JOURNAL OF PROTEOMICS 74 (2011) 2169–2179

available at www.sciencedirect.com

www.elsevier.com/locate/jprot

Proteomic analysis of ontogenetic and diet-related changes in venom composition of juvenile and adult Dusky Pigmy rattlesnakes (*Sistrurus miliarius barbouri*)

H. Lisle Gibbs[a,*], *Libia Sanz*[b], *James E. Chiucchi*[a], *Terence M. Farrell*[c], *Juan J. Calvete*[b,**]

[a]*Department of Evolution, Ecology and Organismal Biology, Ohio State University, 300 Aronoff Laboratory, 318 W. 12th Ave., Columbus, OH 43210-1293, USA*
[b]*Instituto de Biomedicina de Valencia, C.S.I.C., Jaime Roig 11, 46010 Valencia, Spain*
[c]*Biology Department, Stetson University, 421 N. Woodland Boulevard, DeLand, FL 32720, USA*

A R T I C L E I N F O

Article history:
Received 25 April 2011
Accepted 13 June 2011
Available online 22 June 2011

Keywords:
Sistrurus miliarius barbouri
Ontogenetic venom changes
Diet-related venom changes
Snake venom proteomics
Mass spectrometry

A B S T R A C T

Snake venom proteins show high levels of variation at the level of the individual yet the environmental and molecular mechanisms that generate this diversity remain unclear. Here we report the results of a controlled feeding experiment combined with proteomic analyses of periodically collected venom samples to assess the roles of ontogenetic and diet-related effects on venom composition of captive juvenile and adult Dusky Pigmy rattlesnakes (*Sistrurus miliarius barbouri*). Juvenile snakes fed from birth with mice, lizards, or frogs showed little evidence for an ontogenetic shift in venom composition from 5 to 26 months in terms of substantial changes in the relative abundance of major classes of venom toxins. However, there were fine-scale changes in the relative abundance of D49-PLA_2 15, PI-SVMPs, and PIII-SVMP 28, and a decline in the abundance of other PIII-SVMPs. Although juveniles raised on different prey exhibited distinct relative toxin compositional change rates, at 26 months old, their venoms showed similar patterns of protein composition suggesting little effect of diet on the overall make-up of venom in snakes this age or younger. In contrast, adult females raised on different prey over a 26 month period showed prey-related changes in the relative abundance of major protein families from initial to final samples. Specifically, mouse-fed females showed substantial increases in the relative abundance of total PLA_2s and serine proteinases of 95% and >100%, respectively, whereas comparable values for lizard- (42% and −22%) and frog-fed females (2% and 11%) were distinctly smaller in magnitude. Venom from adult snakes fed on different prey also showed distinct changes in the abundance of PLA_2 molecules 15, 19a, and 19, which were, respectively, (>100%, 33%, 63%), (>100%, 0%, 35%), and (71%, 20%, −4%) for the mice-, lizard-, and frog-diet. Venom from snakes raised on frogs contained a small (1.1%) but consistent amount of a PLA_2 molecule (15a) not present in snakes fed on mice or lizards. This work provides evidence that venom composition is somewhat plastic in both juvenile and adult *S. m. barbouri* and that, at least in adults, prey consumed may influence the relative abundance of possibly functionally-distinct classes of venom proteins.

© 2011 Elsevier B.V. All rights reserved.

* *Corresponding author.*
** *Corresponding author:* Tel.: +34 96 339 1778; fax: +34 96 369 08 00.
E-mail addresses: gibbs.128@osu.edu (H.L. Gibbs), jcalvete@ibv.csic.es (J.J. Calvete).

