Toxicon 144 (2018) 42–47

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

The importance of species: Pygmy rattlesnake venom toxicity differs between native prey and related non-native species

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ARTICLE INFO

Article history:
Received 12 November 2017
Received in revised form
29 January 2018
Accepted 30 January 2018
Available online 2 February 2018

Keywords:
Sistrurus
LD_{50}
Dose-response
Native and non-native prey
Model species
Comparative toxicity

ABSTRACT

Venom toxicity assessments are often based upon non-native surrogate prey species that are not consumed in the wild by the venomous predator. This raises questions about the relevance of toxicity results on these "model" prey in addressing ecological or evolutionary questions about venom effects on native prey. We explore this issue by comparing the toxicity of venom from pygmy rattlesnakes (*Sistrurus miliarius*) on taxonomically-diverse sets of model (non-native) and native prey. Specifically, we compared rattlesnake venom toxicity for nine species from three broad taxonomic groups of prey (reptiles, mammals, and amphibians) to determine whether estimates of venom toxicity for the non-native model species of each group was representative of species which were native prey. 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 (*Anolis carolinensis*, *Peromyscus gossypinus*, *Lithobates sphenocephalus*, *Hyla cinerea*, and *Hyla squirella*) 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 in that in each group, non-native models and congeneric native prey showed more similar responses than prey from other genera suggesting that venom may act on common prey targets that result from common ancestry. Second, native prey generally showed higher LD_{50} values than their non-native counterparts, suggesting greater resistance to venom from a predator with which they interact in nature. Our results suggest that researchers should use native prey to generate measures of venom toxicity that are ecologically and evolutionarily relevant. If this is not possible using "model" prey species that are close taxonomic relatives to natural prey may be a reasonable alternative.

1. Introduction

Snake venom plays an essential role in successful foraging by contributing to prey immobilization (Mackessy, 1988; Zimmerman et al., 1990; Richards et al., 2012; Urdaneta et al., 2004; Torres-Bonilla et al., 2016), digestion (Thomas and Pough, 1979, but see Chu et al., 2009), and chemosensory location of prey (Chiszar et al., 1999) following envenomation. These functional effects are the result of the impact of the molecular components of venom on features of prey physiology (Mackessy, 2008). Venom composition varies across multiple taxonomic levels (Chippaux et al., 1991) and is thought to represent adaptive variation that has evolved via natural selection that 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, 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, much of this work has used non-native "model" laboratory species not consumed in the wild as surrogate prey (e.g. Mackessy, 2008). This raises questions about how representative these results from non-native prey are for making inferences about venom toxicity and function relative to native prey which in contrast have a shared ecological and evolutionary history with a given venomous snake.

The most widely used method for assessing prey-specific effects of venom involves constructing a dose-response curve by exposing

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https://doi.org/10.1016/j.toxicon.2018.01.022
0041-0101/

prey to a range of venom doses. 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), allowing estimates of 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 specific 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 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 prey which lack a shared evolutionary history with the snake predator. 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 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 these preceding studies were based on LD_{50} point estimates without confidence intervals. Bénard-Valle et al. (2014) did include confidence intervals in their description of different LD_{50} values for *Micrurus tener* venom on a *Mus* murid model versus the snake *Conopsis lineata*, a sympatric potential, but undocumented (Ernst and Ernst, 2011), prey species. Similarly, Gibbs and Mackessy (2009) documented significant differences in LD_{50} estimates with corresponding error estimates across broad taxonomic groups of prey tested with venoms from four species of *Sistrurus* rattlesnakes. Their results showed that wild caught leopard frogs (*Rana pipiens*) were more resistant to *Sistrurus* venom when compared to non-native brown anoles (*Anolis sagrei*) and house mice (*Mus musculus*).

Although laboratory-raised or commercially available organisms like *Mus* or *Gallus* are readily accessible for toxicity tests, they typically have no recent 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 their resulting evolutionary consequences. As such, native prey may offer more relevant measures of venom toxicity, yet few studies have statistically tested whether this is the case.

