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Snake Population Venomics: Proteomics-Based Analyses of Individual Variation Reveals Significant Gene Regulation Effects on Venom Protein Expression in *Sistrurus* Rattlesnakes

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Abstract Studies of the molecular basis of adaptations seek to understand the relative importance of structural changes in proteins versus gene regulation effects as determinants of phenotype. Amino acid substitutions in gene coding sequences are well documented as causes of variation in snake venom proteins, whereas the importance of gene regulation effects on venom protein abundance and composition is less well known. Here, we use a proteomics-based approach to infer the effects of gene regulation on protein expression by comparing the relative abundance of specific, known venom proteins among different individuals in each of two species of *Sistrurus* rattlesnakes. Variation in the presence or absence, and in the relative amounts, of proteins was high in both species across all major protein families. Based on our empirical criteria for inferring regulatory effects (presence-absence of specific proteins and/or more than threefold variation in abundance) between 51% and 83% of *S. catenatus* individuals and between 40% and 63% of *S. miliarius* individuals showed evidence for gene regulation across the four most abundant proteins (disintegrins, phospholipase A_2's, serine proteinases, and snake venom metalloproteases). Thus, the effects of gene regulation should be considered an important cause of variation in the composition of whole venoms at the intraspecific level. They also suggest the need for testing the adaptive hypothesis for venom plasticity in relation to prey consumed by adult snakes. Finally, the venom variability reported may have an impact in the treatment of bite victims, highlighting the necessity of using pooled venoms as a substrate for antivenom production.

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Introduction

Identifying the molecular basis of adaptations in natural populations is an important yet largely unrealized goal in evolutionary biology (Lewontin 1974; Orr and Coyne 1992; Golding and Dean 1998). Such information is of broad significance because it addresses fundamental questions about the connection between genotype and phenotype for fitness-related traits and, more explicitly, the relative importance of structural variation in proteins versus gene regulatory changes as the basis for adaptive variation in phenotype (Hoekstra and Coyne 2007). Structural changes can be quantified by identifying mutations in the protein coding regions of genes that in turn have functionally verified effects on adaptive variation at the phenotype level. There are an increasing number of examples of this link between genotype and phenotype in a variety of organisms (for review see Hoekstra and Coyne 2007). Likewise, gene regulation effects on adaptive variation, mainly through mutations in the cis-regulatory regions of genes, are increasingly documented (e.g., Whitehead and Crawford 2006), although the precise mutational changes responsible for these effects often remain unclear (but see Shapiro et al. 2004, 2006; Colossimo et al. 2005).

The importance of gene regulation in natural populations has been inferred from measurements of variation in gene expression using a variety of techniques (for review see Wray et al. 2003). The most widely used approach has been to measure variation in mRNA levels for specific genes using microarrays developed for model organisms (review by Whitehead and Crawford 2006). While this approach is powerful, allowing the simultaneous assessment of transcript variation in many genes, it makes the assumption that there is a close link between transcript abundance and protein levels. Further, it requires the existence of a microarray with homologous loci or the resources to generate an array for the species in question. For nonmodel organisms where such resources do not exist, an alternative is to directly measure variation in the amounts of specific proteins as quantified using recently developed proteomics techniques (Burstin et al. 1994; Damerval et al. 1994; de Vienne et al. 2001; Costa and Plomion 1999; Gerber, Fabre, and Planchon 2000; Klose et al. 2002; Enard et al. 2002). Although the specific genetic basis for such variation can be unclear and post-translational modification of expressed proteins can influence protein levels, it has the advantage of directly measuring variation at the level of the phenotype. For well-characterized proteins, for which the genetic basis is established and the importance of postexpression processing understood, variation in amount of a precisely defined protein among individuals can provide a qualitative assessment of protein-specific regulatory effects.

Venom proteins produced by snakes in the family Colubridae are one of the most widely studied types of animal toxins (for general reviews see Meier and Stocker 1995 and Ménez 2002). Venomous snakes in the families Viperidae and Elapidae produce a complex mixture of distinct proteins in specialized venom glands located in the upper jaw, which they inject into prey using fangs (Meier and Stocker 1995). These venoms contain proteins that interfere with the coagulation cascade, the normal hemostatic system, and tissue repair. Despite the fact that viperid venoms may contain well over 100 distinct proteins (Serrano et al. 2005), these proteins belong to only a few major protein families, including enzymes (serine proteinases, Zn^{2+}-metalloproteases, L-amino acid oxidase, group II PLA_2's) and proteins without enzymatic activity (disintegrins, C-type lectins, natriuretic peptides, myotoxins, CRISP toxins, nerve and vascular endothelium growth factors, cystatin, and Kunitz-type protease inhibitors) (Markland 1998; Juárez et al. 2004; Fry and Wüster 2004; Fry 2005; Serrano et al. 2005; Calvete et al. 2007). Venoms represent the critical innovation that allowed advanced snakes to transition from a mechanical (constriction) to a chemical (venom) means of subduing and digesting prey larger than themselves, and as such, venom proteins have multiple functions including immobilizing, paralyzing, killing, and digesting prey. Given the central role that diet has played in the adaptive radiation of snakes (Greene 1983), venom represents a key adaptation that has played an important role in the diversification of these animals.

Analysis of venom proteins using protein gel electrophoresis has consistently shown high levels of intra- and interspecific variation (for review see Chippaux et al. 1991), and there are a small but increasing number of studies that strongly support the idea that this variation reflects local adaptation for feeding on different prey (e.g., Daltry et al. 1996a). There is also indirect evidence that at some of this variation is under genetic control (Chippaux et al. 1982; Mebs and Kornalik 1984; Gregory-Dwyer et al. 1986; Daltry et al. 1996b), possibly as a result of amino-acid-altering substitutions in venom genes (e.g., Chijiwa et al. 2000; Deshimaru et al. 1996; Chang et al. 2000) or the presence or absence of alleles that code for specific venom proteins (Wooldridge et al. 2001).

However, there is also evidence that gene regulation can affect venom composition. Most significantly, a number of snakes show age-related changes in venom composition (e.g., Mackessy 1988; Gutierrez et al. 1990; Andrade and Abe 1999; Guercio et al. 2006; Mackessy et al. 2006; Alape-Girón et al. 2008). Though the functional significance of the observed changes remain unclear, this pattern is interpreted as reflecting ontogenetic changes in gene expression possibly related to diet differences between juvenile (e.g., coldblooded prey such as frogs and lizards) and adults (e.g., warmblooded prey such as mammals) of the same species. More recently, analyses of the venom transcriptome between closely related species has documented differences in which genes are expressed (Pahari et al. 2007), while comparisons of HPLC-generated venom profiles has shown large differences in the amount of a specific protein that is expressed by individual snakes in the same or different populations (Tsai et al. 2001, 2003).