1874-3919/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jprot.2011.06.013

1. Introduction

Venomous snakes in the families Viperidae and Elapidae produce a complex mixture of distinct toxic proteins in specialized venom glands located in the upper jaw, which they inject into prey using fangs [1]. Venom represents a trophic adaptive trait, crucial to the foraging success of the snake. Venom proteins are subjected to accelerated Darwinian evolution [2], and variability of venom composition at the genus, species, subspecies, population, and individual levels may endow snakes with the capability to adapt to different ecological niches. Analysis of snake venom proteins (SVPs) using protein gel electrophoresis has consistently shown high levels of intra- and interspecific variation (for review see [3]) 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 [4]. There is indirect evidence that at some of this variation is under genetic control [5–8] possibly as a result of amino-acid altering substitutions in venom genes [9–11] or the presence or absence of alleles that code for specific venom proteins [12]. However, there is also evidence that individual venom composition can be plastic through time likely due to the effects of gene regulation. Most significantly, a number of snakes show age-related changes in venom composition [13–20]. 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. ectothermic prey such as frogs and lizards) and adults (e.g. endothermic prey such as mammals) of the same species [13]. More recently, analyses of the venom transcriptome between closely related species has documented differences in which genes are expressed [21,22] 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 [23–25].

Much of the work assessing the extent and causes of plasticity in venom composition has relied on indirect evidence such as comparisons of venom composition between age classes of snakes or among individuals that possibly feed on different prey (see above). However, there have been few studies which have used controlled experiments to test the role of specific factors such as age and diet on individual venom composition using a detailed proteomic-based analysis of changes in the abundance of specific proteins. There is evidence from mice that diet can have direct effects on the composition of salivary secretions through the autonomic nervous system and mass spectrometric-based proteomic analyses have recently been used to quantify shifts in specific salivary enzymes in these studies [26,27]. Given that venom glands in snakes are evolutionarily derived from salivary glands in non-venomous ancestral taxa [28] it seems possible that a feed-back loop via autonomic nerves may result in changes in the expression patterns of specific SVPs especially if snakes consistently consume particular prey over long periods of time. To explore this possibility, here, we present results from long-term experiment in which juvenile and adult Dusky Pigmy rattlesnakes (*Sistrurus miliarius barbouri*) were consistently fed different prey over a 26 month period and changes in their venom composition monitored using the proteomic-based approach described by Sanz et al. [29] and Gibbs et al. [25].

Sistrurus m. barbouri is a small North American rattlesnake (average total length of 53.5 cm) found in southern Georgia, all of Florida, and west through southern Alabama, and Mississippi [30,31]. Its diet mainly consists of ectothermic prey such as frogs and lizards although it occasionally also consumes small mammals [32,33]. There is evidence that this snake shows small ontogenetic shifts in venom composition in juveniles [34] and regulatory effects on venom composition in adults [25] but in neither case was the observed plasticity in venom characterized or specifically linked to diet. Here, we use an experimental approach that involves raising snakes on specific diets for long time periods (26 months) to explore the possibility that diet plays a role in mediating this plasticity in venom composition in juveniles and adults. We also use detailed proteomic analyses of the venom (cf. “snake venomics” – [35]) to determine the changes in abundance of major classes of SVPs through time.

2. Experimental section

2.1. *Field collection of snakes and specific prey treatments*

We collected pregnant adult female Dusky Pigmy rattlesnakes in the vicinity of Deland, Florida in August 2008 through searching of open flood plain habitats where these snakes have been previously studied [36]. We transported 6 females back to the lab at Ohio State University where they gave birth to litters of 3–7 juveniles within 1–3 weeks of capture. One female and several juveniles died for unknown reasons over the next few months – *post mortems* showed no evidence for any pathologies or stress. Adults were individually housed in reptile cages while individual young were kept in small plastic containers. All snakes were maintained in the same animal care room under conditions of constant temperature (80 °C) and humidity (75%) and were provided water *ad libitum*. Within two weeks of capture, females were assigned to one of 3 diet treatment: lab mice (*Mus musculus*) (purchased from www.frozenrodents.com), brown anole lizards (*Anolis sagrei*) collected in the vicinity of Deland, Florida, or green tree frogs (*Hyla cinerea*) purchased commercially. All prey fed to snakes were dead but warmed before presenting them to captive animals. Adult females were exclusively fed these prey items at roughly two week intervals from the time of capture onwards for a period of 26 months. Venom samples were collected from each female on the day that they were captured and at roughly 2 month intervals after that date using the procedures outlined by Gibbs et al. [25]. We also collected weight and body length data at the time of each venom extraction. Juveniles were also assigned to specific prey treatments with young snakes initially being fed homogenized prey or parts of prey until they were willing to consume entire prey. Because of their small size, we did not extract venom from juveniles until they were 5 months old. We acknowledge that analyzing venom composition of snakes younger than this age might

