best metric for evolutionary studies of venom. An LD$_{50}$ value is a summary statistic, generating one number from several individual mortality data points. Each mortality response is binomial, having two possible outcomes (dead or alive). Other response variables, such as time to death, can have many possible outcomes making it easier to detect small differences and provide a more sensitive assessment of venom function. As such we strongly encourage researchers conducting evolutionary studies of venom variation to combine the measure of LD$_{50}$ with other measures of function such as time to death that capture additional dimensions of the way venom acts on prey.

*Population specific effects of venom*

Our finding that sympatric venom-prey pairs resulted in more rapid mortality of treefrog prey compared to allopatric combinations of venom and prey is consistent with an antagonistic interaction where the snake predator is locally adapted and evolutionarily ahead of the prey with which it interacts. Our finding that the snake is ahead of the prey is counter to that proposed under the life-dinner principle where selection is predicted to be stronger on prey compared to predators, favoring prey advantage in an arms race (Dawkins and Krebs 1979). The snake predator displaying a pattern of local adaptation (and conversely the prey being locally maladapted) suggests that 1) this predator-prey
interaction has greater fitness implications for the snake and/or 2) it is easier for the snake to modify venom proteins than it is for the prey to modify target molecules that may be biologically conserved for other functions. Future directions for this work include 1) expanding our analyses beyond two populations to include tests of predator and prey populations at a range of spatial scales (Hanifin et al. 2008), 2) using proteomic techniques that isolate venom components to determine the specific venom protein(s) (or synergistic combinations of proteins (Borkow et al. 1993)) that may be responsible for the differences in time to death that we observed (Modahl et al. 2016), and 3) examining if local adaptation of the pigmy rattlesnake to more quickly kill squirrel treefrogs limits its ability to be locally adapted to other prey species that also makeup its diet.

In biological systems the likelihood of local adaptation is influenced by a number of factors including the relative magnitude of gene flow between the interacting species (Hoeksema and Forde 2008), the size of a population through the effect on the amount of standing genetic variation (Leimu and Fischer 2008), and the degree of specificity of the antagonistic relationship in parasite-prey systems (Lajeunesse and Forbes 2002). The amount of habitat divergence (Hereford and Winn 2008) but not geographical scale of a study (Leimu and Fischer 2008) has also been shown to influence the ability to detect local adaptation in the field. In our system selection is likely diffuse because of the broad diet of the pigmy rattlesnake (Gibbs and Mackessy 2009) and multiple predators that feed and hence exert selection on squirrel treefrogs (Binckley and Resetarits 2002; Smith 2005; Toledo et al. 2006). Population sizes of both pigmy rattlesnake predator and
treefrog prey are likely large (May et al. 1996; Farrell et al. 2011; authors’ personal observation), suggesting the effects of chance events such as genetic drift (Kawecki and Ebert 2004) or genetic surfing (Streicher et al. 2016) in the history of these populations is limited.

In addition to documenting the presence of snake local adaptation in this system, we also show that venoms from different populations of snakes have different actions over time on treefrog prey. Specifically, we found a venom origin by dose interaction in our frog mortality data, showing that ANF venom generated a steeper dose-response curve when compared to venom from the WOOD population of snakes. Synergistic effects between different venom proteins (Borkow et al. 1993) could generate this type of a functional venom difference. Also present in our data was a weak pattern showing ANF venom outperforming WOOD venom with regards to the LD$_{50}$ in both populations was present. If venom function is related to diet (Barlow et al. 2009), this trend may indicate a greater reliance on small treefrog prey in the diet of ANF snakes compared to WOOD snakes.

