Functional basis of a molecular adaptation: Prey-specific toxic effects of venom from Sistrurus rattlesnakes

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A B S T R A C T

Understanding the molecular bases of adaptations requires assessing the functional significance of phenotypic variation at the molecular level. Here we conduct such an assessment for an adaptive trait (snake venom proteins) which shows high levels of interspecific variation at the molecular level. We tested the toxicity of venom from four taxa of Sistrurus rattlesnakes with different diets towards 3 representative prey (mice, lizards and frogs). There were significant differences among prey in their overall susceptibility to Sistrurus venom, with frogs being an order of magnitude more resistant than mice or lizards. However, only in mice was there substantial variation in the toxicity of venom from different Sistrurus taxa, with the variation being roughly correlated with the incidence of mammals in the snake's diet. A comparative analysis using published data of the toxicity of rattlesnake and outgroup (Agkistrodon) venoms to mice confirms that both the gain and loss of toxicity to mammals were major modes of venom evolution in Sistrurus catenatus and Sistrurus miliarius. Our findings identify toxicity to mammals as a major axis along which venom evolution has occurred among Sistrurus rattlesnakes, with little evidence for evolutionary changes in toxicity towards the other prey tested. They also emphasize the need to consider ecological and evolutionary factors other than diet alone as causes of variation in venom toxicity.

1. Introduction

Identifying the molecular basis of adaptations in natural populations is an important yet largely unrealized goal in evolutionary biology, despite its potential to address fundamental questions about the role of different types of selective and genetic mechanisms as the basis for adaptive variation in phenotype (Golding and Dean, 1998; Orr, 2005). A key step in this research approach is identifying the functional significance of phenotypic variation at the molecular level. This will be most successful in systems where the possible function of the variation can be narrowly defined due to the nature of the adaptation. In this sense, predator–prey systems offer such a clearly defined phenotypic interface because the functional goals of the traits directly involved in killing the prey by the predator or resisting predation by the prey are clear (Brodie and Brodie, 1999).

Venoms produced by snakes in the Colubroidea are an example of a trait in a predator which shows high levels of variation at the molecular level and which also has a clearly defined function, namely the capture and digestion of prey. Venomous snakes such as rattlesnakes produce a complex mixture of up to 40 distinct proteins of several different families (Mackessy, 2008). Specialized venom glands located in the upper jaw synthesize and store venom which is then injected into prey via long, hollow fangs. Detailed and comprehensive characterization of the genes that underlie this variation and of the proteins they encode are becoming increasingly common for snakes (e.g. Sanz et al., 2005).
2006; Pahari et al., 2007; Gibbs and Rossiter, 2008), but functional characterization of this variation, in terms of effects on prey, still is poorly characterized. In general, the working hypothesis is that the high level of variation in venom at the inter- or intraspecific level (for a review see Chippaux et al., 1991) allows snakes to specialize on different prey (e.g. Mackessy, 1988; Daltry et al., 1996). Support for this hypothesis has come from studies showing a correlation between diet and venom variation in adult snakes (Daltry et al., 1996), associations between ontogenetic shifts in diet and venom composition (Mackessy, 1988), and more rarely, direct tests which have shown that venom produced by a particular age class of snake is most toxic to its preferred prey (e.g. Mackessy, 1988; Andrade and Abe, 1999; Jorge da Silva and Aird, 2001; Urdaneta et al., 2002; Mackessy et al., 2006).

However, studies showing associations between diet and venom have been criticized because they rarely test the key assumption that differences in venom composition are in fact correlated with increased toxicity towards more commonly consumed prey (Sasa, 1999; Mebs, 1999). Other research has also found no association between venom composition and diet (Williams et al., 1988). Further, a few studies with front-fanged snakes (viperids and elapids) that have conducted direct tests of venom toxicity on a range of prey have yielded variable results, ranging from positive associations (see above) to negative associations between toxicity and prey preference (Heatwole and Poran, 1995; Heatwole and Powell, 1998; Mebs, 2001) possibly due to coevolutionary interactions between snakes and their prey. The venom of the Brown Treesnake (Boiga irregularis), a rear-fanged colubrid snake, has been shown to have taxon-specific effects, with preferred prey (lizards and birds) being an order of magnitude more sensitive to venom produced by a particular age class of snake is most toxic to its preferred prey (e.g. Mackessy, 1988; Andrade and Abe, 1999; Jorge da Silva and Aird, 2001; Urdaneta et al., 2002; Mackessy et al., 2006).