have shown even more significant changes in composition over time. As with adults, from that time onward, we extracted venom at roughly 2 month intervals and, at the same time, collected weight and body length measurements, until the juveniles were approximately 26 months old.

2.2. Venom analyses

We analyzed venom composition of adults and juveniles as described [25]. Initially, we analyzed samples in two ways: one, by pooling equal amounts of individual venoms across a particular age-diet treatment and then comparing a single pooled venom profile across treatments or two, analyzing individual venoms separately and then analyzing the information on scored profiles as described [25]. We focus on the results from the pooled samples because of the small sample sizes and more interpretable profiles of the multi-individuals samples. However, the patterns seen in the pooled samples are closely reflected in the individual samples (Gibbs et al. unpublished data).

To analyze venom samples, two mg of crude venom made up of equal amounts of a given set of individual samples were 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 venom proteins were separated by reverse-phase HPLC using a Teknokroma Europa C_{18} (0.4 cm×25 cm, 5 mm particle size, 300 Å pore size) column and an Agilent LC 1100 High Pressure Gradient System equipped with DAD detector and micro-autosampler. The flow-rate was set to 1 ml/min and the column was developed with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B), isocratically (10% B) for 10 min, followed by 10–25% B for 20 min, 25–45% B for 120 min, and 45–70% for 20 min. Protein detection was carried out at 215 nm with a reference wavelength of 400 nm. Chromatographic fractions were assigned to previously characterized *Sistrurus m. barbouri* venom proteins [29] by retention time and SDS-PAGE analysis, sequential N-terminal Edman degradation (using a Procise instrument, Applied Biosystems, Foster City, CA, USA), tryptic peptide mass fingerprinting [29], and isotope-averaged molecular mass determination by electrospray ionization (ESI) mass spectrometry using an Applied Biosystems QTrap™ 2000 mass spectrometer operated in Enhanced Multiple Charge mode in the range m/z 600–1700 [37]. In a small number of cases new proteins were observed and these were identified by N-terminal sequencing and ESI-MS. Amino acid sequence similarity searches were performed against the available databanks using the BLAST program [38] implemented in the WU-BLAST2 search engine at http://www.bork.embl-heidelberg.de. Given that the wavelength of absorbance for a peptide bond is between 190 and 230 nm, protein detection at 215 nm allows to estimate the relative abundances of the different venom toxins (expressed as percentage of the total venom proteins) and 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 "% of total peptide bonds in the sample", which is a good estimate of the % by weight (g/100 g) of a particular venom component. Reverse-phase HPLC runs were consistently superimposable through their X-axes and the acetonitrile gradient profile, and thus all chromatograms of venom samples within and between diet groups were directly comparable to assess changes in the abundance of specific proteins through time.

HPLC venom profiles of individual adults and juveniles were compared both by eye and using the program Metacomps (P. Nuin, in preparation) which matches peaks in different samples on the basis of their retention time (RT) values [25]. This allowed us to assign chromatographic peaks to previously characterized proteins [29] in samples that had been sequentially collected over time. Comparing the RP values for specific known protein peaks allowed us to assess changes in the abundance of a specific protein through time in the pooled venom from individuals in a specific diet treatment. We analyzed both pooled abundance data for five major classes of proteins that make up the majority (~90% - see [29]) of proteins in pigmy rattlesnake venom (see Figs. 1 and 2), including disintegrins (peaks 6–9), phospholipase A_2 proteins (PLA_2s, peaks 15a, 15, 17, 19a, and 19), cysteine-rich secretory protein (CRISP, peak 17), serine proteinases (peaks 20–25), and snake venom Zn^{2+}-dependent metalloproteinases (SVMPs) (peaks 26–31) and variation in select individual protein peaks of PLA_2s (15, 19a, and 19) and SVMPs (28a, 28, 29, and 30) that were prominent and easily scored.

