Venom Composition in Rattlesnakes: Trends and Biological Significance

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ABSTRACT.—Venom glands of rattlesnakes have a largely conserved morphology, and protein products of the gland are often highly homologous across many species. As with snake venoms generally, a primary role of rattlesnake venoms is to incapacitate prey remotely and to facilitate prey handling. However, even within a single species (e.g., Crotalus o. oreganus and C. o. concolor), very prominent differences in absolute composition are observed, suggesting that closely related taxa are utilizing different trophic “strategies.” Using SDS-PAGE, enzyme assays, and toxicity studies, venoms of 27 rattlesnake taxa were analyzed and compared with the intent of placing venom biochemistry into a broader biological context. Venom composition is genetically determined, and therefore is related to phylogeny; however, within specific lineages, venom composition varies considerably. Within the constraints of relatedness of taxa, there is some variability in absolute venom composition, which appears to be related primarily to diet and secondarily to environment. Venom often (but not always) varies ontogenetically, and this variation commonly tracks changes in dominant preferred prey type. For example, both ontogenetically and between subspecies/species, highly toxic venoms lack high concentrations of metalloproteases, enzymes responsible for much of the structural damage resulting from envenomation. Conversely, those with potent metalloprotease activity generally lack presynaptic neurotoxins characteristic of the most toxic venoms. Toxicity versus tissue-damaging effects of rattlesnake venoms follows a predictable inverse trend. The thrombin-like serine proteases and myotoxin A homologs also vary ontogenetically in some species, and higher levels in venoms appear to be directed toward mammalian prey. Further, maintenance of venom complexity is likely driven by adaptive detoxification responses of prey, and the ensuing arms race has led to apparent multiplicity of similar activities and well over 100 distinct molecular entities in a single venom. However, many unanswered questions remain, particularly concerning many Mexican rattlesnake taxa and with regard to comparative toxicity of venom component isoforms to native prey species. Within the rattlesnakes, one finds venom compositional "strategies" which are observed broadly among venomous colubroid snakes, and thus rattlesnakes represent an excellent group for studying venom evolution in squamates.

INTRODUCTION

Produced within the venom gland of snakes is a complex secretion containing dozens of enzymes, toxins, peptides, small organic molecules, and inorganic components (Mackessy and Baxter, 2006). Upon envenomation, homeostatic mechanisms in a myriad of systems are affected rapidly, and in prey animals, the result of envenomation is rapid incapacitation leading to death and initiation (in many cases) of digestion. Among colubrid snakes, there has been some debate about venom occurrence and action in many rear-fanged species, but the recent demonstration of taxon-specific effects (Mackessy et al., 2006) suggests that, depending on the animal model chosen for toxicity assays, effects vary tremendously; differential sensitivity of specific taxa may have profound implications for the biological roles of these venoms. In rattlesnakes (Chiszar et al., 1999), and likely among pit vipers in general (e.g., Stiles et al., 2002; Greenbaum et al., 2003), venom also serves to “tag” or change the odor of, envenomated prey, allowing the snake to relocate prey via strike-induced chemosensory searching.

Venoms, therefore, serve numerous well-documented trophic roles for snakes, but a major unanswered question is why venoms are so complex biochemically, and in particular, why apparent multiplicity of components within specific protein families (e.g., phospholipases A2, serine proteases, metalloproteases, etc.) exists in venom from a single individual. An adaptive response among prey toward detoxification of venom components has been proposed (Mackessy et al., 2003), and an ensuing “arms race” between predator and prey could drive the selection for dynamic evolution of venom composition. Prey-driven evolution of venom is strongly suggested by differential resistance to Crotalus o. oreganus venoms observed among populations of California Ground Squirrels (Spermophilus beecheyi; Poran et al., 1987; Biardi et al., 2000, 2005; Biardi, this volume) and the high level of resistance to C. atrox venom among woodrats (Perez et al., 1978a,b). Additionally, many rattlesnake species feed on a variety of prey (Klauber, 1956; Wallace and Diller, 1990; Holycross and Mackessy, 2002; Holycross et al., 2002), some characteristically at different life stages (Mackessy, 1988; Mackessy et al., 2003), and so venoms may include toxins or other components which act differentially toward specific prey classes. Rattlesnake venom composition often varies with snake age (Minton, 1967;
Reid and Theakston, 1978; Lomonte et al., 1983; Mackessy, 1988; Mackessy et al., 2003), and a recent proteomic study of the venom of a crotaline viperid (Bothrops atrox) demonstrated a larger number of proteins in venoms from juvenile snakes (Guércio et al., 2006).

In a broad sense, venom composition variation does follow phylogenetic trends, particularly at the familial level among the front-fanged snakes (e.g., Tan and Ponnadurai, 1990, 1991). For example, α-neurotoxins (three-finger toxins) are typical and abundant components of elapid venoms (Nirthanan and Gwee, 2004), and although they have recently been documented in the venom gland genome of one species (Lachesis muta; Junqueira-de-Azevedo et al., 2006), they have not yet been demonstrated to be present in secreted viperid venoms. Conversely, viperid venoms are rich sources of hydrolytic enzymes, such as multiple metalloproteases (Bjarnason and Tu, 1978; Bjarnason and Fox, 1989, 1995; Mackessy, 1985, 1993a,b, 1996), many of which are either absent or at low concentrations in elapid venoms. However, patterns within lower level taxa (such as genera) are less clear and have not been analyzed extensively. Rattlesnakes, by virtue of their wide distribution, varied ecology, relatively large venom yields, and robust venom toxinology database, represent an ideal group to investigate venom composition variation and its relation to phylogeny, ecology, and natural history of the animals (c.f. Beaman and Hayes, this volume). One limitation is that, although the venom composition of some species is very well known (e.g., C. atrox; Fox and Serrano, 2005; Serrano et al., 2005; Sistrurus: Sanz et al., 2006), we have little or no information for many other species, particularly for the many species occupying the Mexican highlands (see Campbell and Lamar, 2004).