Our long-term research goal is to understand the molecular mechanisms and evolutionary forces that underlie venom variation in *Sistrurus* rattlesnakes (e.g., Sanz et al. 2006; Gibbs and Rossiter 2008). *Sistrurus* are small North American rattlesnakes (Conant and Collins 1998) which are currently classified as two species, *S. catenatus* and *S. miliarius*, with each species further subdivided into three subspecies (Gloyd 1940). Diet studies show that different taxa of *Sistrurus* rattlesnakes vary in the degree to which they specialize on endothermic versus ectothermic prey (Holycross and Mackessy 2002), which may result in selection for diversification of venom genes and proteins (e.g., Daltry et al 1996a; Chijiwa et al. 2003; Creer et al. 2003). Consistent with this potential we have shown significant differentiation between species in PLA_2

venom genes via positive selection on protein coding regions and gene turnover (Gibbs and Rossiter 2008) as well as substantial differentiation both within and between taxa in venom proteins based on comparisons of one-dimensional PAGE venom profiles (Gibbs et al., unpublished data) and detailed proteomics analyses of venom composition (Sanz et al. 2006). In particular, the proteomic analyses, albeit limited in sample size, revealed variation in whether or not specific proteins were present in different individuals as well as substantial variation in the amount of a specific protein present in multiple snakes. These observations, combined with evidence for limited intra-specific variation in PLA_2 venom genes (Gibbs et al., unpublished data) and evidence for limited but significant ontogenetic changes in venom composition in one species (*S. miliarius* [Deyrup et al. 2000]), have prompted us to explore the role of gene regulation as a cause of variation in whole-venom composition at the individual and population level in these rattlesnakes.

To examine the possible role of regulatory effects, we opted to exploit the detailed proteomics information available from Sanz et al. (2006) to compare levels of specific venom proteins among individuals from two *Sistrurus* taxa: *S. c. catenatus* collected from three geographically distinct locations in eastern and central North America and *S. miliarius barbouri* from a single site in Florida. To this end, we compared individual HPLC profiles using quantitative criteria and then inferred which specific proteins show variation using the detailed analysis of peak identity conducted previously. Our approach closely follows that outlined by de Vienne et al. (2001), with the important addition that, based on the proteomics analyses, we can identify which proteins are associated with specific peaks. The questions that we address are: (i) Based on presence-absence data and variation in abundance, how common were regulatory effects on venom proteins? (ii) Did these effects vary by protein type and/or abundance? and (iii) Were the patterns consistent or different between species?

Methods

Sample Collection

Venom samples were collected from 34 wild-caught *Sistrurus c. catenatus* (hereafter *S. catenatus*) from four sites (Killdeer Plains Wildlife Area [$n = 6$] and Rome Nature Reserve [$n = 4$], both in Ohio; Carlyle Lake State Park, Illinois [$n = 12$]; and Bruce Peninsula National Park, Ontario, Canada [$n = 12$]) and 13 *S. miliarius barbouri* (hereafter *S. miliarius*), which were originally collected in the vicinity of Deland, Florida, and then held in captivity and fed a diet of lab mice for ~3 years. Each animal was constrained using a set of telescoping clear plastic tubes and then induced to bite the top of a 50-ml glass beaker that had been covered with Parafilm. Secreted venom was immediately pipetted into a 2-ml cryovial and stored either in a −80°C freezer or in liquid nitrogen until further processing.

Isolation, Identification, and Quantification of Proteins

Two milligrams of crude venom was dissolved in 100 µl of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile, and insoluble material was removed by centrifugation at 13,000 *g* for 10 min at room temperature. Soluble proteins were separated using an ETTAN HPLC system (Amersham Biosciences) and a Lichrosphere RP100 C_{18} column (250 × 4 mm, 5-µm particle size) eluted at 1 ml/min with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B) (5% B for 5 min, followed by 5–15% B over 20 min, 15–45% B over 120 min, and 45–70% B over 20 min). Protein detection was at 215 nm and peaks were collected manually and dried in a Speed-Vac (Savant). Reverse-phase HPLC runs were consistently superimposable through their *X*-axes and the acetonitrile gradient profile, and thus all chromatograms of venom samples from different specimens from the same species were directly comparable. Given that the wavelength of absorbance for a peptide bond is 190–230 nm, protein detection at 215 nm allows estimation of the relative abundances (expressed as percentage of the total venom proteins – RP) of the different protein families from the relation of the sum of the areas of the reverse-phase chromatographic peaks containing proteins from the same family to the total area of venom protein peaks in the reverse-phase chromatogram. In a strict sense, and according to the Lambert-Beer law, the calculated relative amounts correspond to the "percentage of total peptide bonds in the sample," which is a good estimate of the percentage by weight (g/100 g) of a particular venom component.

Comparison of the HPLC venom profiles of individuals within each *Sistrurus* species, both by eye and using the program metaComps (Nuin 2008), which matches peaks in different samples on the basis of their retention time (RT) values, allowed us to assign chromatographic peaks to previously characterized proteins (Sanz et al. 2006). In a small number of cases new proteins were observed. These new proteins are identified in Fig. 1 (*S. catenatus*) and Fig. 3 (*S. miliarius*) by consecutive numbers that continue the numbering reported, respectively, in Fig. 2 and Table 1 and in Fig. 5 and Table 4 of Sanz et al. (2006) and were identified using our snake venomics approach (Calvete et al. 2007) (Table 1).