Lastly our time to death data indicate that venom origin is an important factor in explaining how quickly a prey item dies. Combined with our detection of local adaptation of venom in this system, we conclude that not only are snake venoms from different populations functioning differently, but they may also act in a way that enhances the snakes’ foraging success on treefrogs in this system. Overall our findings support the hypothesis of Daltry et al. (1996) that the widespread presence of population-level variation in snake venom is adaptive.
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Chapter 5. Venoms from individual pigmy rattlesnakes (*Sistrurus miliarius*) show functional differences in prey mortality

Abstract

Linking variation in molecular phenotypes to adaptive differences in fitness is a goal of evolutionary biology. Rattlesnake-prey systems permit study of the functional consequences of variable venom phenotypes, variation long believed to have important fitness consequences for venomous snakes. In this study, we determined whether individual pigmy rattlesnake venoms (n=32) caused differential mortality in a model prey species, the brown anole. We used a modified lethal toxicity assay followed by binomial regression analyses to test for the significance of individual snake, snake population of origin, venom dose, and snake morphological characteristics (SVL and weight) on the mortality of lizard prey at 48 hours. While snake morphological characteristics were not significant factors contributing to lizard mortality, individual snake ($\chi^2= 155.07$, p<0.001) and snake population of origin ($\chi^2= 12.67$, p=0.049) were both statistically significant predictors of lizard mortality. Venom dose was significant in all binomial regressions ($\chi^2=71.38$, p < 0.001). Individual snake nested within population of origin accounted for 3.6 times more variation in mortality outcome compared to snake population of origin. Our work offers the first rigorous study of individual-level variation in snake venom
function and our findings have significant implications for both venom research and future work on the evolution of adaptations in ecological systems.

Introduction

Identifying the molecular basis of adaptations and how variation in molecular phenotypes impacts fitness in natural populations are two important goals in evolutionary biology (Orr and Coyne 1992; Golding and Dean 1998). Such information is of broad significance because it addresses fundamental questions about the connection between genotype and phenotype for fitness-related traits and how selection operates on such variation. There is increasing information available on the molecular basis of adaptive traits through studies of model and non-model organisms (Hoekstra and Coyne 2007; Storz et al. 2009; Tenaillon et al. 2012; Pallotta et al. 2014; Takuno et al. 2015). However, less information is available that assesses the functional consequences of variation in molecular traits despite its importance in understanding the evolutionary basis of adaptations (Barrett and Hoekstra 2011). Traits that present an opportunity for the \textit{in vitro} assessment of function that can be convincingly linked to possible individual fitness consequences offer powerful systems for linking molecular variation to function (Storz and Wheat 2010).

Snake venom is a phenotypic trait that shows high levels for variation at the molecular level. Venom is made up of diverse molecules including enzymatic proteins, peptides, small organic molecules, and nonorganic components (Mackessy 2008; Casewell 2013;
Fox 2013) for which several in vitro tests of function are available (e.g. Bernardoni et al. 2014). Venom components function together to immobilize (Zimmerman et al. 1990; Richards et al. 2012; Urdaneta et al. 2004; Torres-Bonilla et al. 2016), digest (Thomas and Pough 1979, but see Chu et al. 2009), and locate (Chiszar et al. 1999) prey, as well as defend snakes from predation (Hayes et al. 2002; Jansa and Voss 2011; Voss and Jansa 2012). Variation in venom has been widely documented at higher taxonomic levels (Chippaux et al. 1991; Nawarak et al. 2003; Serrano et al. 2005; Mackessy 2008; Fox and Serrano 2008), among species (Mackessy 2008; Queiroz et al. 2008; Gibbs and Mackessy 2009; Barlow et al. 2009), and within species. Intraspecific venom variation has been observed between subspecies (Calvete et al. 2010; Boldrini-França et al. 2010), geographically separated populations (Glenn et al. 1983; Glenn and Straight 1989; Alape-Girón et al. 2008; Gibbs and Chiucchi 2011; Margres et al. 2015a), snake age classes (Mackessy 1988; Mackessy et al. 2006; Alape-Girón et al. 2008; Calvete et al. 2010; Margres et al. 2015b; Wray et al. 2015), and between male and female snakes (Menezes et al. 2006). A genetic basis is known for some changes in snake venom composition and function (Wooldridge et al. 2001; Li et al. 2005; Dagda et al. 2013). However, despite venom variation being nearly ubiquitous, the functional consequences of this variation remain largely unknown, particularly in terms of their impact on native prey. Some work exists on the toxicity of interspecific venom variation on non-model organisms (Jorge da Silva and Aird 2001; Gibbs and Mackessy 2009; Richards et al. 2012), but fewer studies have focused on the functional consequences of within-species variation (but see Dagda et al. 2013; Holding et al. 2016; Smiley-Walters et al. submitted). Research on functional
consequences of intraspecific venom variation would be valuable because it would clarify the selection pressures acting on genes that encode venom proteins and therefore provide insight on the evolutionary forces shaping genetic variation within populations. Intraspecific work would complement studies that show evidence of positive selection on snake venom genes at the species-level and above (Lynch 2007; Gibbs and Rossiter 2008; Rokyta et al. 2011) and account for plasticity in expression levels (Rokyta et al. 2015) that remain for this molecular trait.