Although rattlesnake venoms are known to vary as a function of numerous intrinsic and extrinsic factors, many components are common to venoms of most species, and a high degree of sequence homology exists among specific components, such as the phospholipases (Kini, 1997) and small myotoxins (Mebs and Ownby, 1990). However, activity levels of many enzymes (and therefore, presumably, content levels) may vary tremendously. Common rattlesnake venom components include a variety of enzymes, including several different nuclease, hyaluronidase, L-amino acid oxidase, several to many metalloproteases, serine proteases, phospholipases, and other less abundant enzymes. These catalytic proteins may have both specific and generalized effects, as demonstrated by the activity of several metalloproteases toward numerous protein components of the basement membrane defining the capillary endothelium. Hydrolysis of this layer results in loss of capillary integrity, producing hemorrhage (Fox and Serrano, 2005). Metalloproteases also hydrolyze various other structural proteins (such as collagen), and so are important to tissue degradation (Gutiérrez and Rucavado, 2000), one of the “predigestion” effects of venoms. One subclass of the phospholipases has evolved from hydrolytic enzymes into potent presynaptic β-neurotoxins responsible for the high toxicity of venoms of several species (Aird and Kaiser, 1985; Aird et al., 1985; Bieber et al., 1990; Weinstein and Smith, 1990; see also the chapters in this volume by Powell et al., Powell and Lieb, and Werman), including the Tropical Rattlesnake (C. durissus terrificus), the Mohave Rattlesnake (C. s. scutulatus), the Midget-faded Rattlesnake (C. oreganus concolor), and the Tiger Rattlesnake (C. tigris). Rattlesnake venoms also contain specific non-enzymatic peptides, typically with potent biological activities, such as the myotoxins, disintegrins, and bradykinin-potentiating peptides, and a variety of less well characterized smaller organic and inorganic compounds. Many of these common venom components and their likely biological roles are listed in Table 1.

Venom composition may vary ontogenetically, sometimes with profound effects on the overall pharmacology of the venom. Previously, we have demonstrated distinct ontogenetic changes in venom composition, which appear to be related to concomitant changes in diet (C. o. oreganus and C. o. helleri; Mackessy, 1988). Toxicity and metalloprotease activity levels were inversely related, with neonate venoms more toxic and adult venom containing much higher levels of metalloproteases. However, in a related subspecies (C. o. concolor), these shifts in venom composition were not seen, and adult venoms were very toxic and showed low metalloprotease activity, in spite of similar ontogenetic shifts in diet (Mackessy et al., 2003). These two rather disparate “venom composition strategies” appear to be a broader reflection of trends in rattlesnake venoms of different species, as well as among venomous snakes in general, and the observations from these studies have led to the investigations detailed below. In this paper, venoms which show higher levels of metalloprotease activity and lower toxicity (>1.0 μg/g mouse body weight) will be referred to as type I venoms, whereas those with low metalloprotease activity and higher toxicity (<1.0 μg/g mouse body weight) will be referred to as type II venoms.

The goal of the present study is to begin to identify patterns of protein component occurrence in different rattlesnake venoms and to evaluate trends in venom composition variation. New data are presented and integrated with data collected from the literature. Because several phylogenetic hypotheses concerning relationships among the rattlesnakes exist (e.g., Murphy et al., 2002; Wüster et al., 2005; Castoe and Parkinson, 2006), one can begin to set these trends in venom composition in a phylogenetic context and ask whether or not trends observed follow evolutionary lineages. This paper does not attempt to define absolutely the composition of rattlesnake venoms in general; rather, it should be seen as a step toward understanding the complex interactions between composition, phylogeny, and biological roles of rattlesnake venoms.

**Materials and Methods**

*Reagents.—NuPage 12% acrylamide gels, Novex Mark 12 standards, and related supplies were obtained from Invitrogen, Inc. (San Diego, California, USA). All other bio-*
Table 1. Common components of rattlesnake venoms and general characteristics.

<table>
<thead>
<tr>
<th>Component Name</th>
<th>Approximate Mass (kDa)</th>
<th>Function</th>
<th>Biological Activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Phosphodiesterase</td>
<td>94-140</td>
<td>Hydrolysis of nucleic acids and nucleotides</td>
<td>Depletion of cyclic, di- and tri-nucleotides;</td>
<td>Mackessy, 1998; Aird, 2002</td>
</tr>
<tr>
<td>5’-nucleotidase</td>
<td>53-82</td>
<td>Hydrolysis of 5’-nucleotides</td>
<td>Nucleoside shock (?)</td>
<td>Rael, 1998; Aird, 2002</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>73</td>
<td>Hydrolysis of interstitial hyaluronan</td>
<td>Decreased interstitial viscosity – diffusion of venom</td>
<td>Tu and Kudo, 2001</td>
</tr>
<tr>
<td>L-amino acid oxidase (homodimer)</td>
<td>85-150</td>
<td>Oxidative deamination of L-amino acids</td>
<td>Induction of apoptosis, cell damage</td>
<td>Tan, 1998</td>
</tr>
<tr>
<td>Snake venom metalloproteases: M12 reprolysins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-IV</td>
<td>48-85</td>
<td>Hydrolysis of many structural proteins, including basal lamina components, fibrinogen, etc.</td>
<td>Hemorrhage, myonecrosis, prey digestion</td>
<td>Fox and Serrano, 2005</td>
</tr>
<tr>
<td>P-III</td>
<td>43-60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-II</td>
<td>25-30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-I</td>
<td>20-24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine proteases:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin-like</td>
<td>31-36</td>
<td>Catalysis of fibrinogen hydrolysis</td>
<td>Rapid depletion of fibrinogen; hemostasis disruption</td>
<td>Markland, 1998; Swensen and Markland, 2005</td>
</tr>
<tr>
<td>Kallikrein-like</td>
<td>27-34</td>
<td>Release of bradykinin from HMW kininogen; hydrolysis of angiotensin</td>
<td>Induces rapid fall in blood pressure; prey immobilization</td>
<td>Nikai and Komori, 1998</td>
</tr>
<tr>
<td>“Arginine esterase”</td>
<td>25-36</td>
<td>Peptidase and esterase activity</td>
<td>Uncertain; predigestion of prey (?)</td>
<td>Schwartz and Bieber, 1985</td>
</tr>
<tr>
<td><strong>Non-enzymatic proteins/peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine-rich secretory proteins (CRiSPs)/ helveprins</td>
<td>21-29</td>
<td>Possibly block cNTP-gated channels</td>
<td>Induced hypothermia; prey immobilization (?)</td>
<td>Yamazaki and Morita, 2004</td>
</tr>
<tr>
<td>PLA2-based presynaptic neurotoxins (2 subunits, acidic and basic)</td>
<td>24</td>
<td>Blocks release of acetylcholine from axon terminus</td>
<td>Potent neurotoxicity; prey immobilization</td>
<td>Aird et al., 1985; Ducancel et al., 1988; Faure et al., 1994</td>
</tr>
<tr>
<td>C-type lectins</td>
<td>27-29</td>
<td>Binds to platelet and collagen receptor</td>
<td>Anticoagulant, platelet-modulator</td>
<td>Leduc and Bon, 1998</td>
</tr>
<tr>
<td>Disintegrins</td>
<td>5.2-15</td>
<td>Inhibit binding of integrins to receptors</td>
<td>Platelet inhibition; promotes hemorrhage</td>
<td>Calvete et al., 2005</td>
</tr>
<tr>
<td>Myotoxins – non-PLA2</td>
<td>4-5.3</td>
<td>Modifies voltage-sensitive Na channels; interacts with lipid membranes</td>
<td>Myonecrosis, analgesia; prey immobilization</td>
<td>Laure, 1975; Fox et al., 1979; Bieber and Nedelhov, 1997</td>
</tr>
<tr>
<td><strong>Smaller peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradykinin-potentiating peptides</td>
<td>1.0-1.5</td>
<td>Increases potency of bradykinin</td>
<td>Pain, hypotension; prey immobilization</td>
<td>Wermelinger et al., 2005</td>
</tr>
<tr>
<td>Tripeptide inhibitors</td>
<td>0.43-0.4</td>
<td>Inhibit venom metalloproteases and other enzymes</td>
<td>Stabilization of venom components</td>
<td>Francis and Kaiser, 1993; Munekiyo and Mackessy, 2005</td>
</tr>
<tr>
<td><strong>Smaller organic compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purines and pyrimidines (AMP, Hypoxanthine, Inosine)</td>
<td>AMP = 0.347</td>
<td>Broad effects on multiple cell types (?)</td>
<td>Hypotension, paralysis, apoptosis, necrosis (?); prey immobilization</td>
<td>Aird, 2002, 2004</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.192</td>
<td>Inhibition of venom enzymes</td>
<td>Stabilization of venom</td>
<td>Francis et al., 1992; Freitas et al., 1992</td>
</tr>
</tbody>
</table>