Our goals in this study were as follows: using test animals that were representative of the major classes of prey consumed by different Sistrurus taxa, we conducted LD50 studies to determine (1) if prey-specific effects are present and if so, how they vary across taxa in relation to diet, and (2) if toxicity towards different prey covaries (which would support trade-offs in toxicity towards different prey as a mechanism underlying patterns of toxicity among taxa). Finally, using published data and comparative analyses, we address the question of how toxicity towards mammals evolved in these rattlesnakes.

2. Materials and methods

2.1. Sistrurus venom samples

Venoms were extracted manually from single adult snakes from three subspecies of S. catenatus and from S. m. barbouri using standard methods (Mackessy, 1988). Snakes were from the following locations: S. c. catenatus, Killdeer Plains Wildlife Area, Wyandot County, Ohio; S. c. tergeminus, Cheyenne Bottoms Wildlife Area, Barton County, Kansas; S. c. edwardsii, Lincoln County, Colorado and S. m. barbouri, central Florida. Protein concentration of each venom sample was assayed in triplicate according to Bradford (1976) as modified by BioRad Inc., using bovine gamma globulin as a standard.

2.2. Diet analyses

Diet information for the four taxa analyzed here was consolidated from several previously published or
unpublished sources (Holycross and Mackessy, 2002; Farrell and May, unpublished data). Prey items were classified into six categories (mammals, lizards, anurans, snakes, birds, centipedes) and plotted as percent of total diet for all samples for each taxon collected across all populations.

2.3. Test animals

We determined the toxicity of venoms towards three different animals which are representative of major prey classes (Mammalia, Reptilia, and Amphibia) in Sistrurus diets (see above): NSA mice (obtained from UNC Animal Facility breeding stock), wild-caught Brown Anoles (Anolis sagrei) from Florida (purchased from Quality Pets, Florida) and wild-caught Northern Leopard Frogs (Rana pipiens) from Ohio. Our goal in this study was to gain a broad picture of toxicity across classes of prey that characterize diet variation in these snakes. We recognize that in the wild, Sistrurus prey on a diversity of species in each of these general categories, and so our assumption is that patterns of toxicity we observe for each of these “types” of prey will be broadly representative of the response of other types of small mammals, lizards and frogs. This assumption could be tested in future studies but for logistic reasons was beyond the scope of this work.

We measured LD₅₀ for each venom-prey combination using the general procedures outlined in Munekiyo and Mackessy (1998) and Mackessy et al. (2006). Briefly, venom doses were delivered intraperitoneally (IP) in sterile saline, with doses adjusted to individual animal body masses. Three animals per dose were utilized, and all animals were monitored for 24 h. Lethality was expressed as micrograms venom per gram body mass (=mg/kg) producing 50% mortality after 24 h and was calculated (along with 95% confidence intervals) from the raw mortality-dose data using the Trimmed Spearman–Karber (TSK) program version 1.5 (U.S. Environmental Protection Agency, 1990). Our methods make a careful attempt to minimize the number of animals used in these assays. All procedures with vertebrates have been evaluated and approved by the University of Northern Colorado-IACUC (protocol #9401).