Two general approaches are often used to test venom function — enzymatic assays and venom lethal toxicity assays (Chippaux et al. 1991; Jorge da Silva and Aird 2001; Modahl et al. 2016). Enzymatic assays measure the biochemical activity of individual venom components such as snake venom metalloproteases (Biardi and Coss 2011; Holding et al. 2016; Modahl et al. 2016), phospholipase A2 (Debono et al. 2016; Modahl et al. 2016), or L-amino acid oxidase (Jorge da Silva and Aird 2001; Modahl et al. 2016). Additionally, more general venom function can also be quantified including venom proteolytic, hemolytic, or fibrinolytic activity (Biardi and Coss 2011). Venom lethal toxicity assays typically have used injections to estimate the median lethal dose (LD₅₀) of whole venom in house mice (Mus musculus) (Chippaux et al. 1991), determining the weight-adjusted dose of venom that elicits a 50% mortality response. These assays use multiple individual (survival/mortality) responses across a range of venom doses to estimate a comparative LD₅₀ value, a summative measure that indicates a dose that is likely harmful to a typical prey. Modifications to lethal toxicity assays include injecting
mice with sera from native prey prior to injecting them with snake venom (Poran et al. 1987) or performing assays with native prey or close taxonomic relatives of prey instead of white mice (Mackessy 1988; Urdaneta et al. 2004; Gibbs and Mackessy 2009). Each approach for testing venom function has benefits and drawbacks; enzymatic assays can provide information about which venom components cause functional differences in venom performance and also use a procedure that reduces whole-organism mortality which is a challenge with lethal toxicity tests. However single enzyme assays ignore possible synergistic actions of toxins (Borkow et al. 1993) as they act on prey tissues. Additionally, venom functional differences must occur in vivo to have evolutionary implications in a predator-prey interaction. Thus, lethal toxicity assays likely offer the most realistic measure of whole venom function as would occur in a real-world prey envenomation.

Tests of venom function are one aspect of snake venom research, a field with a history centered on medical diagnosis and clinical effects of snake bite (Warrell 2013), antivenom development (Hawgood 2001; Winkel et al. 2006), and creation of novel toxin-based drugs (McCleary and Kini 2013). Today many venom researchers have expanded their focus to improve basic biological knowledge of venom including how this complex molecular trait interacts with snake ecology, evolution, and natural history. However, to date, much of snake venom research has relied on a methodology that was developed for medical antivenom needs, including a reliance on pooled venoms over individual venoms. A pooled venom is a solution made up of multiple individual snake
venoms of the same species. Recognition of the pooled versus individual venom alternatives for research is not a new idea (see Chippaux et al. 1991). Pooled venoms are well suited for anti-venom development because they create an average venom as a target against which to mount anti-body defenses. Despite their usefulness in medical research, pooled venoms are not ideal for studies focused on snake ecology and evolution where individually variable traits are the target of natural selection. In addition, individual venoms are highly ecologically relevant because venom from one snake enters the prey during an envenomation. Research conducted with individual venoms make the study of the individual component of a variable trait possible. Thus, individual venoms are most appropriate to use when addressing evolutionary questions such as what processes drive venom variation.