Mass in kilodaltons (kDa). Note that this list is not all-inclusive and that masses, functions, and activities do not apply to all compounds isolated from all rattlesnake venoms. Specific rattlesnake venoms may not contain all components. (?) – indicates hypothetical function and/or activity.
Table 2. Enzyme activities and toxicity of rattlesnake venoms.

<table>
<thead>
<tr>
<th>Species</th>
<th>Thr (nmol/min/mg)</th>
<th>Kal (nmol/min/mg)</th>
<th>MPr (ΔA342 nm/ min/mg)</th>
<th>PLA₂ (nmol/min/mg)</th>
<th>PDE (ΔA400 nm/ min/mg)</th>
<th>LAAO (nmol/min/mg)</th>
<th>Toxicity (Mouse LD₅₀, μg/g)</th>
<th>General Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Crotalus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. adamanteus</em></td>
<td>1012.2</td>
<td>90.4</td>
<td>0.333</td>
<td>22.60</td>
<td>1.36</td>
<td>49.7</td>
<td>2.0</td>
<td>Florida</td>
</tr>
<tr>
<td><em>C. atrox</em></td>
<td>671.9</td>
<td>902.7</td>
<td>1.341</td>
<td>18.01</td>
<td>1.04</td>
<td>31.8</td>
<td>3.5</td>
<td>Arizona: Cochine Co.</td>
</tr>
<tr>
<td><em>C. basiliscus</em></td>
<td>898.1</td>
<td>937.6</td>
<td>2.522</td>
<td>52.37</td>
<td>0.82</td>
<td>23.0</td>
<td>2.8</td>
<td>SW Mexico</td>
</tr>
<tr>
<td><em>C. durissus terrificus</em></td>
<td>211.0</td>
<td>796.4</td>
<td>0.071</td>
<td>5.80</td>
<td>1.55</td>
<td>4.0</td>
<td>0.13</td>
<td>Brazil</td>
</tr>
<tr>
<td><em>C. (durissus) tzaban</em></td>
<td>972.9</td>
<td>145.4</td>
<td>1.496</td>
<td>20.71</td>
<td>1.34</td>
<td>69.4</td>
<td>2.5</td>
<td>Mexico: Quintana Roo</td>
</tr>
<tr>
<td><em>C. enyo enyo</em></td>
<td>764.5</td>
<td>69.0</td>
<td>0.825</td>
<td>33.65</td>
<td>0.71</td>
<td>37.6</td>
<td>2.8</td>
<td>Mexico: Baja California Sur</td>
</tr>
<tr>
<td><em>C. horridus (atraudatus)</em></td>
<td>897.4</td>
<td>221.9</td>
<td>0.285</td>
<td>42.66</td>
<td>0.36</td>
<td>24.9</td>
<td>1.0</td>
<td>Arizona: Cochise Co.</td>
</tr>
<tr>
<td><em>C. horridus horridus</em></td>
<td>1073.4</td>
<td>849.1</td>
<td>1.315</td>
<td>27.84</td>
<td>2.10</td>
<td>70.8</td>
<td>3.0</td>
<td>New York</td>
</tr>
<tr>
<td><em>C. lepidus lepidus</em></td>
<td>971.7</td>
<td>1032.6</td>
<td>1.070</td>
<td>61.50</td>
<td>0.38</td>
<td>65.2</td>
<td></td>
<td>Texas: Val Verde Co.</td>
</tr>
<tr>
<td><em>C. lepidus klauberi</em></td>
<td>1194.4</td>
<td>983.4</td>
<td>1.320</td>
<td>79.30</td>
<td>0.45</td>
<td>61.9</td>
<td>1.55</td>
<td>Arizona: Cochine Co.</td>
</tr>
<tr>
<td><em>C. michelli pyrrhus</em></td>
<td>315.9</td>
<td>781.6</td>
<td>1.242</td>
<td>21.79</td>
<td>1.31</td>
<td>22.4</td>
<td>2.5</td>
<td>California: Riverside Co.</td>
</tr>
<tr>
<td><em>C. molussus molossus</em></td>
<td>657.3</td>
<td>602.5</td>
<td>0.924</td>
<td>13.11</td>
<td>0.36</td>
<td>26.4</td>
<td>2.7</td>
<td>Arizona: Cochine Co.</td>
</tr>
<tr>
<td><em>C. oreganus oreganus</em></td>
<td>981.7</td>
<td>1034.1</td>
<td>1.572</td>
<td>15.05</td>
<td>0.62</td>
<td>34.3</td>
<td>2.8</td>
<td>California: San Luis Obispo Co.</td>
</tr>
<tr>
<td><em>C. oreganus concolor</em></td>
<td>621.6</td>
<td>621.8</td>
<td>0.135</td>
<td>29.27</td>
<td>0.69</td>
<td>34.4</td>
<td>0.46</td>
<td>Wyoming: Sweetwater Co.</td>
</tr>
<tr>
<td><em>C. oreganus helleri</em></td>
<td>847.4</td>
<td>551.8</td>
<td>1.460</td>
<td>22.76</td>
<td>0.39</td>
<td>43.2</td>
<td>1.5</td>
<td>California: Los Angeles Co.</td>
</tr>
<tr>
<td><em>C. polystictus</em></td>
<td>985.4</td>
<td>841.5</td>
<td>1.379</td>
<td>27.15</td>
<td>0.64</td>
<td>7.8</td>
<td>3.4</td>