2.4. Comparative analyses

Initial analyses identified toxicity of Sistrurus venom to mammals as a key axis along which whole venom toxicity varies in this group. To gain a broader evolutionary perspective on the evolution of venom toxicity to mammals in rattlesnakes, we reconstructed ancestral values for venom-related traits and body length at key nodes of the rattlesnake phylogeny by constructing a phylogeny using published mtDNA gene sequences (Castoe and Parkinson, 2007) and information from the literature on IP LD₅₀ values published mtDNA gene sequences (Castoe and Parkinson, 2007) and information from the literature on IP LD₅₀ values. To generate a phylogeny to serve as the framework for the comparative analyses, we used a concatenated data set (provided by T. Castoe) consisting of 2306 nucleotides of aligned sequences from mitochondrial 12 and 16S RNA, tRNA, cytochrome b, and ND4 gene regions from 19 Crotaulus, 2 Sistrurus, and 2 Agkistrodon species (see Appendix A) to construct a tree based on maximum likelihood. To choose the substitution model which best fit the combined data set, we used the online version of MODELLTEST 3.7 (http://darwin.uvigo.es/software/modeltest.html – see Posada and Crandall, 1998) in combination with PAUP 4.0b10 (Swofford, 2003) to choose among different models using an AIC criteria. We then used PAUP to estimate the phylogenetic relationships among gene sequences using heuristic searches (TBR [tree–bisection–reconnection] branch swapping and random sequence addition) under maximum likelihood (ML) criteria using the parameter values from ‘best-fit’ model of sequence evolution as identified by MODELLTEST. We rooted this tree using the concatenated sequences from A. contortrix and A. piscivorus and estimated a single “best fit” tree under the ML criterion. We then used the estimated branch lengths and tree topology as inputs into the analyses in Compare.

2.5. SDS-PAGE

To compare toxicity results with potential variation in venom sample composition, the same venoms used in the toxicity assays were analyzed by SDS-PAGE as described previously (Mackessy and Baxter, 2006), with 24 μg venom (reduced with DTT) loaded per lane. All materials for SDS-PAGE (MES and sample buffers, Novex 12% acrylamide NuPage gels, Novex Mark 12 standards) were obtained from Invitrogen, Inc. (San Diego, CA, USA). Typical protein families of resolved bands were identified based on mass and prior analyses (Mackessy, 2008). Identification of
crotoxin homologs in *S. catenatus* venoms was based on comparison with venom (type A) from *Crotalus scutulatus scutulatus*, which contains Mojave toxin (Mackessy, 2008) and the proteomic data of Sanz et al. (2006).

3. Results

3.1. Prey preference

Differential utilization of prey is apparent for the four taxa of *Sistrurus* (Fig. 1). Mammals are the main prey type taken by *S. c. catenatus*, whereas lizards and frogs (anurans) are the primary prey of *S. m. barbouri*. For *S. c. tergeminus*, mammals followed by lizards are the preferred prey, respectively). Overall, as a species, *S. catenatus* includes a greater proportion of mammals in its diet than does *S. miliarius* (63% vs. 15%, respectively).

3.2. Prey-specific effects

Table 1 shows IP LD$_{50}$ values (±95% confidence intervals) from tests of four different *Sistrurus* venoms on the three representative types of prey. Three major patterns are present. First, frogs are 1–2 orders of magnitude more resistant to the effects of *Sistrurus* venom (mean LD$_{50}$ across venom types: 94.2 ± 6.3 sd) than are mice (2.4 ± 3.3) or lizards (0.79 ± 0.44). However, there is little variation in the effects of venoms from different *Sistrurus*: the coefficient of variation (CV) for mean LD$_{50}$ is 6.6% and 95% CIs for the LD$_{50}$s of all venoms overlap.

Second, although on average, *Sistrurus* venoms are most toxic to lizards, again, as for frogs there is limited variation in toxicity among different *Sistrurus*. The CV for LD$_{50}$ is higher (55%) largely due to a high LD$_{50}$ for *S. c. tergeminus*, but for all cases except one (*S. c. edwardsii* vs. *S. c. tergeminus*), the 95% CIs overlap, indicating no significant differences in LD$_{50}$ values for lizards for most venoms.

Finally, the mean toxicity of *Sistrurus* venom towards mice is intermediate between values for lizards and frogs. However, most striking is the high level of variation present, and it roughly correlates with the relative importance of small mammals in the diets of different snakes. The CV for mouse LD$_{50}$ values (137%) is 2.5 times greater than that for lizards and more than an order of magnitude greater than that for frogs. In addition, the LD$_{50}$ for the small mammal specialist (*S. c. catenatus*) is significantly lower than that for the other 3 taxa, whereas the LD$_{50}$ for the ectotherm specialist (*S. m. barbouri*) is significantly higher. Values for the other two *Sistrurus* which consume mixed diets are intermediate, although we note that the venom of *S. c. tergeminus* is significantly less toxic to mice than that of *S. c. edwardsii*, despite the fact that it includes more mammals in its diet. However, the venom of *S. catenatus* as a species is more toxic to mammals (mean LD$_{50}$ averaged across three subspecies: 0.57) than that of *S. miliarius* (7.19) and is positively associated with the overall importance of mammals in their respective diets (see above).