Our goal in this study was to determine if individual snake venoms have functional consequences in a naturalistic prey species. We addressed this objective using individual venoms from the pigmy rattlesnake (*Sistrurus miliarius*). We measured individual venom function with a modified venom lethality assay on a model prey, the brown anole (*Anolis sagrei*), which is consumed by *S. miliarius* in the wild. Our assay methodology is novel to the venom community and combines the strengths of the whole-organism response offered by lethal toxicity assays with a more powerful regression analysis technique. Using this approach, we were interested in whether individual (Gibbs et al. 2009) and population-level variation in venom composition (Smiley-Walters unpublished...
data; D. Rokyta personal communication), which has been documented for this species, results in differences in venom performance as measured by lizard mortality.

Methods

Snakes and Venoms

We located pigmy rattlesnakes (n=32) in the field between 2011 and 2015; the majority (n=18) of individuals were captured in 2013. Our study used snakes collected from seven different field locations in Florida including Lake Woodruff National Wildlife Refuge (WOOD, n=12), Lake George Wildlife Management Area (LAGE, n=10), Lake Monroe Conservation Area (LAMK, n=5), two disjunct locations in Ocala National Forest (OCFA, n=1, and OCFB, n=2), Merritt Island National Wildlife Refuge (MEIS, n=1), and Apalachicola National Forest (APNF, n=1) (Figure 9). Except for the Apalachicola National Forest, all of these sites were located in close proximity to one another in central Florida. The three sites where we sampled the majority (n=27) of our snakes are separated by a Euclidean distance of approximately 60 km. At each study site, we located adult snakes by visual survey between late May and late October. We transported captured snakes back to Stetson University (DeLand, FL) and recorded their snout-vent-length (SVL) and weight. We then extracted venom by coaxing each snake to bite a parafilm-covered beaker. The average snout-vent length of snakes from which venom was obtained was 41.7 cm (range: 31.0 – 49.9 cm), while the average weight was 63.7 g (range: 30.0 – 123.0 g). We stored whole venoms at -80° C until they were used in toxicity trials.
We did not pool venoms in this study, but worked with venoms from individual snakes. Because snake venoms have high protein concentrations (~225-250 mg/mL) (Mackessy 2008), we diluted each venom using saline solution (Scholar Chemistry). We created three stock solutions for each snake venom and estimated the protein concentration of each in duplicate or triplicate using the Bio-Rad Protein Assay Kit (Bio-Rad) and a bovine gamma globulin standard. For all but one snake, we used all three stock solutions as venom sources in toxicity trials to minimize the likelihood that lizard deaths resulted from poor protein quantification of a single solution; the remaining snake venom used two of the three stock solutions.

**Prey and Toxicity Trials**

We used brown anoles as a model prey species because they are an invasive species in the state of Florida (Kolbe et al. 2007), easy to obtain, preyed upon by pigmy rattlesnakes where the species co-occur (SSW and TF personal observation), and they are congeneric to a native prey species, the green anole (*Anolis carolinensis*). We collected brown anoles by hand in DeLand, FL (29.035°N, -81.300°W) and housed them in individual plastic containers. We weighed each lizard and only used anoles at least 1.0 g mass in venom toxicity trials.

We conducted toxicity trials in 2014 and 2015 using the 32 individual snake venoms. We determined initial dosage for our trials based on the LD$_{50}$ for the brown anole estimated
from pooled venom (Gibbs and Mackessy 2009; Smiley-Walters unpublished data). Each
diluted venom was injected into 12 brown anoles at mass-dependent doses ranging from
0.6 to 2.2 mg protein per kg lizard body weight. We used the same doses (0.6, 0.8, 0.9,
1.2, 1.5, 1.8, 2.0, 2.05, 2.1, 2.15, 2.2, and 2.2 mg/kg) for each snake venom (Figure 10).
We conducted toxicity trials with 25 snake venoms in 2014 and an additional 7 snake
venoms in 2015. Within each year, we performed venom injections in paired rounds so
that all venoms were injected at the same dose into all lizards on the same day. A round
included all venoms tested within that year. In each round of toxicity testing, we injected
each lizard with venom from a randomly assigned snake (e.g. one of 25). Additionally,
as a control, we included one to four saline controls each day toxicity trials occurred. We
used an injection volume of 20 µl for each lizard; this venom-saline solution was
delivered intraperitoneally on the ventral side. We closely monitored lizards in the hours
following venom injection. We recorded the status of each lizard (alive or dead) at 24
and 48 hours after injection. In total, we injected 384 brown anoles with a venom
solution (32 snake venoms x 12 lizards each) and 37 brown anoles with a saline control
solution.