The complications associated with drawing toxicity inferences using tests on model 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. Even within mammals, the house mouse may be a poor model as it has substantially lower resistance to venom compared to native mammals such as the woodrat (*Neotoma micropus*) and California ground squirrels (*Otospermophilus beecheyi*) as evidenced by traditional LD_{50} or serum-protective LD_{50} tests (Perez et al., 1978; Poran et al., 1987). Additionally, in invertebrates, captive bred desert locusts (*Schistocerca gregaria*) were poor proxies for assessing saw-scaled viper (*Echis* sp.) venom toxicity and performance on natural scorpion prey (Richards et al., 2012). It remains to be seen whether these previous results are generalizable to other species and taxonomic groups of snake prey (for example, amphibians).

In this study, we build on previous work examining whether non-native "model" organisms are representative of native species in terms of their susceptibility to venom from pygmy rattlesnakes (*Sistrurus miliarius*). 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 pygmy rattlesnakes (*Sistrurus miliarius*) on different prey species representative of three broad taxonomic groups (reptiles, mammals, and amphibians) eaten 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 pygmy rattlesnake venom toxicity of different broad taxonomic groups and comparing model organisms to natural prey, respectively. Our work furthers previous comparative research on non-native versus native prey species by using quantitative statistical tests and native prey field-captured from regions of co-occurrence with the snakes.

2. Materials and methods

2.1. Collection and processing of rattlesnake venom

We collected pygmy rattlesnakes (*Sistrurus miliarius*) using visual surveys 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. 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.

2.2. Prey acquisition and lethal toxicity assays

We obtained LD_{50} estimates for pygmy rattlesnake venom on nine different native and non-native prey species from both the laboratory and the literature. Five native prey species (identified on the basis of diet data from Gibbs and Mackessy, 2009 and Farrell and May [unpublished data]) were captured in the vicinity of Lake Woodruff National Wildlife Refuge: the green anole (*Anolis carolinensis*), the cotton mouse (*Peromyscus gossypinus*), the southern leopard frog (*Lithobates sphenocephalus*), the green treefrog (*Hyla cinerea*), and the squirrel treefrog (*Hyla squirella*). We captured non-native brown anoles (*Anolis sagrei*) on the grounds of Stetson University (DeLand, FL, USA). We purchased non-native house mice (*Mus musculus*) from a local commercial supplier. The laboratory methods used for assessing toxicity for these seven species are detailed below. We supplemented our laboratory data with data available from a previous study on *Sistrurus* venom toxicity (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 a second set of identically analyzed parameters (LD_{50} and standard error estimates) on the house mouse and brown anole as well as initial estimates on a species not tested with our laboratory methods, the non-native northern leopard frog (*Lithobates pipiens*). Finally, we also used an LD_{50} estimate for pygmy rattlesnake venom reported on the non-native 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 pygmy 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 non-native prey 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-h mortality status (alive or dead).

2.3. 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 genera (in frogs only). 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. (2017), 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 in mortality responses between native and non-native species within each broad taxonomic group (reptiles, mammals, and amphibians) using the dose-response data. In the two species (*M. musculus* and *A. sagrei*) for which data were available from Gibbs and Mackessy (2009) and this study, we first tested 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 non-native *Anolis sagrei* ($n = 47$) and native *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 by comparing non-native *M. musculus* ($n = 18$, data only from this study) versus native *P. gossypinus* ($n = 42$). In the frogs, we 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 native *H. cinerea* ($n = 40$) versus native *H. squirella* ($n = 28$) and non-native *L. pipiens* ($n = 18$) versus native *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.

3. Results

Overall, our results demonstrate significant species-specific toxicity of pygmy rattlesnake venom on prey. In the reptiles, we found no statistically significant effect of study on mortality ($z = 0.966$, $p = 0.334$, $df = 44$). Therefore, we combined data from 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*, ($LD_{50} = 0.81$ mg/kg) was approximately four times more susceptible to pygmy rattlesnake venom in our analysis than the native species, *A. carolinensis* ($LD_{50} = 3.83$ mg/kg) (Fig. 1).