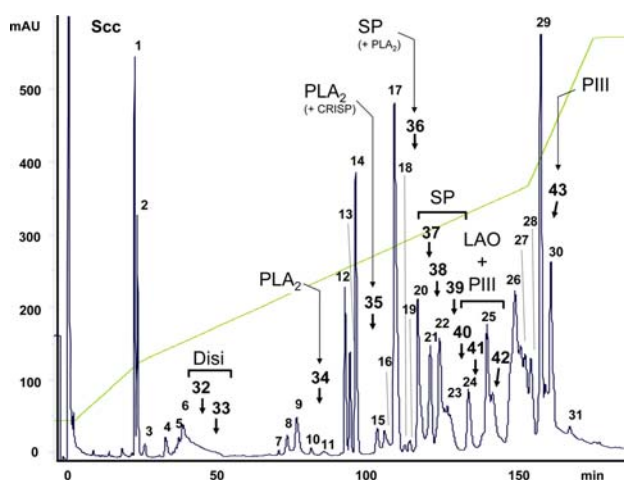

Fig. 1 Reverse-phase HPLC profile of *S. catenatus* venom. Protein fractions 1–31 correspond to those previously identified by Sanz et al. (2006). Peaks not found in this sample but found in other Scc venoms are numbered 32–42; their elution positions are indicated by arrows, and their identities are displayed. Disi, disintegrin; PLA_2, phospholipase A_2; SP, serine proteinase; LAO, L-amino acid oxidase; PIII, snake venom metalloprotease of class PIII

Assessing the Possible Effects of Gene Regulation on Protein Levels

One of our major interests was to use within-species venom composition variation data to assess the relative importance of regulation of genes encoding specific proteins. To this end we used two measures. First, we determined the presence or absence of a protein found in at least one other sampled individual. Individuals with no detected protein were assumed to represent snakes in which the genes for a particular protein were present but not expressed, although alternative explanations are possible (e.g., Wooldridge et al. 2001; see below for discussion). Second, for snakes that had detectable levels of a given protein we sought to determine which individuals had expression levels that were higher than expected due to variation in the number of alleles (one or two) expressed at a given locus. Our goal was to use a measure based on relative levels of protein abundance that was analogous to measurements of variation in gene expression based on relative levels of mRNA for specific genes (cf. Whitehead and Crawford 2006). In such studies, gene regulation is inferred to occur when there is more than a twofold variation in quantities of mRNA hybridizing to a particular cDNA sequence (e.g., see Cavalieri et al. 2000; Whitney et al. 2003).

To calculate a comparable measure, we first defined the baseline level of expression as the lowest positive RP value observed across all snakes. We then calculated a relative peak ratio (RPR) by dividing all nonzero RP values for a specific peak by the baseline value to obtain a value ≥ 1 that reflects the relative abundance of a specific protein in a particular snake. Individuals that showed no evidence of a protein peak were assigned an RPR value equal to 0, indicating no expression. Based on the logic outlined above, RPR values ranging from 1 to 2 indicate levels of gene expression consistent with allelic variation in the corresponding protein coding gene. However, to be conservative we used an upper cutoff value of 3 for this category, and so RPR values >3 were classified as variation consistent with overexpression due to possible effects of gene regulation. This measure assumes a roughly one-to-one relationship between particular venom loci and specific venom proteins that are produced. There is evidence of such a relationship for at least some PLA_2 locus-protein combinations in both *S. catenatus* and *S. miliarius* (Gibbs and Rossiter 2008).

Finally, we estimated correlations between RP values for all individual proteins shown in Figs. 1 and 3 within each species using Spearman rank correlations and associated *p*-values. Because of the large number of pairwise comparisons we arbitrarily set the cutoff for significant correlations at $p < 0.01$. We were interested in detecting two types of correlations: (1) significant negative correlations between different proteins of the same family, which may indicate instances of posttranslational modification in which one protein was converted into another of the same type; and (2) significant positive correlations between proteins, which might indicate common regulation of abundance by the same transcription factor(s).

Results

Abundance and Variation of Specific Venom Proteins

To investigate within-species venom variability, the reverse-phase HPLC protein profiles of venoms from 34 specimens of *S. catenatus* and 13 *S. miliarius* were compared. The HPLC profiles and relative amounts of different proteins were similar to those found in a previous study (Sanz et al. 2006) of venom composition in these rattlesnakes which was based on much smaller sample sizes (two *S. catenatus* and a single *S. miliarius*). However, each venom exhibited distinct features in both qualitative (protein peaks not observed in other venoms) and quantitative (variation in the relative proportions of components shared between venoms) features. Figures 1 and 3 display representative chromatograms for *S. catenatus* and *S. miliarius* venoms, respectively, showing previously characterized protein fractions (labeled 1–31 as in Figs. 2 [Scc] and 5 [Smb] of Sanz et al. [2006]). Novel protein fractions are labeled 32–43 and their identities were established using our venomics approach (Calvete et al. 2007). These comparisons revealed that *S. catenatus* venoms exhibit a higher qualitative variation than *S.*