<td>Mexico: Jalisco</td>
</tr>
<tr>
<td><em>C. pricei pricei</em></td>
<td>1267.4</td>
<td>1226.8</td>
<td>1.180</td>
<td>70.10</td>
<td>0.05</td>
<td>33.8</td>
<td>1.25</td>
<td>Arizona: Cochine Co.</td>
</tr>
<tr>
<td><em>C. pusillus</em></td>
<td>960.6</td>
<td>918.7</td>
<td>1.575</td>
<td>24.70</td>
<td>0.74</td>
<td>12.2</td>
<td></td>
<td>Mexico: Michoacan</td>
</tr>
<tr>
<td><em>C. ruber ruber</em></td>
<td>670.9</td>
<td>919.8</td>
<td>2.263</td>
<td>5.40</td>
<td>0.29</td>
<td>50.2</td>
<td>3.8</td>
<td>California: Riverside Co.</td>
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<tr>
<td><em>C. scutulatus scutulatus</em></td>
<td>860.9</td>
<td>781.8</td>
<td>0.021</td>
<td>36.76</td>
<td>1.04</td>
<td>49.2</td>
<td>0.2</td>
<td>Arizona: Cochine Co.</td>
</tr>
<tr>
<td><em>C. tigris</em></td>
<td>968.4</td>
<td>18.9</td>
<td>0.052</td>
<td>28.77</td>
<td>0.16</td>
<td>1.95</td>
<td>0.07</td>
<td>Arizona: Pima Co.</td>
</tr>
<tr>
<td><em>C. viridis viridis</em></td>
<td>981.4</td>
<td>971.8</td>
<td>0.900</td>
<td>41.30</td>
<td>0.60</td>
<td>49.2</td>
<td>1.8</td>
<td>Colorado: Weld Co.</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>853.9</strong></td>
<td><strong>695.4</strong></td>
<td><strong>1.06</strong></td>
<td><strong>31.84</strong></td>
<td><strong>0.77</strong></td>
<td><strong>36.5</strong></td>
<td><strong>2.0</strong></td>
<td></td>
</tr>
<tr>
<td><strong>1 SE</strong></td>
<td><strong>252.3</strong></td>
<td><strong>359.9</strong></td>
<td><strong>0.69</strong></td>
<td><strong>19.5</strong></td>
<td><strong>0.51</strong></td>
<td><strong>20.5</strong></td>
<td><strong>1.2</strong></td>
<td></td>
</tr>
<tr>
<td><em>Sistrurus</em></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. catenatus catenatus</em></td>
<td>593.9</td>
<td>428.5</td>
<td>0.21</td>
<td>41.7</td>
<td>0.134</td>
<td>62.1</td>
<td>0.9</td>
<td>Wisconsin</td>
</tr>
<tr>
<td><em>S. catenatus edwardsii</em></td>
<td>396.3</td>
<td>533.4</td>
<td>1.32</td>
<td>14.9</td>
<td>0.257</td>
<td>88.5</td>
<td>1.35</td>
<td>Colorado: Lincoln Co.</td>
</tr>
<tr>
<td><em>S. catenatus tergeminus</em></td>
<td>642.8</td>
<td>537.7</td>
<td>1.02</td>
<td>16.1</td>
<td>0.137</td>
<td>86.9</td>
<td>2.6</td>
<td>Kansas: Barton Co.</td>
</tr>
<tr>
<td><em>S. miliarius barbouri</em></td>
<td>676.4</td>
<td>253</td>
<td>1.1</td>
<td>38.8</td>
<td>0.078</td>
<td>56.0</td>
<td>4.5</td>
<td>Florida</td>
</tr>
<tr>
<td><em>Crotalus (Sistrurus) ravus</em></td>
<td>999.7</td>
<td>1000</td>
<td>0.853</td>
<td>31.2</td>
<td>0.063</td>
<td>19.7</td>
<td>3.2</td>
<td>Mexico: Puebla</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>661.8</strong></td>
<td><strong>550.5</strong></td>
<td><strong>0.901</strong></td>
<td><strong>28.5</strong></td>
<td><strong>0.134</strong></td>
<td><strong>62.6</strong></td>
<td><strong>2.51</strong></td>
<td></td>
</tr>
<tr>
<td><strong>1 SE</strong></td>
<td><strong>97.4</strong></td>
<td><strong>123.7</strong></td>
<td><strong>0.19</strong></td>
<td><strong>5.6</strong></td>
<td><strong>0.034</strong></td>
<td><strong>12.5</strong></td>
<td><strong>0.65</strong></td>
<td></td>
</tr>
</tbody>
</table>

* Enzyme activities: thrombin-like (Thr), kallikrein-like (Kal), caseinolytic metalloprotease (MPr), phospholipase A₂ (PLA₂), phosphodiesterase (PDE), and L-amino acid oxidase (LAAO).
Venom composition in rattlesnakes

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chemicals (analytical grade or better) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA).