We controlled for multiple sources of variation in our toxicity trial methodology
including reducing temporal variation by conducting injections in paired trials. However,
other sources could not be removed from our study such as variation in resistance
exhibited by individuals within our lizard population. This variation is part of any natural
predator-prey system. Thus, while lizard individual variation increases the noise in our
dataset it does not bias our findings (venom treatment was randomly assigned to individual lizards) and is a real component of this ecological system.

Data Analysis

To visualize our raw data, we used R version 3.3.1 (R Core Team 2016) including generating a heat map showing the outcome of each lizard toxicity trial for each venom. To compare our toxicity data with other studies, we estimated the median lethal dose ($LD_{50}$) of our complete dataset at 24 and 48 hours in R using logistic regressions (glm function) with venom dose as an independent variable followed by the dose.p function. In all subsequent analyses, we disregarded the saline controls and focused on the 48 hour endpoint of our observations. In general, we tested for the significance of variables using likelihood ratio tests (function anova or function lrtest of the epiDisplay package) to compare full and reduced logistic regression models that we created with the glm function in R. We included venom dose in all binomial regressions. The first of these likelihood ratio tests was to determine the significance of individual snake, comparing a binomial model including both dose and individual snake to one including only dose. To test for the significance of individual variation within our two largest populations, we repeated this test in each of two subsets of data (WOOD and LAGE). Lastly, to determine if individual snake was significant when nested within population of origin, we used JMP version 10.0.0 to run a logistic model with nested parameters.
To determine if other parameters besides individual snake influenced 48 hour lizard mortality, we compared additional full and reduced models with likelihood ratio tests (anova function). However, here we used mixed logistic regression models (glmer function in package lme4) because individual snake was significant in our above analyses, necessitating the use of mixed models with individual snake as a random effect. We tested for the significance of venom dose, venom population of origin, snake snout-vent length (SVL), and snake weight in predicting lizard morality in this manner. Lastly, to partition the variation in our data, we performed an ANOVA in JMP on the mortality data comparing the percent variation explained by population of origin to the percent explained by individual snake nested within population of origin.

Results

Of the 384 lizards injected with a venom solution, 129 (33.6%) were dead after 24 hours and 135 (35.2%) were dead after 48 hours. Thus, most of the deaths occurred in the first 24 hours following injection. None of the saline treatment controls died in the 48 hours following injection. Using the entire dataset, we found the median lethal dose of individual pigmy rattlesnake venoms on *Anolis sagrei* to be 2.16 mg/kg (SE = 0.088) at 24 hours and 2.09 mg/kg (SE = 0.079) at 48 hours using a logistic regression model. Given that the most complete representation of the data is based on mortality at 48 hours, we report the 48 hour results for the remainder of our analyses.
Individual snake was significant in determining lizard mortality ($\chi^2 = 155.07$, p<0.001) (Figure 10) based on a likelihood ratio test comparing logistic regression models. When limiting the dataset to a subset from each of the two largest populations, individual snake remained significant in both the WOOD population ($\chi^2 = 40.41$, p<0.001) and the LAGE population ($\chi^2 = 55.09$, p<0.001). Similarly, individual snake nested within population of origin was a significant predictor of mortality in a logistic regression run in JMP ($\chi^2 = 113.17$, p<0.001). At 48 hours, snake venoms displayed a bi-modal distribution with respect to the number of lizards killed (Figure 11). This finding suggests that two broad classes of venoms exist with respect to toxicity to lizards: those with high and those with low toxicity.