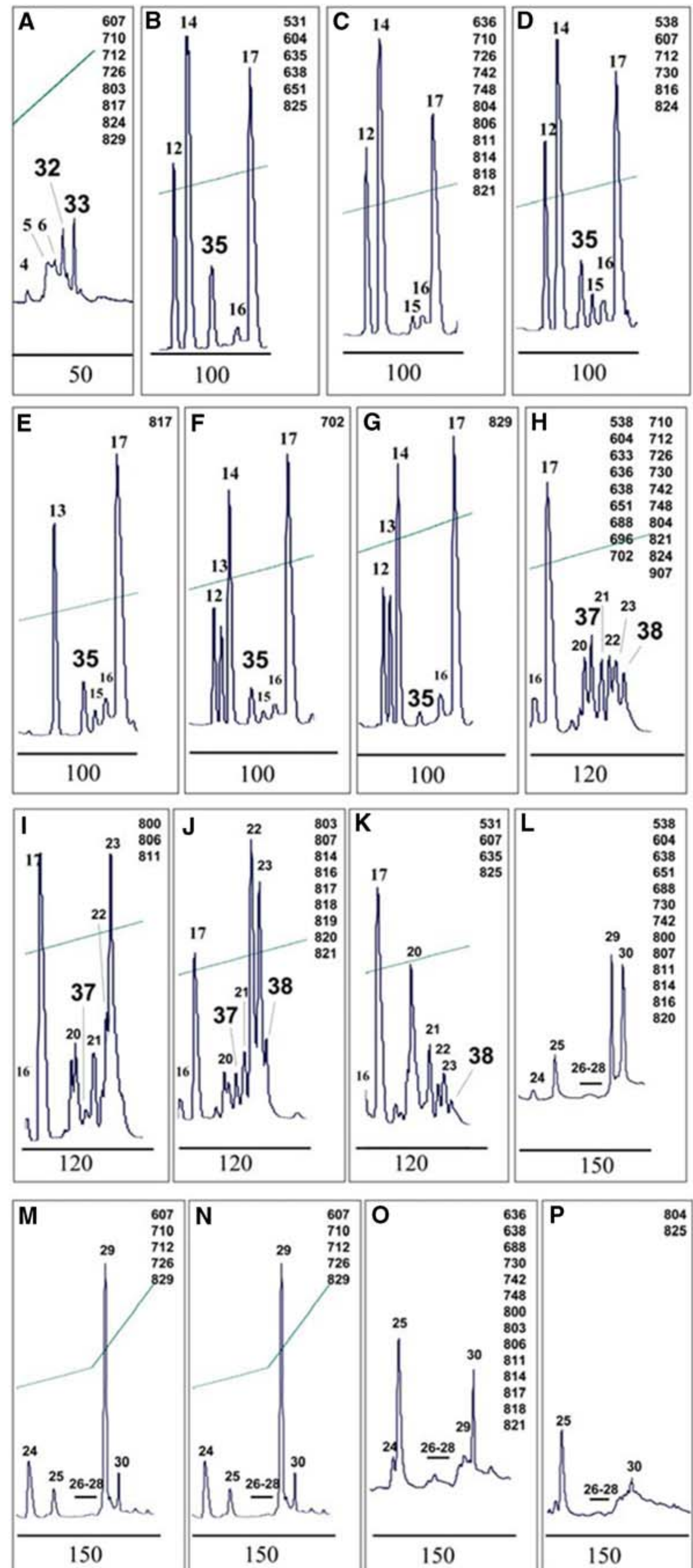

Fig. 2 Intraspecific individual variation in the composition of venoms from *S. catenatus*. Panels **a**–**p** display details of the reverse-phase HPLC chromatograms of the venoms from individual *S. catenatus* showing representative qualitative and quantitative differences in the concentrations of venom components labeled as in Fig. 1. Numbers in the upper-right corner of each panel correspond to individual *S. catenatus* snakes exhibiting the displayed venom profile

Table 1 Details for measures of relative percentage (RP) and relative percentage ratio (RPR) (see text) for different protein venom families of *S. catenatus*

Protein family	RP (mean ± SD)	CV (×100)	Individuals with zero protein	Range of nonzero RP values	RPR (mean ± SD)	CV (×100)	Maximum RPR
Disintegrin	1.48 ± 0.69	99.6	6 (17.6%)	0.31–3.4	6.23 ± 3.47	70.9	15.0
PLA_2	4.99 ± 1.74	114.0	8.6 (25.2%)	3.03–9.5	3.60 ± 2.55	55.5	9.9
Serine proteinase	3.44 ± 3.07	121.5	10.1 (29.8%)	1.14–13.2	5.09 ± 3.83	66.6	13.8
SVMP	1.04 ± 1.60	164.6	13.3 (39.2%)	0.14–7.2	10.85 ± 11.95	107.6	47.5
Myotoxin	0.58 ± 0.25	43.1	0	0.18–1.1	3.22 ± 1.41	43.7	5.8
NGF	0.21 ± 1.6	76.2	8 (24%)	0.08–0.6	3.43 ± 1.48	43.2	7.0
CRISP	0.71 ± 0.66	92.3	8 (24%)	0.27–3.0	3.44 ± 2.23	64.8	11.1
PLA_2/SP	1.13 ± 0.99	87.6	4 (12%)	0.07–2.9	18.27 ± 13.62	74.6	41.0
LAO/SVMP	3.13 ± 1.37	43.8	0	0.41–5.9	7.63 ± 3.35	43.9	13.3

Note: *CV* coefficient of variation; *PLA_2* phospholipase A_2; *SVMP* snake venom metalloproteases; *NGF* nerve growth factor-like protein; *CRISP* cysteine-rich secretory protein; *PLA_2/SP* protein fractions containing mixture of serine proteinase (SP) and *PLA_2*; *LAO/SVMP* protein fractions containing mixture of L-amino acid oxidase and SVMP. Values represent means averaged across the individual proteins in each family as shown in Fig. 1 except for CV, which is based on mean and SD values given in the table

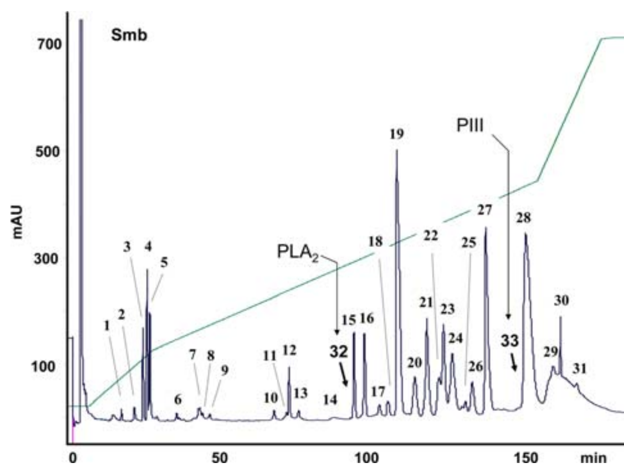

Fig. 3 Reverse-phase HPLC profile of *S. miliarius* venom. Protein fractions 1–31 correspond to those previously identified by Sanz et al. (2006). Peaks absent from this sample but found in other Smb venoms are numbered 32 and 33; their elution positions are indicated by arrows, and their identities are displayed. PLA_2, phospholipase A_2; PIII, snake venom metalloprotease of class PIII

miliarius venoms. On the other hand, both *Sistrurus* venoms exhibit a substantial degree of qualitative variation involving all major toxin classes (Figs. 2 and 4, Tables 1 and 2). As pointed out by de Vienne et al. (2001) the coefficient of variation (CV = standard deviation/mean) is a useful comparative measure of variation in protein amounts because the magnitude of the standard deviation is expressed independent of the mean. Thus, CV allows direct comparisons of the relative variation of different proteins independent of their mean percentage abundance.

The relative levels of variation vary widely both among the major protein families of *S. catenatus* venom and between specific proteins within each family (range of CV values for disintegrins, 34–214%; phospholipase A_2's [PLA_2's], 12–417%; serine proteases [SPs], 32–255%; snake venom metalloproteases [SVMPs], 124–194%) (data not shown). Mean CV values for each protein class capture this variation, with values ranging from a low of 34.9% (PLA_2 s) to a high of 153.8% for SVMPs (Table 1). *S. miliarius* venom proteins are also variable but the magnitude of the variation is less (Table 2). Mean CVs for the major protein families vary from 84.7% (SVMP range, 21–105%) to 118% (PLA_2's, 32–133%), with disintegrins (40–137%), and SPs (30–244%) exhibiting intermediate mean CV values of 105% and 100%, respectively (Table 2).