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 mg/kg, $n = 15$) than our non-albino mice purchased commercially which had an estimated LD_{50} of (15.18 mg/kg, $n = 18$). 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 h ($z = 2.35$, $p = 0.019$, $df = 57$). The overall LD_{50} estimate for *M. musculus* ($LD_{50} = 12.89$ mg/kg) was one third of the LD_{50} estimated for *P. gossypinus* ($LD_{50} = 33.89$ mg/kg) and comparable to that of *R. norvegicus* ($LD_{50} = 9.72$ mg/kg) (Fig. 2). Thus, our data on mammals suggests that *M. musculus* is a poor model for the native rodent (*P. gossypinus*) in our rattlesnake-prey system.

Finally, in amphibians, we found large differences in the LD_{50} estimates for the four frog species tested (Fig. 3). *Lithobates sphenocephalus* had the highest LD_{50} ($LD_{50} = 130.51$ mg/kg), followed by *L. pipiens* ($LD_{50} = 94.69$ mg/kg), *H. cinerea* ($LD_{50} = 36.04$ mg/kg), and *H. squirella* ($LD_{50} = 8.42$ mg/kg). Our probit regression analysis found genus to be a significant predictor of frog mortality at 24 h

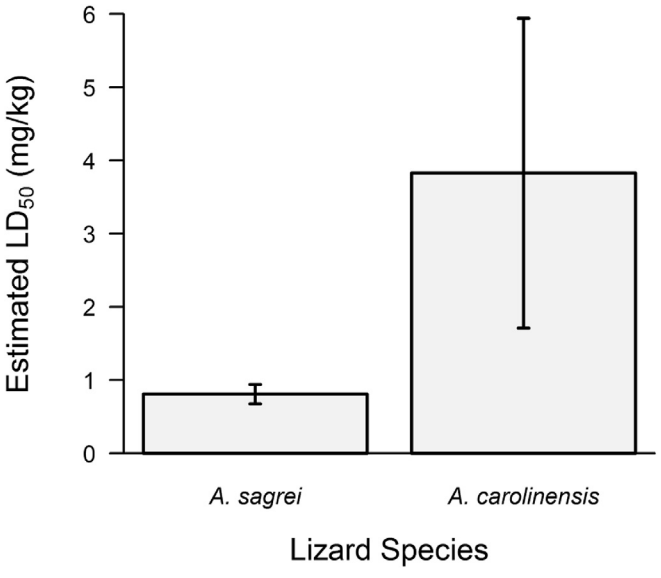

Fig. 1. Estimated median lethal dose (LD_{50}) for two congeneric lizard species. Error bars represent the 95% confidence interval.

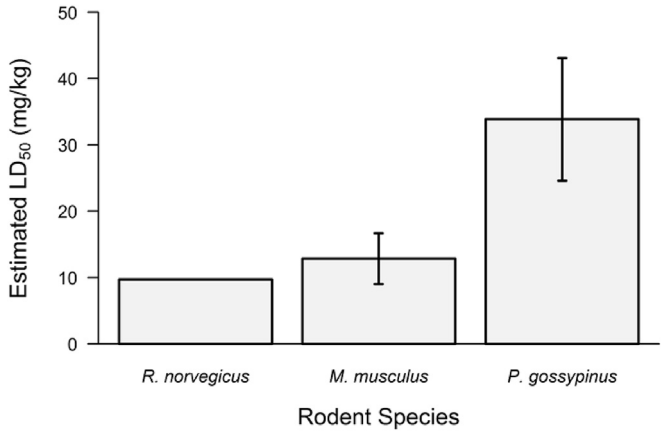

Fig. 2. Estimated median lethal dose (LD_{50}) for three rodent species. Error bars represent the 95% confidence interval. The estimate for *R. norvegicus* was taken from Assi and Nasser (1999). The estimate for *M. musculus* is based on combined data from both Gibbs and Mackessy (2009) and this study.

($z = 4.14$, $p < 0.001$, $df = 104$) with *Hyla* being more susceptible to pygmy rattlesnake venom than *Lithobates*. Within *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 *Hyla*, we found species to be a significant predictor of frog mortality ($z = 2.48$, $p = 0.013$, $df = 65$). *Hyla squirella* were more susceptible than *H. cinerea* to pygmy rattlesnake venom (Fig. 3). Our data on amphibians suggest that *L. pipiens* may be a reasonable model species for the congeneric *L. sphenocephalus* but not for all frogs as it is a poor proxy for treefrogs (*Hyla* sp.).