As expected, venom dose was a significant predictor of lizard mortality in our mixed logistic regression ($\chi^2 = 71.38$, p < 0.001). Snake SVL ($\chi^2 = 0.34$, p = 0.561) and snake weight ($\chi^2 = 0.44$, p = 0.508) were not significant predictors of lizard mortality. However, snake population of origin was a significant predictor ($\chi^2 = 12.67$, p = 0.049). Snake venoms from WOOD were the most lethal to lizards, followed by LAMK, LAGE, and OCFB (Figure 12). Individual snake, nested within population of origin, accounted for 3.6 times more variation in lizard mortality in an ANOVA than snake population of origin suggesting much higher levels of functional variation at the level of individual compared to that of population.
Figure 9. Snake collection sites. Snakes venoms were sampled from seven populations in Florida; each population is indicated by a red circle. A) Site APNF (n=1) and central Florida region indicated by red square. B) Enlarged region of central Florida showing sites OCFA (n=1), LAGE (n=10), WOOD (n=12), OCFB (n=2), LAMK (n=5), and MEIS (n=1). The linear distance between LAGE and LAMK is approximately 60 km.
Figure 10. The outcome of each venom-dose combination for the toxicity trials. The end status of each lizard is represented by a shaded rectangle: dark grey represents anoles that died by 24 hours, medium grey represents those that died by 48 hours, and whitish-grey represents those that were alive after 48 hours. Snake venoms are ordered so as to cluster snakes from the same population together (e.g. LAGE) and to sort snakes within a population by their venom lethality.
Figure 11. Histogram of the number of lizards killed during the 48 hour post-injection period out of the 12 that were tested for each individual snake venom. Snake venoms show a bimodal distribution with many snake venoms killing either a low (0-3) or high (6-9) number of lizards. Few venoms resulted in a number of kills near the average mortality rate of 35.2% which corresponds to 4.2 lizards killed. Five venoms resulted in 50% lizard mortality.
Figure 12. The average percent mortality for lizards is variable across the four populations represented by multiple venom samples. Averages are shown for the populations of WOOD (n=12), LAMK (n=5), LAGE (n=10), and OCFB (n=2). Error bars indicate +/- 1.0 SEM.
Discussion

Our study provides the first quantitative assessment of the functional significance of individual snake venom variation. We show that variation at the level of individual snake explains >3.5 times the variation in lizard mortality compared to population level variation in this rattlesnake species. While regional geographic differences in venom composition (Glenn et al. 1983; Glenn and Straight 1989; Alape-Girón et al. 2008; Gibbs and Chiucchi 2011; Margres et al. 2015a) and function (Glenn and Straight 1989; Glenn et al. 1994; Holding et al. 2016) have been described in several snake species, there are only anecdotal reports of variation in whole venom function at the individual level. For example, a range of values of lethal toxicity from individuals of the same venom type (A, B, or A +B) were reported in *Crotalus scutulatus* by Glenn and Straight (1989).

Elsewhere, results of specific enzymatic assays have described intra-specific venom variation but fail to conduct associated significance tests (Dagda et al. 2013) or fail to describe how this variation compares to that which exists at the level of population (Dagda et al. 2013; Holding et al. 2016). As a whole, individual variation has been largely overlooked and focus has remained on larger biological scales. If generalizable to other venomous animal species, our work suggests the importance of assessing functional variation among individual venoms and developing ecological and evolutionary explanations for the origin and maintenance of this variation.

One of the shortcomings of our study is that we worked with highly diluted volumes of venom. It is important to recognize that if our toxicity tests used venom protein amounts
similar to those of natural pigmy rattlesnake envenomations, we would likely have seen complete lizard mortality. By diluting venom, we were able to inject multiple prey items (n=12) with a single venom, as well as perform accompanying protein quantifications.

We conducted toxicity assays near an area of the logistic curve with maximal variation in lizard mortality. Thus, we used *Anolis sagrei*, a species very susceptible to pigmy rattlesnake venom, as a model for how variation in venom components functions in a whole organism. The variation in venom function that we documented likely has greater ecological relevance in more venom-tolerant prey species such as leopard frogs (Gibbs and Mackessy 2009; Smiley-Walters unpublished data) or in species which have moderate susceptibility to pigmy rattlesnake venom but experience an imperfect strike that results in a reduction in venom volume successfully delivered.