Variation in the presence and/or absence of proteins and variation in their relative amounts both contribute to the high overall CV values observed in the two *Sistrurus* species. In terms of presence and/or absence variation, the percentage of sampled *S. catenatus* individuals not expressing a specific protein is significantly higher than that of *S. miliarius* specimens (Tables 1 and 2, Figs. 1–4). For the major protein types the mean value averaged across individual proteins of *S. catenatus* venoms ranges from 18% (disintegrins) to 39% (SVMPs) (Table 1), whereas in *S. miliarius* mean values for major proteins range from 7% (SVMPs) to about 13% (SPs) (Table 2).

There is also substantial variation in the amount of protein present in snakes that express a given protein. Among major types of proteins, there is a 3-fold (PLA_2's) to 51-fold (SVMPs) ratio between the mean minimum and the mean maximum RP values in *S. catenatus* (Table 1), while for *S. miliarius*, these values range from a threefold difference for SVMPs to an eightfold difference for disintegrins (Table 2). Thus, based on extreme values, some individuals express proteins at levels that are substantially higher than can be explained by allelic dosage effects alone. However,

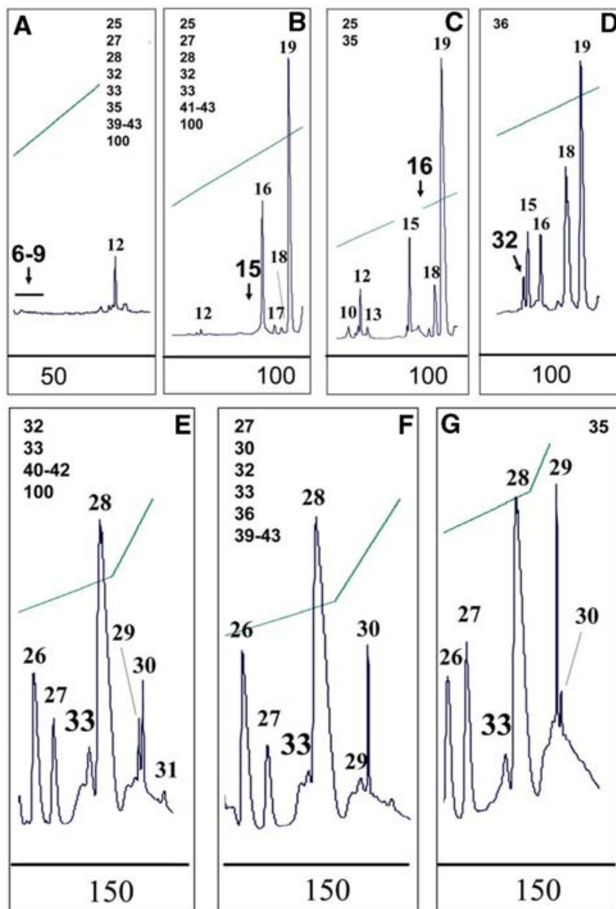

Fig. 4 Intraspecific individual variation in the composition of venoms from *S. miliarius*. Panels (**a**–**g**) display details of the reverse-phase HPLC chromatograms of the venoms from individual *S. miliarius* showing representative qualitative and quantitative differences in the concentrations of venom components labeled as in Fig. 3. Numbers at the top of each panel correspond to individual *S. miliarius* snakes exhibiting the displayed venom profile

although ratios calculated from extreme values show the potential for gene regulation effects, they do not inform us about their general importance.

To explore this issue, we calculated RPR ratios for the venom proteins of each individual *S. catenatus* and *S. miliarius* snake (Table 1). Most proteins show evidence for the effects of regulation in *S. catenatus*: all major protein types have mean RPR values >3, ranging from a mean of 5.1 for SPs to 10.9 for SVMPs, and many individual proteins show the same pattern (protein type and number [%] of individual proteins with mean PRP >3: disintegrins, 3 of 3 [100%]; PLA_2's, 5 of 10 [100%]; SPs, 6 of 8 [75%]). For *S. miliarius*, mean RPR values are lower but many are still >3 (disintegrins, 1 of 3 [33%]; PLA_2's, 2 of 4 [25%]; SPs, 3 of 7 [43%]; SVMPs, 1 of 3 [33%]) and mean values for each major protein type are >3 (Table 2). In summary, on average individual snakes of both species show levels of expression for many proteins that are greater than the threshold we use to identify regulatory effects.

To quantify the relative importance of each effect (lack of expression or regulation effects) on abundance, we categorized RPR values for each snake across all proteins into one of three groups: 0 (lack of expression); 1–3 (consistent with gene dosage effects alone); and >3 (consistent with gene regulation effects). The proportion of values in the 0 and >3 categories estimate the number of individuals showing the potential effects of gene regulation (broadly defined) on venom protein abundance. Table 3 shows that in *S. catenatus* the proportion of RPR values in these two categories combined ranges from 50% (MYO) to 83% (SVMPs) in all snakes. In *S. miliarius*, SVMPs show the lowest proportion of RPR values in this combined category (50%), whereas the highest value is 86% for the single CRISP venom protein (Smb17; Fig. 3).

These values also can be used to evaluate the relative importance of regulatory effects among different proteins within a species and for comparable proteins in different species. Differences in the relative number of values in different categories would argue for differences in the relative importance of regulation effects on protein abundance. To simplify this analysis we pooled RPR values into

Table 2 Details for measures of relative percentage (RP) and relative percentage ratio (RPR) (see text) for different protein venom familes in *S. miliarius*

Protein family	RP (mean ± SD)	CV (×100)	Individuals with zero protein	Range of nonzero RP values	RPR (mean ± SD)	CV (×100)	Maximum RPR
Disintegrin	0.94 ± 0.71	75.5	1.7 (11.9%)	0.33–2.7	3.07 ± 3.23	105.2	9.5
PLA_2	6.28 ± 2.94	46.8	1.25 (8.9%)	3.2–12.5	6.64 ± 7.83	117.9	27.9
Serine proteinase	2.56 ± 1.33	52.0	1.86 (13.3%)	1.1–5.7	3.07 ± 3.06	100.0	11.7
SVMP	4.33 ± 3.39	78.2	1 (7%)	5.0–14.8	4.85 ± 4.11	84.7	12.9
BPP	0.19 ± 0.19	97.5	3 (21%)	0.1–0.7	1.94 ± 1.89	97.4	6.6
CRISP	0.27 + 0.26	97.0	5 (36%)	0.1–0.8	2.73 ± 2.65	97.1	8.2