To summarize, *Sistrurus* venoms show strong prey-specific effects on frogs, lizards, and mice. However, there is substantial variation in LD$_{50}$ values among different snakes for mice only, and it is roughly correlated with the frequency with which mammals are consumed.

3.3. Comparative analyses

Fig. 2 shows the ancestral character value reconstructions for (a) LD$_{50}$ values for mice (b) total LD$_{50}$ doses for mice and (c) adult snake body length. For comparison, we also provide species-specific values for (d) LD$_{50}$ and (e) total doses. In Fig. 2a–c, we collapsed the clade containing the *Crotalus* species and present only a range of values for species in this group because our interest was in values for the node which is basal to the two main groups of rattlesnakes (*Sistrurus* and *Crotalus*) relative to values for *Sistrurus* and not in details of character evolution among species of *Crotalus*. For LD$_{50}$ values, the reconstructed value for the ancestor of all rattlesnakes was 3.59 and for the ancestor of *Sistrurus*, 3.96. Thus, in *S. catenatus*, the per unit toxicity of venom towards mammals has increased by an order of magnitude, whereas in *S. miliarius* it has decreased.

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**Table 1**

<table>
<thead>
<tr>
<th>Venom source</th>
<th>Mouse LD$_{50}$ (mg/kg)</th>
<th>Lizard LD$_{50}$ (mg/kg)</th>
<th>Frog LD$_{50}$ (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scc</td>
<td>0.23 (0.13, 0.43)</td>
<td>0.70 (0.42, 1.15)</td>
<td>95.3 (79.7, 113.8)</td>
</tr>
<tr>
<td>Sct</td>
<td>1.48 (1.33, 1.63)</td>
<td>1.41 (0.84, 2.37)</td>
<td>86.2 (79.6, 93.4)</td>
</tr>
<tr>
<td>Sce</td>
<td>0.60 (0.45, 0.81)</td>
<td>0.39 (0.23, 0.68)</td>
<td>101.4 (93.7, 109.9)</td>
</tr>
<tr>
<td>Smb</td>
<td>7.19 (5.71, 9.06)</td>
<td>0.66 (0.48, 0.92)</td>
<td>93.9 (72.6, 121.5)</td>
</tr>
</tbody>
</table>

| Mean (sd)   | Frog (0.44) | 94.2 (6.3) |
| CV          | 137.0%      | 55.2%      | 6.6%      |

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**Fig. 1.** Diet of *Sistrurus* rattlesnake taxa analyzed in this study. Data for all *S. catenatus* is from Holycross and Mackessy (2002). Data for *S. miliarius barbouri* is from Farrell and May (unpublished data) for snakes in Florida. Sample sizes are: *S. c. catenatus*: $n = 139$; *S. c. tergeminus*: $n = 111$; *S. c. edwardsii*: $n = 163$; *S. m. barbouri*: $n = 103$. 

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**Fig. 2.** Lethal toxicity (24 h LD$_{50}$) of venom from four *Sistrurus* rattlesnake subspecies towards three potential prey. Venom sources were as follows: Scc: *Sistrurus catenatus catenatus*; Sct: *S. c. tergeminus*; Sce: *S. c. edwardsii*; Smb: *S. miliarius barbouri*. Mouse: *Mus musculus*; Lizard: *Anolis sagrei*; Frog: *Rana pipiens*. 

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675
by approximately one-half. Among rattlesnakes as a whole, both values represent extremes, with the LD\(_{50}\) value for *S. catenatus* being among the top three most toxic values, and the *S. miliarius* value is the least toxic that is observed (Fig. 2d).

When dose equivalents are used as a measure of toxicity, the patterns are generally the same, although the loss of toxicity to mammals in *S. miliarius* is more striking than the gain in *S. catenatus*. The value for the ancestor of all rattlesnakes and for *Sistrurus* alone was \(1840;\) this value increases to 3000 for *S. catenatus* but drops to 221 for *S. miliarius*. This value for *miliarius* is the second smallest observed for any rattlesnake, whereas the value for *catenatus* is intermediate (Fig. 2e).