Venoms.—Snakes were collected from a variety of locations in the western United States under permits from state and federal authorities (see acknowledgements), and several were received as donations or from law enforcement confiscations. Venoms were obtained via manual extraction as described previously (Mackessy, 1988), and were centrifuged (to remove solids), frozen, lyophilized, and stored frozen until used (this material is referred to as crude venom). All venoms were initially reconstituted in nanopure water (Millipore Ultrafiltration system; Millipore Corporation, Billerica, Massachusetts, USA). Venoms from several species were gifts from C. Ownby (C. h. horridus, C. d. terrificus) and the late S. Minton (C. polyxystus, C. pusillus, C. ravus). All venoms analyzed in this study were from individual adult snakes.

Electrophoresis.—NuPage 12% polyacrylamide gels (17 lane, 1.0 mm thick) were run as suggested by the manufacturer (Invitrogen). Briefly, venom samples at a final concentration of 2.0 mg/ml were prepared in sample buffer containing 15 mM DTT (final concentration). Samples were heated at 70°C for 10 min, and 12 μL was added to each lane. Electrophoresis was conducted using denaturing MES (morpholino-ethane sulfonic acid) buffer (50 mM MES, 50 mM Tris-HCl, 0.1% SDS, 1.0 mM EDTA, pH 7.3) for approximately 40 min at 200 V. Gels were then stained in 0.1% Coomassie Brilliant Blue R-250 overnight and destained. Gels were photographed using an Alpha Imager (Alpha Inotech, San Leandro, California, USA), and clarity was optimized using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, California, USA). Individual bands were not digitally quantitated, and qualitative comparisons were based on presence/absence and relative band intensity of a given molecular mass class of proteins.

Enzyme assays.—Crude venoms were assayed for six common enzyme activities, including thrombin-like (Thr act), kallikrein-like (Kal act), caseinolytic metalloprotease (MPR act), phospholipase A2, (PLA2 act), phosphodiesterase (PDE act), and L-amino acid oxidase (LAAO act), as described previously (Munekiyo and Mackessy, 1998). All enzymes were assayed in triplicate and corrected for substrate blanks. Protein concentration was determined (in triplicate) using BioRad Coomassie brilliant blue reagent as described by the manufacturer. Enzyme activities were expressed as specific activities (nanomoles product formed per minute per milligram protein, nmol/min/mg) or as a change in absorbance per min per milligram protein (ΔAU/min/mg).

Toxicity and other venom properties.—Toxicities (mouse LD₅₀) of venoms were compiled from published reports (Glenn and Straight, 1982, 1985; Minton and Weinstein, 1984; Weinstein and Smith, 1990; Khole, 1991); several species for which reliable LD₅₀ values were not available (Sistrurus catenatus edwardsii, S. c. tergeminus, C. lepidus klauberi, C. p. pricei) were determined using intravenous (caudal vein) delivery and standard methods (e.g., Mackessy et al., 2006). Potential relationships between venom toxicity and enzyme activities were analyzed using Spearman rank correlations. Significant differences were determined at the level of P < 0.05. Identification of electrophoretic bands was accomplished by comparative electrophoretic analysis with previously purified venom proteins (e.g., Mackessy, 1993a,b, 1996; Sanz et al., 2006) and by comparison with published masses of specific venom components (see also ExPaSy Proteomics Server, http://us.expasy.org/).

As an initial analysis of the relationship between phylogeny and venom composition, venom type (I or II) was mapped onto the relevant portion of the Bayesian MCMC phylogenetic analysis of Castoe and Parkinson (2006: Fig. 3). This hypothesis was chosen because it contains a large number of the taxa analyzed in the present study and because their analysis of phylogenetic data is particularly robust.

Results

Rattlesnake venoms contained large amounts of protein (~225-250 mg/mL), and protein concentration of lyophilized venom was 90-93%. Venom yields for all species increased exponentially with size, as has been noted in larger series of samples from individual species (Mackessy, 1988; Mackessy et al., 2003). Venoms of most species were clear yellow; a notable exception was venom from C. tigris, which was colorless. Species sampled and general localities are listed in Table 2.

Electrophoresis.—One-dimensional electrophoresis demonstrated that venoms contained 20-30 proteins (Figs. 1-3); however, this is an underestimate of the total number of proteins, as all molecules with similar masses (± 10%) and carbohydrate modifications may migrate on gels as apparently single entities. Regardless, relative positions of bands allowed identification of specific protein families in individual samples, in turn providing a comparison of functional components present in various species’ venoms. Eight groups of functionally important activities are highlighted on gels (Figs. 1-3). It should be noted, however, that within each of the designated functional groups, additional non-related proteins likely exist.

Levels of metalloproteases appeared to vary considerably between species, based on relative band intensity differences (large dense bands contain greater amounts of protein than thin, more diffuse bands); structural classifications of venom metalloproteases (P-I through P-IV) refer to increasing domain complexity (Bjarnason and Fox, 1995; Fox and Serrano, 2005). High molecular mass metalloproteases (PIII), which are typically hemorrhagic and have masses of 52-55 kDa, were present in most venoms, but were very faint in those from C. d. terrificus, C. o. concolor, C. s. scutulatus, and C. tigris. Another distinctive class of metalloproteases (PI), with a mass of approximately 21-23 kDa, were present in many venoms, but again were conspicuously absent from C. d. terrificus, C. o. concolor, C. s. scutulatus, and C. tigris venoms, as well as from those
Figure 1. SDS-PAGE analysis of venoms from twelve taxa of *Crotalus*. Approximate molecular masses (M, in kiloDallons) are given on the left, and protein classes of major constituents, and their major actions, are given on the right. All venoms showed considerable complexity, but note that PIII metalloprotease levels are very low in *C. scutulatus*, *C. tigris*, and *C. durissus terrificus* venoms, and PI metalloproteases are absent from these and *C. horridus atricaudatus* and *C. enyo* venoms. CRISP, cysteine-rich secretory protein; LAAO, L-amino acid oxidase.