Our results raise several relevant explanations concerning generating mechanisms and repercussions of venom variation at the individual level. We found that pigmy rattlesnakes differed in their toxicity to lizards; some snakes had venom that was “hot” toward killing lizards while others had “cold” venom. The presence of different phenotypes within populations rather than dominance of one superior phenotype presents questions concerning trade-off constraints in this system. It is possible that venoms that have lower toxicity toward brown anoles are better adapted to killing other taxa of prey compared to snakes with venom of higher anole toxicity. These conclusions are plausible based on assays with snake venom metalloproteases showing that fractions differ in their reactivity with sera from mammals versus birds (Bernadoni et al. 2014). The high levels
of functional variation in our system also suggest a mechanism for maintaining this variation in the predator population over time to be some form of frequency-dependent selection (Christiansen 1988; Endler and Greenwood 1988; Levin et al. 1988; Bolnick and Lau 2008) or an inducible offensive strategy (Kopp and Tollrian 2003a; Kopp and Tollrian 2003b; Levis et al. 2015). We feel that the next step in this line of research is to determine if the differential mortality that we documented for pigmy rattlesnake venoms on the brown anole can be correlated with specific proteins or combinations of proteins in the venoms of these snakes. Additionally, examining if venom function changes or is constant over time in individual snakes would be useful in informing the stability of phenotypic traits in this system.

Our findings have implications for venom research study design. First, we estimated the 24 hour LD_{50} of pigmy rattlesnake venom on brown anoles to be 2.16 mg/kg (SE = 0.088) in this study using individual venoms. This number is considerably higher than those found using the same species but with pooled venoms where the 24 hour LD_{50} was estimated at 0.66 mg/kg by Gibbs and Mackessy (2009) and near 0.8 mg/kg in our studies (Smiley-Walters unpublished data). These differences in LD_{50} estimates suggest that functional assays done with pooled venom may reflect the more toxic individuals included in them rather than an averaging of component venoms. More work is needed to determine how functional properties of venom change with different pooled combinations of individuals. However, we can currently say that summary metrics of pooled and individual venoms are not identical.
A second implication relates to the fact that intraspecific and individual-level variation along with the causes and consequences of this variation have recently seen renewed focus in various scientific sub-disciplines including general ecology (Bolnick et al. 2003; Araújo et al. 2011; Wolf and Weissing 2012), behavioral ecology (Sih et al. 2004; Réale et al. 2010; Dall et al. 2012; Wolf and Weissing 2012), community ecology (Bolnick et al. 2011; Violle et al. 2012; Hart et al. 2016), trait-based plant ecology (Albert et al. 2011), evolutionary ecology (Dall et al. 2012; Wolf and Weissing 2012), epidemiology (Lloyd-Smith et al. 2005), and endocrinology (Williams 2008). Collectively, these studies indicate that individual variation has important implications in ecology and evolution and that a lack of appreciation for this variation can lead to oversimplification of biological systems. Our results and these interdisciplinary insights should inform venom research. An appreciation for and focus on individual variation will open new doors for research and allow collection of more appropriate data in venomous animal systems and other fields where measures of central tendency remain the primary focus. For example, intraspecific diet specialization has been documented in many species (Bolnick et al. 2003; Woo et al. 2008; Araújo et al. 2011) and individual-level research could inform whether this process is contributing to maintenance of intraspecific variation in venomous species with diverse diets such as the pigmy rattlesnake (Gibbs and Mackessy 2009). Focus on the individual will lead to more appropriate information to address questions that center on snake ecology (e.g. predator-prey dynamics) and evolution (e.g. selection on venom proteins) where Darwinian selection operates on the
individual. We recommend the venom community recognize that for non-medically relevant research questions, individual venoms should be used whenever possible. By placing emphasis on the individual, we can continue to build a scaffold connecting complex molecular traits to their molecular basis, fitness implications, and ultimately repercussions at the level of the ecological community.