Finally, there seems to have been little change in body size (at least when estimated by body length) along the *Sistrurus* lineage, as both *S. miliarius* (60 cm) and *S. catenatus* (77 cm) are similar in size to the value reconstructed for the ancestor of all rattlesnakes (61 cm). In summary, these results confirm the importance of variation in toxicity in mammals among *Sistrurus* and suggest that diet shifts in relation to body size have not played a role in venom evolution in this group, because the body size of *Sistrurus* is similar to that reconstructed for the ancestor of all rattlesnakes.

### 3.4. SDS-PAGE analysis of venoms

Several differences in protein banding patterns were observed for the four venoms (Fig. 3). PI metalloproteases appear to be absent from *S. c. edwardsii* venom, and this venom contains only a single (acidic) PLA\(_2\) band, whereas the other venoms also contain an N6-PLA\(_2\), as described previously (Sanz et al., 2006; Gibbs and Rossiter, 2008); however, only *S. c. catenatus* venom has this potent toxin in significant quantities. Variation in the relative amounts of CRiSPs and disintegrins is also apparent.

### 4. Discussion

#### 4.1. Experimental issues

Our demonstration of strong prey-specific effects of *Sistrurus* venoms is dependent on a number of important assumptions related to the design of our study. In particular, because our goal was an initial survey of toxicity across broad categories of prey that are representative of animals consumed by *Sistrurus*, we limited our tests to easily-obtained animals that may or may not show sensitivities to venom that are representative of other species of that type. This assumption needs to be tested by estimating LD\(_{90}\)s to *Sistrurus* venom for other prey within these broad classes (small mammals, lizards, and frogs) that are consumed by these snakes in the wild. In particular, it would be of great interest to compare the toxicity of venom towards closely related prey species which were either sympatric or allopatric with the snakes that were the source of the test venoms, because geographic associations between potential prey and venomous snakes have been shown to influence the response of the prey to venom (Heatwole and Poran, 1995; Heatwole and Powell, 1998). In addition, some native mammals, such as *Neotoma* woodrats, show...
remarkable resistance to venoms of rattlesnakes (Perez et al., 1978), suggesting coevolutionary adjustments with these potential predators.

Finally, to limit any confounding effects of inter-individual variation, we restricted our sources of venom to a single individual from each Sistrurus taxon. Yet our recent proteomics-based analyses have shown that venom composition can vary between individuals (Sanz et al., 2006) which raises the question of how consistent the toxic effects of venom from different individuals are on the same prey. All these issues need to be addressed in future work – we see the results presented here as an important first step in this line of research through our demonstration of significant prey-specific effects that are especially variable in mammals. Our work strongly reinforces the view that understanding the function of venom composition in snakes requires an ecological perspective in terms of effects of venom on varied potential prey, rather than a mammal-biased focus on animal models that are relevant to human health.

4.2. Comparisons of toxicity values with other studies

No other studies have conducted toxicity analyses in Sistrurus towards a range of prey types. However, our results are broadly similar in documenting differences in the toxicity of S. catenatus vs. S. miliarius venom towards mice. In particular, both Minton (1956) and Githens (1935) found relatively low LD50 values for S. catenatus, similar in magnitude (0.22 and 0.90, respectively) to values reported here, whereas for S. miliarius, Githens (1935) and Kocholaty et al. (1971) found LD50 values that were much higher (6.00 and 6.84, respectively), also similar to our values. Thus there is previous support for our finding that toxicity to mammals varies substantially among Sistrurus. While we could find no data on toxicity of Sistrurus venoms to other prey types, there is evidence from two other pit vipers for a low toxicity of venom towards frogs compared to mammals. In particular, LD50 values for mice for venom from A. contortrix and A. piscivorus are substantially lower (~5–10) than recorded for bullfrogs (Rana catesbeiana) (125 and 82, respectively) (Heatwole et al., 1999). This suggests that the limited effectiveness of venom towards amphibians relative to mammals may be a general feature of pit vipers, although additional toxicity tests of a range of venoms and prey are required to confirm this.