Figure 2. SDS-PAGE analysis (composite gel) of venoms from four taxa of *Sistrurus* and eight taxa of *Crotalus*. Relevant portions of individual gels were aligned using protein standards run on each gel, and labels are as in Fig. 1. It is apparent that many proteins are shared between *Sistrurus* and *Crotalus*.
of *C. enyo*, *C. l. klauberi*, *C. pricei*, and *S. c. edwardsii*; in rattlesnakes, only PI metalloprotease(s) are observed at this position in gels. All venoms contained at least one band of ~14 kD, and phospholipases A2 are the most common proteins of this mass in venoms. Variation in number and intensity of bands was apparent for many other components, particularly serine proteases, disintegrins, and myotoxins. Myotoxins were apparently absent from venoms of *C. enyo*, *C. l. klauberi*, *C. pricei*, *C. tzabcan*, and some *S. c. edwardsii*. Myotoxin levels also varied ontogenetically in *C. o. concolor* venoms (Fig. 3).

**Enzyme assays.**—Venoms from all species showed activity in all assays (Table 2), though several species varied notably from average values. Thrombin-like activity was greatest in *C. pricei* venom and lowest in *C. d. terrificus* venom. Kallikrein-like activity was greatest in *C. pricei* venom and lowest in *C. tigris* venom. Metalloprotease activity was greatest in *C. basiliscus* venom and lowest in *C. tigris* venom. Phospholipase A2 activity was greatest in *C. l. klauberi* venom and lowest in *C. ruber* venom. Venom phosphodiesterase activity was greatest in *C. h. horridus* venom and lowest in *C. (Sistrurus) rarus* venom. L-amino acid oxidase activity was greatest in *S. c. edwardsii* venom and lowest in *C. tigris* venom. The most toxic venoms tended to have lower enzyme activities, but with the exception of metalloprotease activity (see below), no relationships (concordance or discordance) among relative enzyme activity levels were apparent.

**Toxicity.**—Mouse LD50 data for venoms from adult *C. l. klauberi*, *C. pricei*, and *S. c. tergeminus* were determined in the author’s lab using NSA mice; all others are from literature reports (Table 2). Venom toxicity varies considerably with species, but there are two basic groups: species with venom LD50s below 1.0 μg/g, and those with LD50s of ~1-5 μg/g (Fig. 4). All high toxicity venoms contain phospholipase-based presynaptic β-neurotoxins (Bieber et al., 1987, 1990), which are highly homologous with crotoxin (isolated originally from the venom of *C. d. terrificus*). This toxin also appears in venoms from some populations of species which typically produce venom of lower toxicity (*C. lepidus*; Rael et al., 1992; *C. o. helleri*; French et al., 2004), and the presence of crotoxin homologs greatly increases toxicity (see Wooldridge et al., 2001). Among rattlesnakes, production of highly toxic venoms is apparently dependent upon expression of the crotoxin homolog gene in the venom proteome. However, myotoxin a and homologues have an important role in rapid prey immobilization (Ownby et al., 1988), and this small toxin is also typically present in the most toxic venoms.

The four most toxic venoms also showed the lowest metalloprotease activity, and venom toxicity for all data showed an inverse correlation (r² = 0.65; P = 0.005) with metalloprotease activity (Fig. 5). However, this correlation was driven by the high toxicity venoms, and when type I and type II venoms are analyzed separately, the corresponding correlation of toxicity with metalloprotease activity was 0.15 (P = 0.55) for 19 type I venoms and 0.82 (P = 0.04) for the six type II venoms. None of the other enzyme activities showed an apparent relationship with toxicity (data not shown).

**Occurrence of type I and type II venoms and phylogeny.**—The occurrence of type I or type II venom among rattlesnake species investigated was mapped onto the phylogenetic hypothesis of Castoe and Parkinson (2006; Fig. 6). Both venom types occur in single species clades (e.g., *C. mitchelli*, *C. oreganus*, *C. scutulatus*) and among species groups (e.g., *C. durissus* and related species), and both venom types occur in basal (*Sistrurus*) and the most derived lineages. Based on the broad occurrence of both type I and type II venoms, neither venom type appears to be a basal vs. a derived trait, suggesting that this apparent dichotomy is related to other factors affecting venom composition.

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**Figure 3.** SDS-PAGE analysis of venoms from adult and neonate *Crotalus oreganus concolor*, a subspecies with type II venom (Mackessy et al., 2003). For this subspecies, PIII metalloproteases are very faint, and all of these venoms appear to lack the PI metalloprotease(s) found in many rattlesnake venoms. Myotoxins are one of the components which vary ontogenetically, and levels in neonate venoms are very low (note very faint bands relative to adult venoms).
**DISCUSSION**

Rattlesnake venoms are complex glandular secretions with a diversity of activities, both enzymatic and non-enzymatic (e.g., Table 1). Like most viperids, rattlesnake venoms have a greater number of higher molecular mass enzymes (~14-140 kDa) than lower molecular weight non-enzymatic toxins (<15 kDa), while elapid venoms generally show the opposite trend. These components also comprise the majority of venom mass, respectively. Many components are common to most species of rattlesnakes, likely a reflection of their common evolutionary history, but relative amounts vary widely, and some venom proteins, such as the crototoxin homologs, appear to be expressed only in a limited number of lineages or in particular populations of a single species/subspecies (c.f. Werman, this volume). This differential expression of the venom genome (including post-translational modifications) leads to much of the variation observed in the venom proteome of rattlesnakes, which in turn yields venoms with very different biological properties and effects (Sanz et al., 2006; Pahari et al., 2007).

Venoms are trophic adaptations which allow for a chemical (rather than a mechanical) means of dispatching prey. This chemical mechanism, envenomation, involves a complex series of biochemical and physiological events resulting from the disruption of many homeostatic mechanisms of prey. Because venom proteins are injected at very high concentrations in relatively large amounts, these disruptions happen very rapidly, leading to prey incapacitation and death. Although this process involves many components which are injected simultaneously, there are some compounds which act very rapidly and others which have a slower onset of gross effects. One can therefore envision a “diffusion wave” emanating from the injected venom bolus, with smaller toxins generally among the fast-acting components and larger enzymes among the slower-onset components (Fig. 7). It should be stressed that, although this hypothesis is consistent with diffusion rates as a function of particle size, it has not been experimentally confirmed, so this figure should be viewed as a conceptual representation of envenomation.