Note: *CV* coefficient of variation; *PLA_2* phospholipase A_2; *SVMP* snake venom metalloproteases; *BPP* bradikinin-potentiating peptide; *CRISP* cysteine-rich secretory protein. Values represent means averaged across the individual proteins in each family as shown in Fig. 2 except for CV, which is based on mean and SD values given in the table

Table 3 Proportion of individual relative percentage ratio (RPR) values for distinct protein families from individual *S. catenatus* and *S. miliarius* venoms

Protein family	*S. catenatus*				*S. miliarius*			
	N	0	1.0–3.0	>3	*N*	0	1.0–3.0	>3
Disintegrin	102	0.19	0.29	0.52	42	0.11	0.37	0.38
PLA_2	340	0.25	0.49	0.25	56	0.09	0.57	0.34
Serine proteinase	272	0.30	0.28	0.42	98	0.13	0.53	0.34
SVMP	204	0.39	0.17	0.44	56	0.07	0.60	0.32
CRISP	34	0.24	0.38	0.38	14	0.36	0.14	0.50
Myotoxin	34	0.00	0.50	0.50				
NGF	34	0.24	0.35	0.41				
BPP					14	0.21	0.50	0.29

Note: *PLA_2* phospholipase A_2; *SVMP* snake venom metalloproteases; *CRISP* cysteine-rich secretory protein; *NGF* nerve growth factor-like protein; *BPP* bradikinin-potentiating peptide. RPR value equal to zero indicates no expression of this protein. RPR values ranging from 1 to 3 may indicate levels of gene expression consistent with only allelic variation of the corresponding protein coding gene. RPR values >3 correspond to variation consistent with protein overexpression due to gene regulation effects

just two categories: from 1 to 3 (no regulatory effects) and 0 or >3 (consistent with regulatory effects). For *S. catenatus* there is a highly significant difference in proportion of RPR values in each of these categories across all proteins listed in Table 3 ($\chi^2 = 272$; df = 13; $p < 0.001$) and for the major proteins (disintegrin, PLA_2's, SPs, and SVMPs) only ($\chi^2 = 462$; df = 7; $p < 0.001$). The same pattern holds for *S. miliarius* (Table 3) (all proteins, $\chi^2 = 59.4$, df = 11, $p < 0.001$; disintegrin, PLA_2's, SPs, and SVMPs, $\chi^2 = 10.7$, df = 7, $p = 0.013$). Comparisons of the distribution of RPR values in each category between *S. catenatus* and *S. miliarius* showed significant differences for three of four major proteins (disintegrin, $\chi^2 = 5.5$, df = 3, $p = 0.019$; SPs, $\chi^2 = 19.4$, df = 3, $p < 0.001$; SVMPs, $\chi^2 = 44.1$, df = 3, $p < 0.001$) but not for PLA_2 ($\chi^2 = 0.05$, df = 3, $p = 0.82$). Thus, the effect of gene regulation on venom protein abundance varies between proteins within the same species and between most major protein types between species.

Correlations Between Protein Abundance and Variation

Examining the relationship between overall abundance of a specific protein and different measures of variation will indicate if either more abundant or rarer proteins have higher levels of variation. To do this, for each species, we tested for significant correlations between the average RP for the individual proteins and the CV for RP. For both species there was a significant negative relationship between these variables (*S. catenatus*, $r = –0.50$, $p < 0.01$; *S. miliarius*, $r = -0.53$, $p < 0.01$), demonstrating that more abundant venom proteins showed less variation between individuals. To determine which factors underlie these relationships, we also determined if there was a correlation between the proportion of individuals with no protein present (measure of relative importance of nonexpression) and the mean RPR for each protein (measure of the magnitude of the effect of gene regulation). For both species, there was a significant negative relationship between the proportion of nonexpressing individuals (*S. catenatus*, $r = -0.50$, $p < 0.01$; *S. miliarius*, $r = -0.43$, $p < 0.05$) but not between mean RPR (*S. catenatus*, $r = -0.27$, $p > 0.05$; *S. miliarius*, $r = -0.26$, $p > 0.05$). Thus variation in the relative frequency with which a protein is expressed, but not variation in the relative level of expression, seems to be the reason for the relationship. In other words, more abundant proteins show less variation among individuals, likely because the genes producing them are more consistently turned on.

Geographic Variation in Gene Expression

To see if there was any variation in gene expression among snake from different populations, we compared the proportion of individuals that expressed a given protein among the three *S. catenatus* populations that were sampled. There was little evidence for geographic variation in most proteins. Only six proteins showed significant differences in the proportions of individuals with and without the protein present, and only one (serine proteinase Scc39 in Fig. 1) remained significant after adjusting the critical *p*-value for the number of tests performed (Table 4).

Correlations Between Abundances of Individual Proteins

In *S. catenatus*, there were five pairs of proteins of the same type whose abundances were significantly negatively correlated with each other: serine proteinase (SP) 1 (peak Scc20 in Fig. 1) with each of SP 2 (Scc37) ($r = -0.53$; $p = 0.0014$), SP 5 (Scc22) ($r = -0.56$; $p = 0.0006$), and SP 7 (Scc23) ($r = -0.58$; $p = 0.0003$); SP 3 (Scc21) with SP 4 (Scc38) ($r = -0.47$; $p = 0.0047$); and PLA_2 (Scc9) with PLA_2 6 (Scc13) ($r = -0.61$; $p = 0.0001$). These pairs represent just 4.8% of the total number of possible pairwise comparisons (5/104) between individual proteins and each type with two or more proteins present. In *S. miliarius*, only 2 of 26 (7.6%) possible correlations were significant: disintegrins (DISIs) 1 (Smb6-9) (Fig. 3) and 2 (Smb11-12) ($r = -70$; $p = 0.0056$) and PLA_2's Smb15 and Smb16) (Fig. 3) ($r = -0.69$; $p = 0.0067$). If we assume that these correlations represent possible instances of posttranslational modifications of proteins, these results suggest that