4.3. Venom evolution in Sistrurus

Our results have implications for understanding the evolution of venom function in snakes. First, they do not
support a “trade-off” model of venom function implied by some models of venom evolution (e.g. Daltry et al., 1996), whereby an increase in the overall toxicity of venom towards a particular prey class is correlated with a decline in toxicity towards a different type of prey. Rather they suggest that within this group of closely related snakes that vary in diet, prey-specific toxic effects are independent of each other. For example, all snakes produce venom that is quite toxic to lizards and weakly toxic to frogs despite the fact that some snakes (S. c. catenatus) eat no lizards and few frogs while others (S. miliarius barbouri) include a high proportion of both prey types in their diets. The low toxicity of S. miliarius venom to frogs raises the issue of how this species immunoizes this commonly-eaten prey type and suggests that frogs may be killed by using hunting methods not involving venom (e.g. simple grasp and swallow) or that S. miliarius venom may be more toxic to prey frogs that are sympatric with this species (see above).

The second major implication is that our results suggest that toxicity to mammals alone is one axis along which overall venom toxicity has evolved in these snakes. Comparative analyses indicate that this has involved both an increase in toxicity in S. c. catenatus and a loss in toxicity in S. miliarius. If tests on other prey species uphold this pattern, then it identifies the key functional characteristic of Sistrurus venom (toxicity to mammals) at the interspecific level which must be considered as an important selection pressure in any explanation of venom gene and protein evolution in Sistrurus. For example, one phenotypic pattern that was recently documented through proteomic analyses was the increased overall diversity of venom proteins in all three S. catenatus taxa as compared to S. miliarius. Our results suggest the testable hypothesis that the functional consequence of this increase in venom protein complexity might be due to selection for additional venom proteins that allow the three S. catenatus taxa to “add” mammals to their diets through evolutionary time.

This trend towards greater venom toxicity and increasing reliance on mammalian prey is most pronounced at the extremes (S. c. catenatus, a mammal specialist, vs. S. m. barbouri, an ectotherm specialist). An unanswered question is why S. c. tergeminus, which also utilizes a large percentage of mammals in its diet, produces a venom which is significantly less toxic towards mammals than S. c. catenatus. The proximate reason for this difference may involve the relative quantities of a major component of viperid venoms which greatly increases toxicity to mammals, namely, crototoxin homologs, which are 2 subunit presynaptic neurotoxins based on a PLA2 scaffold (i.e., Bieber et al., 1990). Although this component is present in venoms from both S. c. catenatus (Sanz et al., 2006) and S. c. tergeminus (Chen et al., 2004), it is a major component only in S. c. catenatus venom (see Fig. 3; also Sanz et al., 2006).

However, our results also indicate that toxicity to specific prey is likely not the only determinant of venom variation in these snakes. Toxicity of venom towards NSA mice in S. c. tergeminus and S. c. edwardsii show that ecological and evolutionary factors in addition to selection in relation to diet can potentially influence venom composition in different species (e.g. Sasa, 1999; Wüster et al., 1999; Mebs, 2001). Although these subspecies are closely related phylogenetically (Kubatko and Gibbs, unpublished data), adult size and habitats utilized (Conant and Collins, 1991) are more similar among S. c. catenatus and S. c. tergeminus than either is to S. c. edwardsii, and these factors may lead to greater utilization of mammals by S. c. tergeminus despite having less toxic venom. Alternatively, toxicity of venom towards NSA mice may simply underestimate sensitivity of native mammalian prey of S. c. tergeminus to its venom, leading to the lack of the observed association between diet and toxicity in this subspecies. Our results emphasize that a challenge for future research is to understand the relative importance of selection in relation to diet relative to other ecological and evolutionary factors, and the effects of experimental constraints (such as species for toxicity testing) as explanations for variation in venom toxicity in these rattlesnakes.

Acknowledgements

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Appendix. Data used for analyses with Compare

<table>
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<tr>
<th>Species</th>
<th>IP LD50 (mg/kg)</th>
<th>Source</th>
<th>Mean venom yield (mg)</th>
<th>Source</th>
<th>Total LD50 doses</th>
<th>Mid-length (cm)</th>
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Sources: 1: Consroe et al. (1992); 2: Mackessy (2008); 3: www.venomdoc.com; 4: This study; 5: Ernst (1979); 6: Glenn and Straight (1981); 7: Behler and King (1979); 8: Klauber (1972).
Conflicts of interest

None declared.

References