**General trends in venom composition among rattlesnakes.**—Patterns of occurrence and levels of various venom components vary considerably between groups and even within species, but one basic trend is apparent: venoms which are characteristically high in metalloprotease activity typically are less toxic (type I), and those with high toxicity typically show low levels of metalloprotease activity (type II). It is important to keep in mind that these venom types refer to general features of adult rattlesnake venoms, as there may also be ontogenetic shifts in composition which further complicate this general dichotomy.

Type I venoms are characteristic of many of the larger-bodied rattlesnakes, and this venom type contains high to very high metalloprotease activity and moderate lethal toxicity, commonly showing an LD50 of 2-5 μg/g body mass (inbred mice). This venom type appears to be more common among rattlesnakes, and species with type I venom include *C. atrox*, *C. basiliscus*, *C. cerastes*, *C. d. durissus*, *C. h. horridus*, *C. mitchelli pyrrhus*, *C. molossus molossus*, *C. o. oreganus*, *C. r. ruber*, *C. t zabcan*, *C. v. viridis* (possibly also *C. polystictus* and *C. pusillus*), *S. c. tergeminus*, and *S. miliarius barbouri*.
Type II venom is characterized by high to very high toxicity (less than 1.0 μg/g body mass, inbred mice) and very low to non-detectable metalloprotease activity. This venom type appears to be less broadly distributed among rattlesnakes, and because of the gross similarity to venoms of juvenile snakes which show ontogenetic shifts in composition (see below), it has been suggested that type II venom represents a paedomorphic trait (Mackessy et al., 2003). Species showing type II venom include *C. d. terrificus*, *C. h. atricaudatus*, *C. l. klauberi* (some), *C. o. concolor*, *C. o. helleri* (some), *C. s. scutulatus*, *A. tigris*, *S. c. catenatus*, and perhaps *S. c. edwardsii*. Species not analyzed in this study which also show Type II patterns include *C. d. collilineatus* (Lennon and Kaiser, 1990) and *C. vegrans* (Kaiser and Aird, 1987).

Note that within several well-defined species clades, such as *C. durissus* and *C. oreganus*, both venom types occur, suggesting that this aspect of venom composition is independent of phylogeny. The presence of type I or type II venoms, when mapped on a recent phylogenetic hypothesis of relationships among rattlesnake species (Castoe and Parkinson, 2006), again shows no apparent trend with phylogeny (see Fig. 6; c.f. Werman, this volume). Even among different populations of the same subspecies (*C. l. klauberi*: Rael et al., 1992; *C. o. helleri*: French et al., 2004; *C. s. scutulatus*: Glenn and Straight, 1978, 1989; Glenn et al., 1983), striking differences in toxicity were noted, indicating that type I or II variants can occur within a single subspecies. At present, the functional significance of this population-level variation is unknown, but it may be related to other important aspects of the snakes’ biology (see below).

**Venom ontogeny.**—In addition to the trends in adult rattlesnake venom composition discussed above, many venoms, perhaps most, show some aspects of ontogenetic shifts in venom composition and/or toxicity (Minton, 1967; Fiero et al., 1972; Reid and Theakston, 1978; Lomonte et al., 1983; Minton and Weinstein, 1986; Mackessy, 1988; Gutiérrez et al., 1991; Mackessy et al., 2003). Many of these ontogenetic patterns of variation also appear to follow the type I/type II dichotomy discussed above. For type I species, neonate and juvenile snakes produce a more toxic venom with low metalloprotease activity, low serine protease (thrombin-like, kallikrein-like, plasmin-like) activity, and often high phospholipase A2 activity. Conversely, venoms from adult snakes are less toxic but have much higher levels of metalloproteases and serine proteases (Mackessy, 1993a,b). Venoms from type II species have been suggested to be paedomorphic because both the neonate/juveniles and the adults have highly toxic venoms with low metalloprotease activity, similar to the venoms of the neonate/juveniles of type I species (but not the adult snakes). However, venoms from type II species also show some aspects of venom ontogenetic shifts in composition: activities of several of the serine proteases (thrombin-like, plasmin-like) and levels of the non-enzymatic myotoxins, potent inducers of immobilization in rodents, are very low in neonate venoms and quite high in venoms from adult snakes (Mackessy et al., 2003). Therefore, the type I/type II dichotomy probably oversimplifies the actual underlying complex patterns of variation.

So what is the functional significance of these general patterns of venom composition? One difference is the differential effect on prey. If venoms are of high toxicity, prey will be killed rapidly and the bulk of the venom bolus, containing the myriad enzymes found in many venoms, will remain localized. For small snakes feeding on prey with a relatively high surface-to-volume ratio, a low predigestion quality to venom may not be important, because stomach enzyme activity will lead to rapid penetration of the prey peritoneum and neutralization of gut flora (see Mackessy, 1988, for a fuller discussion). In particular, if prey abundance is limited,

![Diagram of rattlesnake venom types](image)

**Figure 6.** Adult rattlesnake venom type mapped onto the rattle-snake portion of the phylogenetic hypothesis of Castoe and Parkinson (2006, Fig. 3). All symbols are from the original figure: posterior probability estimates, numerals, and black boxes; circles indicate posterior probability support of 100%. Venom type does not appear to be related to phylogeny, as neither basal nor derived taxa within any clade groupings show one type preferentially. Both types are also seen several times in subspecies of a single species (*C. durissus, C. oreganus, C. mitchelli*).
the most important selective advantage conferred by venom is to immobilize/kill prey rapidly and with certainty. However, for large snakes feeding on large prey, typically mammalian or avian, putrefaction of prey via gut flora activity is a potential problem (e.g., Thomas and Pough, 1979), and the preponderance of metalloproteases in venoms of Type I species obviates this concern. Venoms of these adult snakes are somewhat less toxic than venoms of juvenile snakes, but the high levels of kallikrein-like proteases (Mackessy, 1993b), which promote vascular tone collapse via liberation of bradykinin, and the common occurrence of myotoxin a homologues (Bober et al., 1988), assure that prey will be rapidly immobilized. Extended survival of prey while immobilized can allow for venom components to be delocalized from the site of injection, increasing systemic effects of lytic components such as metalloproteases.