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Chapter 6: Conclusions

My dissertation research focused on the pigmy rattlesnake-prey system in order to broaden knowledge of snake-prey interactions in general and explore the functional consequences of adaptive trait variation, in the form of venom variation, in this system. Pigmy rattlesnakes can reach very high densities in the southeastern United States (May et al. 1996) and are seasonally the most abundant snake in road surveys (Dalrymple et al. 1991; SSW personal observation). Despite being common, very little is known about interactions between this snake and its many species of prey. Additionally, information concerning the effect of ambush-foraging predators, such as rattlesnakes, on prey species in general is rare compared to predators using other modes of foraging (Pressier et al. 2007). As introduced in Chapter 1, my dissertation work used a multi-faceted approach to fill this knowledge gap in a system where the predator uses venom as an offensive weapon to consume a diverse set of prey.

Specifically, in Chapter 2, I explored the foraging behavior of a native mouse in the presence and absence of a rattlesnake predator. My work documented no change in cotton mouse foraging behavior in response to the presence of pigmy rattlesnakes but that the novelty of the environment created by the experimental design was a much larger predictor of seed consumption than the presence of a predator. My research raises
questions concerning whether pigmy rattlesnakes may evade detection by their prey by being chemically cryptic, in addition to visually camouflaged, as documented in other systems (Miller et al. 2015). My findings also may place increased emphasis on the aspects of predation that involve interactions related to venom in this system rather than behavioral interactions between these rattlesnakes and their prey.

In Chapter 3, I present the first of three chapters that explore the function of venom in the pigmy rattlesnake-prey system. I explored venom function at multiple biological scales. In Chapter 3, I started at the broadest scale by examining venom toxicity to different species within broad taxonomic groups of prey. My research had the specific goal of comparing venom toxicity in native prey species to non-native prey species. I demonstrated that not all species within a taxonomic group are equally susceptible to pigmy rattlesnake venom but that, in most species pairs compared, the native species was more resistant to venom than was the non-native species in the same taxonomic group. Additionally, as best illustrated with the toxicity data on frogs, congeneric species perform more similarly to one another, in terms of their venom susceptibility, than do more distantly related species in the same broad taxonomic group. Besides providing a baseline of information for my work in the subsequent chapters of my dissertation, my research in this chapter has important implications for ongoing research in the venom community. I suggest that native species should be used for venom toxicity testing, especially when the goals of the research are to address evolutionary or ecological
questions. I also argue for the use of congeners of native species in toxicity studies when native species are not available.

In Chapter 4, I used population-level data from pigmy rattlesnake venom on co-occurring squirrel treefrog prey to test for the presence of local adaptation in this system. Using the local versus foreign definition of local adaptation (Kawecki and Ebert 2004), I found that the signal of local adaptation was present in one response metric used in this system, but absent in the other. Specifically, the response of time to death of the squirrel treefrog prey after injection with venom displayed a signal of local adaptation, but the more traditional measure, frog mortality at 24 hours, did not show the signal of local adaptation. I argue that the time to death measure is a much more biologically relevant measure of venom effectiveness for a strike-and-release venomous snake compared to the 24 hour mortality measure. My findings from this chapter have implications for studies on local adaptation and the hypotheses concerning evolution of venom variation. First, my research suggests that not all response measures are equal for detecting local adaptation in ecological studies. This is important because research using only one response variable may fail to detect local adaptation if that variable is not the most biologically relevant to the species interaction. Second, my work offers additional evidence that venom is an adaptive trait and suggests that venom variation may result from adaptive rather than neutral processes.
In Chapter 5, my research addressed the population and individual-level variation in function of pigmy rattlesnake venom using individual rather than pooled snake venoms. This is the first study to rigorously examine individual-level function in a venomous snake system. Working with a common introduced lizard species (brown anole) that is congeneric with a native prey species, I injected a total of 384 lizards with venom and assessed their mortality rates. Based on this data set, I found that individual variation is very important in determining lizard mortality in this system. The venom of some individual snakes had a much greater effect on lizards than the venom of other individual snakes within the same population, although population-level differences in venom performance were also observed. My results provide strong evidence that individual-level variation in venom function is important in populations of venomous snakes.

In summary, broadly speaking, my results demonstrate that venom function varies at multiple biological scales in both rattlesnakes and in their prey. My research offers methodological guidelines for future work on venom function, especially for research asking ecological and evolutionary questions about this complex adaptive trait.


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