Table 4 *S. catenatus* venom proteins showing significant ($p < 0.05$) variation between populations in the proportion of individuals expressing or not expressing the protein

Protein	Population	Individuals expressing protein	Individuals not expressing protein	*p*-value
Scc 11 (NGF)	BP	3	9	0.022
	IL	0	12	
	OH	5	5	
Scc 35 (PLA_2)	BP	2	10	0.028
	IL	7	5	
	OH	7	3	
Scc 36 (SP)	BP	4	8	0.000*
	IL	0	12	
	OH	8	2	
Scc 38 (SP)	BP	4	8	0.019
	IL	5	7	
	OH	9	1	
Scc 39 (SP)	BP	2	10	0.003*
	IL	9	3	
	OH	8	2	
Scc26 (PIII-SVMP)	BP	6	6	0.021
	IL	6	6	
	OH	0	10	

Note: BP—Killdeer Plains Wildlife Area and Rome Nature Reserve, both in Ohio; IL—Carlyle Lake State Park, Illinois; BP—Bruce Peninsula National Park, Ontario, Canada. After adjusting the critical *p*-value for the total number of tests performed using the Benjamini-Yekutieli (2001) False Discovery Rate correction, only those *p*-values marked with an asterisk remained significant. Numbering as in Fig. 1

this process is uncommon in these snake venom proteins, affecting <10% of all possible combinations of proteins. However, when it does occur it affects select PLA_2 proteins in both species and specific serine proteases in *S. catenatus* and certain disintegrins in *S. miliarius.*

Finally, we also looked for instances of positive correlations between sets of proteins that might suggest groups of individual proteins under common regulatory control. We found examples of positively correlated networks in both species (Fig. 5). In *S. catenatus* there were five sets of proteins, all of which showed positive correlations in abundance with each other. Three involved proteins from the same family (SVMPs, PLA_2's, and SPs) while the other two consisted of multiple types of venom proteins. In *S. miliarius*, three sets of proteins (all containing different protein types) showed positive correlations in abundance with each other (Fig. 5).

Discussion

This study is the first to estimate the effects of gene regulation on venom protein expression on a "venome"-wide level in a snake. Our results suggest that these effects are important across all proteins in two species of snakes and thus need to be considered in mechanistic and evolutionary explanations for venom variation among rattlesnakes at the individual, population and species levels.

We make several important assumptions in our analyses. First, we assume that the absence of a protein detected in other individuals represents nonexpression of that protein in that snake. As discussed above two alternative explanations are that the protein is expressed but then is posttranslationally modified, appearing in the chromatogram as a different peak and/or that protein-specific alleles are absent in snakes which the protein is also absent (cf. Wooldridge et al. 2001). We feel that both phenomena are unlikely to account for the absence of specific proteins in

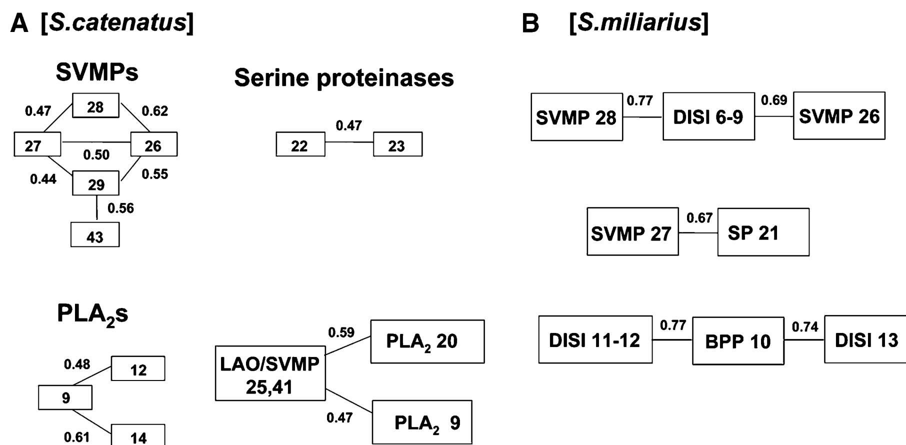

Fig. 5 Individual venom proteins which show significant positive correlations in abundance in (**a**) *S. catenatus* and (**b**) *S. miliarius*. Values for the Spearman rank correlation coefficients are shown next to the line connecting individual proteins. All correlations were significant at $p < 0.01$. Peak numbers correspond to those displayed in Fig. 1 (*S. catenatus*) and Fig. 3 (*S. miliarius*). SVMP, snake venom metalloprotease; BPP, bradykinin-potentiating peptide. For other abbreviations see the legend to Fig. 1

many individuals. The low number of significant negative correlations between the abundances of individual proteins of the same type suggest that posttranslational modifications are rare in these venom proteins. Although limited to a single type of protein, surveys of variation in a small number of PLA_2 loci among *S. catenatus* populations suggest that all individuals have the same array of loci and allelic variation at single loci is not present (Gibbs et al., unpublished data). However, additional information on variation in the presence and absence of venom loci and on allelic variation within loci for gene encoding a wider range of proteins is necessary for us to be confident that the absence of a specific protein is due to nonexpression of alleles at an extant locus.

We also assume that the minimum amount of a protein detected in any individual represents an empirical benchmark for the minimum expression expected across individuals and that more than a threefold increase over this benchmark level indicates evidence for the effects of expression. Similar benchmarks have been used in other proteomic-based studies of expression (de Vienne et al. 2001), and as described above a more than twofold increase in transcription levels has been interpreted as evidence for expression effects in microarray studies (Cavalieri et al. 2000; Whitney et al. 2003). These benchmarks assume an additive model for the effects of gene copy number on protein and mRNA expression levels across all proteins with, for example, no positive or negative epistatic genetic effects on protein expression. While there are increasing numbers of studies which are establishing links between the transcriptome-proteome levels for venom proteins (e.g., Junqueira-de-Azevedo and Ho 2002; Fry 2005; Junqueira-de-Azevedo et al. 2006; Pahari et al. 2007; Sanz et al. 2008), none have been comprehensive enough to evaluate these assumptions about the relationships between gene number and protein levels for any venom protein and so our conclusions remain tentative until such evaluations can be made. Studies in other taxa have shown that pleiotropic effects on protein expression occur but are uncommon, affecting <10% of the proteins in the system studied (Gottlieb and de Vienne 1988).