3. Results

3.1. Ontogenetic changes in venom composition

Figs. 1 and 2 present HPLC profiles showing changes in venom composition in pooled samples from 5 to 26 months for mouse- (Fig. 1) and lizard- and frog-fed juveniles (Fig. 2). Table 1 summarizes changes between the first and last venom samples for juveniles in all diet treatments in terms of relative changes in the abundance of toxins, both in terms of major classes and specific proteins. There were no large changes (arbitrarily defined as ≥50% change) in the overall abundance of any of the major classes of proteins with the possible exception the CRISP toxin, which showed consistent declines across all treatments, and an increase of >100% of the pooled disintegrins in the frog-fed juvenile. However, individual toxin abundances showed substantial changes: specifically, PLA_2 15 peak showed a substantial increase across all treatments as did PI-SVMPs and the PIII-SVMP identified with peak 28. The amount of change varied across diet-treatments with the increase in PLA_2 15 being highest for the frog-fed juveniles (from 0.1 to 5.6%), intermediate for the snakes raised on lizards (0.1 to 2.5%), and lowest (1.8 to 4.4%) for the mouse-fed snakes. Like-wise the change in relative size of the PIII-SVMP 28 peak was greatest for the frog-fed juvenile and less for the other two treatments. Noteworthy, although in the three diet-treatments the increment of PIII-SVMP 28 occurred concomitantly with decreasing relative abundances of all other PIII-SVMPs, the rate of this shift was different between snakes fed with endothermic and ectothermic prey. Hence, peak 28 represents the major PIII-SVMP in the profiles of venom collected from 9-month old snakes fed from birth with mice