4. Discussion

4.1. Measures of venom toxicity

Our study indicates that many commercially available non-native prey species may be poor proxies for the toxicity responses of native prey species. In each of the three 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 one another 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 affinity 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.

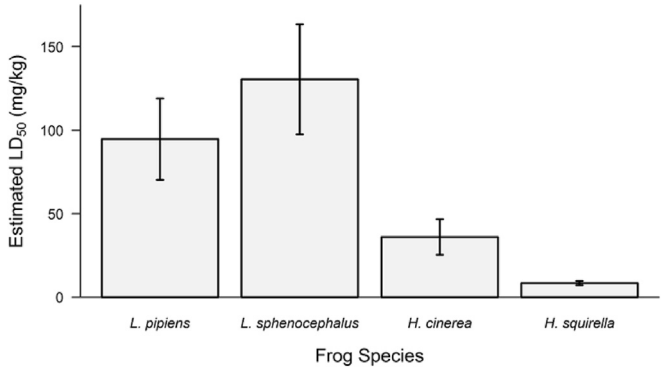

Fig. 3. Estimated median lethal dose (LD_{50}) for four frog species. Error bars represent the 95% confidence interval. The estimate for *L. pipiens* is based on data from Gibbs and Mackessy (2009).

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 pygmy rattlesnake (Smiley-Walters et al., 2017) and the different studies used venom of different geographic origin and, hence, potentially composition. However, these differences would fail to explain why this study effect was limited to the mammals because results for *Anolis* and *Lithobates* did not differ between studies. It is possible that there was a venom difference that had a differential impact on 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 pygmy 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 coat colors and were not albino mice like those used by Gibbs and Mackessy (2009) suggesting they were a different strain.

4.2. 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 were between the two frog genera. While *L. sphenocephalus* was only 1.38 times more resistant to pygmy 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 pygmy rattlesnake venom of *H. squirella*. One reason that *Hyla* treefrogs may be so much more susceptible to pygmy 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 rather than at ground-level where pygmy rattlesnakes forage. Although most treefrogs 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 *Hyla squirella* 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 another frog species with respect to their estimated LD_{50}, making the choice of a single appropriate model to represent this taxonomic group problematic. Species differences within larger taxonomic groups have been shown for specific venom components (Heyborne and Mackessy, 2013).

Second, comparisons of toxicity estimates for native prey and related non-natives suggest evidence for the evolution of venom resistance in native prey which have extended interactions with the rattlesnake which is the source of the venom used in the tests. Specifically, our results show several examples of species pairs (e.g. *Anolis* lizards) where a native species has a greater resistance (higher LD_{50} estimate) to co-occurring rattlesnake venom than a naïve model species. The predominate use of non-native model species in venom toxicity studies probably has resulted in a consistent overestimation of venom toxicity. Although these repeated outcomes could be caused by chance, the repeated pattern suggests the occurrence of selection for increased resistance to pygmy 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., 2017). 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.

Ethics statement

All animal experiments complied with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and all appropriate institutional permits were obtained for field collections and toxicity tests (permit numbers provided in the Acknowledgements section).

Acknowledgements

Financial support for this study was provided by grants from the East Texas Herpetological Society and the American Society of Mammalogists to SASW. Additional support for our work came from The Brown Visiting Teacher-Scholar Fellows Program at Stetson University. We collected pygmy rattlesnake venoms under a License to Possess or Exhibit Venomous Reptiles and/or Reptiles of Concern from the Florida Fish and Wildlife Conservation Commission with the following license renewal identifications (year): 411-75193 (2011-2012), 411-95180 (2012-2013), 411-104419 (2013-2014), and 411-115575 (2014-2015). Native prey species were caught under a Scientific Collecting Permit from the Florida Fish and Wildlife Conservation Commission (permit number LSSC-11-00067A) and Lake Woodruff National Wildlife Refuge Special Use Permits (2012-002, 2013-004, and LW2014-500R). Our study methodology was approved by Stetson University IACUC (protocol # 2011TF101). We thank Steve Mackessy and Callie Wolfe for help with developing and conducting toxicity assays, respectively. We are also thankful to the numerous colleagues, visiting researchers, and Stetson University students who helped locate snakes in the field.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.toxicon.2018.01.022.

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