Evidence for Regulatory Effects in Other Snakes

Although limited, there are data from at least two other species of venomous snakes showing evidence of regulatory effects on venom abundance. First, Tsai et al. (2001) compared HPLC profiles of four distinct PLA_2 venom proteins isolated from five individual *Calloselasma rhodostoma*, each collected from a different location in Southeast Asia. A reanalysis of the data in their Table 1 shows that three of four proteins had ratios of minimum-to-maximum %RP values >3.2 and that one individual did not express a protein (H1E6) found in the other four snakes. Second, Tsai et al. (2003) also used HPLC profiles to quantify variation in seven PLA_2 venom proteins isolated from seven individual *Crotalus viridis* collected throughout their range in the western United States. Based on data from their Table 2, min/max ratios for %RP values for six of the seven proteins detected in more than one snake ranged from 1.6 to 19.3, with five of six being >2.7, and five of six proteins were not detected in one or more snakes. Although the number of individuals compared was small and the venom proteins examined were limited to PLA_2's, these results suggest that our results may be general, with the caveats that geographic and individual variation may be confounded in these studies of small numbers of snakes from different locations and that the presence/absence variation in specific proteins could have explanations other than regulatory effects (see above).

Molecular and Evolutionary Implications

Within each species, there is variation in the importance of regulatory effects for different proteins. For example, for major proteins in *S. catenatus*, the percentage of individuals that showed possible regulatory effects ranged from 51% across all PLA_2's to 83% for SVMPs. In *S. miliarius*, the overall values are lower and the proteins which show high (disintegrins; 63%) and low (SVMPs; 40%) values are different. Hence, even between these two closely related congeneric species, there is substantial variation in the relative importance of regulatory effects on the expression of different proteins in whole venom.

One factor that correlates with variation in whether a protein is expressed in different individuals is overall abundance: proteins that are more abundant show less variation in expression through presence-absence variation than those which are less abundant. A possible interpretation of this pattern is that abundant, highly expressed venom proteins may be analogous to highly expressed "housekeeping" proteins which perform a variety of essential cellular functions (Gygi et al. 2000; Warrington et al. 2000). Under this idea, abundant venom proteins may perform generic killing and digestive functions that are not prey specific, whereas less abundant proteins may be more plastic in either evolutionary or ecological timescales and serve to "customize" an individual snake's venom to feeding on particular prey requiring a specific venom protein (Mackessy et al. 2006). This could be tested experimentally either by monitoring changes in venom in snake feed over long periods of time on distinct diets (see below) or by comparing rates of evolution of the genes or regulatory pathways underlying these less abundant proteins with those of abundant proteins (cf. Winter et al. 2004).

We see two important evolutionary implications of our results for studies of snake venom evolution. First, the effects of gene regulation must now be considered a potentially important explanation for the variation in abundance of different proteins in whole venom. To date, much attention has been focused on how structural differences in proteins due to positive diversifying natural selection in relation to diet contribute to inter- and intra-specific variation in whole venom (e.g., Daltry et al. 1996a; Kordiš and Gubenšek 2000; Ohno et al. 2003; Gibbs and Rossiter 2008) and strong indirect evidence that such genetic effects are important (see above). Based on this evidence it is clear that structural changes in venom proteins must be responsible for a significant portion of the high levels of phenotypic variation in venom proteins at the inter- and intraspecific levels. However, there is now evidence for regulatory effects on venom expression for different proteins in different species based on a variety of methodologies (proteomics-based analyses [Tsai et al. 2001, 2003; this work]; mRNA expression levels [Ma et al. 2001, 2002]). This evidence argues that regulation now needs to be considered an equally important mechanism causing phenotypic variation in whole-venom composition as is the case in other taxa (*Conus* snails) in which differential expression of different venom loci is important in producing species-specific venom that allows species to capture specific prey (Duda and Palumbi 2004).

Given the insights arising from studies of the molecular evolution of venom gene coding regions, direct study of intra- and interspecific polymorphism in the promoter region upstream of coding region of specific genes would be useful in exploring the possible genetic basis for regulatory effects (e.g., Shapiro et al. 2004, 2006; Colossimo et al. 2005). The promoter regions of some venom protein genes which contain cis-regulatory elements are reasonably well characterized (for review see Chang 2007). Insights into whether these regions are undergoing selection could come from studies which determine levels of polymorphism in specific regulatory elements or more general tests of whether sequence in promotor region is evolving under selection through comparisons of levels of polymorphism in the promoter region versus intronic regions (cf. Haygood et al. 2007).

Finally, if gene regulation effects prove to be as common as the evidence to date suggests, then the adaptive significance of this plasticity in this key trophic adaptation in snakes needs to be addressed. Past work has shown strong evidence for ontogenetic changes in venom composition as snakes grow (cf. Mackessy 1998). The interpretation is that changes in venom composition reflect changes in diet related to shifts in body size. Following this logic we propose the hypothesis that changes in venom gene expression in adult snakes may be related to changes in diet through time. Highly expressed proteins may allow individuals to target the overall effectiveness of their venom to particular prey through the expression of prey-specific venom proteins (cf. Mackessy et al 2006). This hypothesis could be tested by experimentally altering the diet of captive *Sistrurus* and then monitoring changes in venom composition and testing the effectiveness of the more highly expressed proteins on the most commonly consumed prey. The proteins with positively correlated abundances shown in Fig. 5 represent possible sets of proteins which may covary with changes in diet.

We plan to conduct such experiments to test this hypothesis as a cause of gene expression effects in *Sistrurus*.

Biomedical Considerations

Besides ecological and taxonomical implications, the venom variability reported here may have an impact in the treatment of bite victims and in the selection of specimens for antivenom production. The occurrence of intraspecies variability in the biochemical composition and symptomatology after envenomation by snakes from different geographical locations and of different ages has long been appreciated by herpetologist and toxinologists, and appears to be a general feature of venoms (Chippaux et al. 1991). Taborska and Kornalik (1985) reported considerable individual variability in both pathophysiological and enzymatic activities between parents and siblings of a family of *B. asper* snakes. The disparity of symptoms in victims of the same species of snake has long alerted clinicians to the requirement for more specific antivenoms (Jiménez-Porras 1964; Chippaux et al. 1991). Thus, knowledge of the individual variability in venoms, as reported here, could be relevant for antivenom production. However, detailed comparative proteomic analyses are scarce and limited to a few species. Our study represents an effort in that direction and highlights the necessity of using pooled venoms as a representative substrate for antivenom production.

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