A second significant effect may be freedom from constraints on prey size. Large bulky prey species which are covered by inert epidermal/dermal derivatives are difficult to digest whole, and forced egestion (due to prey putrefaction) appears to be poorly tolerated by many species, particularly among neonates (unpubl. obs.). Conversely, the caloric value of a single large meal is significant (Greene, 1997). Rattlesnakes are gape-limited predators, and maximum prey size possible is directly related to gape, but physical and mechanical limits are likely only part of the defining factors. If large prey cannot be digested efficiently and sufficiently, then capacity to ingest large prey is futile. Initiation of chemical degradation of prey before swallowing is a major biological role for metalloproteases, and these increase with increasing gape size for Type I species. A prediction which follows from this hypothesis is that a constraint on maximum prey size typically consumed by species with Type II venoms should be observed, and prey on average should be significantly smaller than maximum gape size would permit. Some supportive evidence has been provided by diet and venom data for C. o. concolor (Mackessy et al., 2003), which most often consumes relatively small prey, whereas other subspecies of C. oreganus, such as C. o. oreganus and C. o. helleri, may consume very large prey (Klauber, 1956; Mackessy, 1988).

A third functionally significant effect of venom compositional variation may relate to relaxation of constraints on foraging activities. Ectotherm digestive rates will fluctuate with ambient temperature (cf. Dorcas et al., 1997, 2004), which at higher latitude or altitude may be significantly variable within the span of a single day. It has been suggested that venomous snakes generally will ingest prey

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**Enzyme-mediated:**
- Hyaluronidase – decreased interstitial viscosity → diffusion of other venom components
- Kallikrein-like – rapid fall in BP → hypotension

**Toxin mediated:**
- Myotoxin – disruption of sodium ion gradients → muscle dysfunction, immobilization
- PLA₂-based β-neurotoxin – inhibition of ACh vesicle fusion → neuromuscular paralysis

**Enzyme-mediated:**
- Thrombin-like – hydrolysis of fibrinogen → coagulopathy
- Hemorrhagic MPr – hydrolysis of basal lamina of endothelium → hemorrhage

**Toxin mediated:**
- Disintegrins – interfere with platelet-fibrinogen binding → promote hemorrhage

**Enzyme-mediated:**
- LAAO – generation of peroxide radicals → generalized apoptotic events
- Phosphodiesterase – hydrolysis of cNMPs → depletion of 2nd messengers & disruption of cNMP-gated channels
- Phospholipase A₂ – hydrolysis of PLs → hemolysis, nephrotoxicity etc.
- Metalloproteases – hydrolysis of structural proteins → inflammation, necrosis, predigestion

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**Figure 7.** Hypothetical sequence of events following envenomation. Note that all activities of all enzymes and toxins are not included. “Immediate events” are most likely responsible for rapid incapacitation of prey, whereas “long-term events” contribute significantly to the predigestion roles of venom. Abbreviations: ACh, acetylcholine; BP, blood pressure; cNMP, cyclic nucleotide monophosphate; LAAO, L-amino acid oxidase; MPr, metalloprotease; PLA₂, phospholipase A₂; PL, phospholipid.
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killed by venoms regardless of the absolute composition of those venoms (i.e., dead is dead: Mebs, 1999), and observations of scavenging by rattlesnakes (summarized in DeVault and Krochmal, 2002) demonstrate that snakes do not have to envenomate prey in order to digest it efficiently. It is possible that under optimal conditions (e.g., low daily temperature variation, approximately 20-32°C), metalloproteases may not confer a significant selective advantage of increasing digestion efficiency (McCue, 2007). However, animals rarely operate under optimal conditions, and particularly at extremes of latitude and/or altitude, environmental thermal conditions can be quite variable, and the advantage of highly degrading venoms under these conditions may be critically important. Vipers generally are among the few heavy-bodied snakes to occur at high elevations and/or latitudes (e.g., Thomas and Pough, 1979; Carlsson and Tegelström, 2002; Campbell and Lamar, 2004), and for the few which have been investigated, venom shows type I characteristics (Mackessy, 1988). It has been hypothesized that, particularly in these extreme environments, the selective advantage conferred by tissue-degrading metalloproteases is greatest, and these abundant venom components may permit broader ranges of foraging (and therefore digesting) temperatures.

Advances in genomics and proteomics are greatly accelerating the rate at which one can analyze venoms, and techniques in these areas promise to provide near-complete compositional data in the near future (c.f. Serrano et al., 2005; Sanz et al., 2006). However, for many species, detailed aspects of the natural history and ecology, such as diet, foraging, and general activity patterns, and even distributional limits, are incompletely known. These data are critical for understanding relationships between venom composition and the selective impetus favoring particular compositional trends, such as the type I/type II trends noted above. The tremendous number of molecular tools which can be applied to evolutionary and ecological questions have largely overshadowed many other techniques and methods, but robust natural history data are required to provide a meaningful biological context to the biochemical data (e.g., Jorge da Silva and Aird, 2001). There is a paucity of data available on the venoms of many Mexican species of rattlesnakes, particularly the smaller montane species, and an integrative approach toward these species and their venoms, currently in progress, should provide important information concerning the evolution of venoms in rattlesnakes.

ACKNOWLEDGMENTS

Many friends and colleagues contributed venom samples, helped collect snakes and venoms, and/or allowed access to animals in their care; their help was critical to the results presented here and is greatly appreciated. These contributors include J. Badman, D. Chiszar, A. Holycross, B. Jennings, C. Montgomery, S. Minton, T. Moisi, C. Ownby, R. Reisner, R. Seigel, B. Starrett, S. Sweet, and B. Tomberlin. The assistance of many students at UNC is also gratefully acknowledged: A. Boswell, C. Csizmadi, B. Heyborne, J. Honea, A. Kaye, J. Kreps, R. Malecki, A. Malone, R. Milne, S. Munekiyo, N. Sixberry, H. Sommers, A. Wang, A. Wastell, and K. Williams. Scientific collecting permits for snakes or/and venom samples were granted by the Arizona Game and Fish Department (MCKSY000221), Colorado Division of Wildlife (0456, 06HP456), Kansas Wildlife and Parks (SC-147-96), and the New Mexico Game and Fish Department (1984). Financial support for projects which contributed to this work was provided by the Colorado Division of Wildlife and by the UNC Sponsored Programs and Academic Research Center.

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