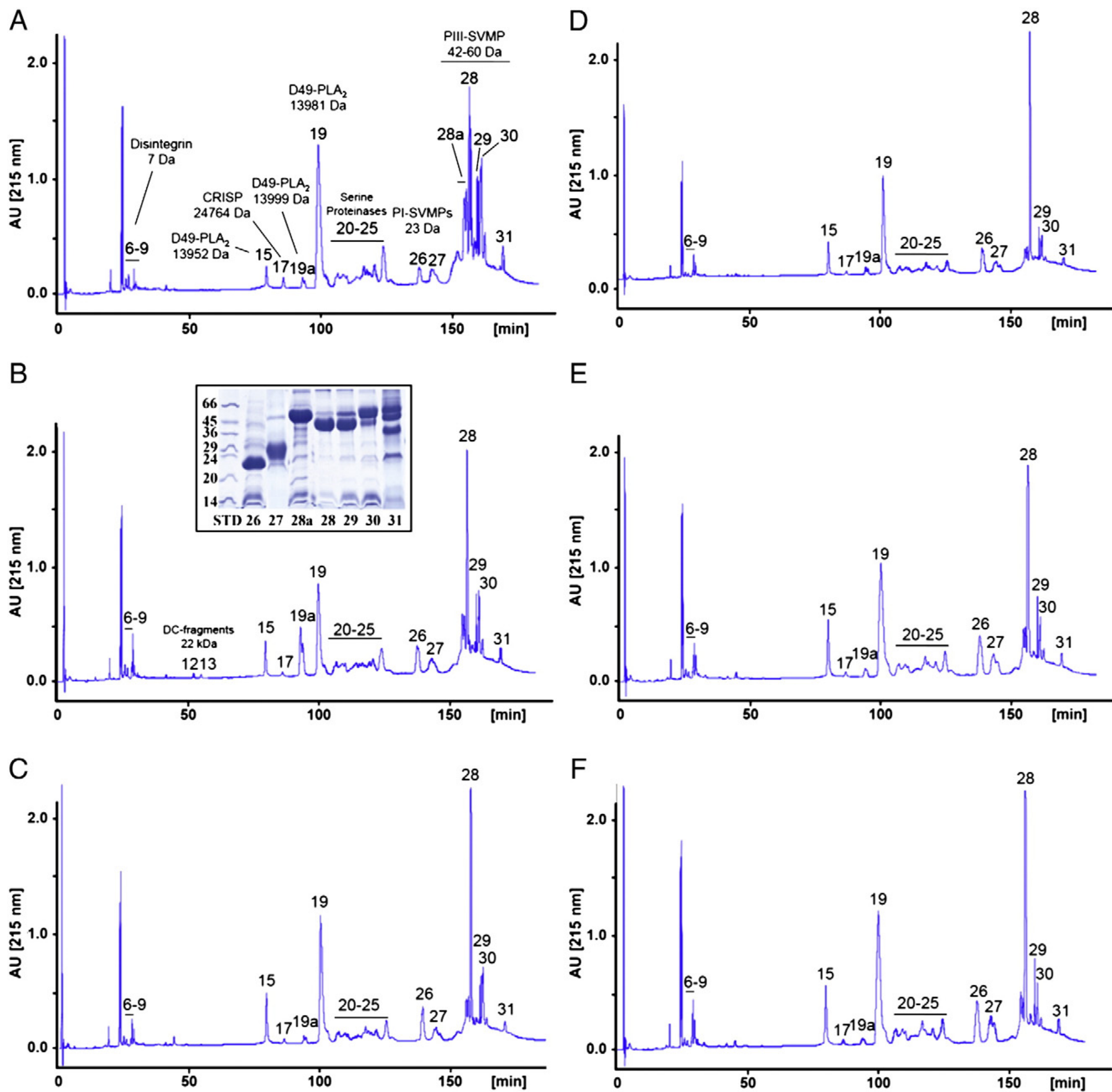

Fig. 1 – Ontogenetic changes in the venom of *Sistrurus m. barbouri* fed from birth with endothermic prey. Reverse-phase HPLC whole venom profiles showing ontogenetic changes in pooled venom composition of juvenile pigmy rattlesnakes raised from birth on a diet of mice (n = 8) (Table 1). Panels illustrate profiles of venom collected at 6 (A), 9 (B), 15 (C), 17 (D), 23 (E) and 26 (F) months after birth. Chromatographic peaks are labeled using the same numbering as in Fig. 5 of Sanz et al. [27] and their relative changes over time are listed in Table 1. The distribution of major venom proteins is highlighted in panels A and B . The insert in panel B shows an SDS-PAGE analysis of PI- and PIII-SVMPs.

(Fig. 1), but only after 15 month in the venom of snakes raised on lizards (Fig. 2).

When averaged across values for all toxin classes (general and specific) the frog-fed juvenile showed the highest absolute value for % change (47%) followed by the mouse (36%) and lizard-fed snakes (30%) (Table 1). Finally, the ratio of abundances of PI to PIII metaloproteases showed increases in all diet treatments over time but where greatest in the mouse-fed juveniles (mouse: 0.11 (initial) to 0.30 (end); lizard: 0.11 to 0.20; frog: 0.07 to 0.11). Overall, our results demonstrate that ontogenetic changes in *Sistrurus miliarius* venom do not involve substantial changes in the overall make-up of broad classes of toxins but rather more fine-scale shifts in the relative proportion of specific components within each broad class. Further, there appear to be some diet-related differences in these fine-scale changes, although our sample sizes are limited.

The identities of the chromatographic fractions initially assigned by retention time and SDS-PAGE analysis to previously characterized venom proteins [29], were verified by N-terminal sequencing, tryptic peptide mass fingerprinting, and ESI-MS (Fig. 3). In addition, peak 19a, which had not been described in the pigmy rattlesnake venom proteome [29], and

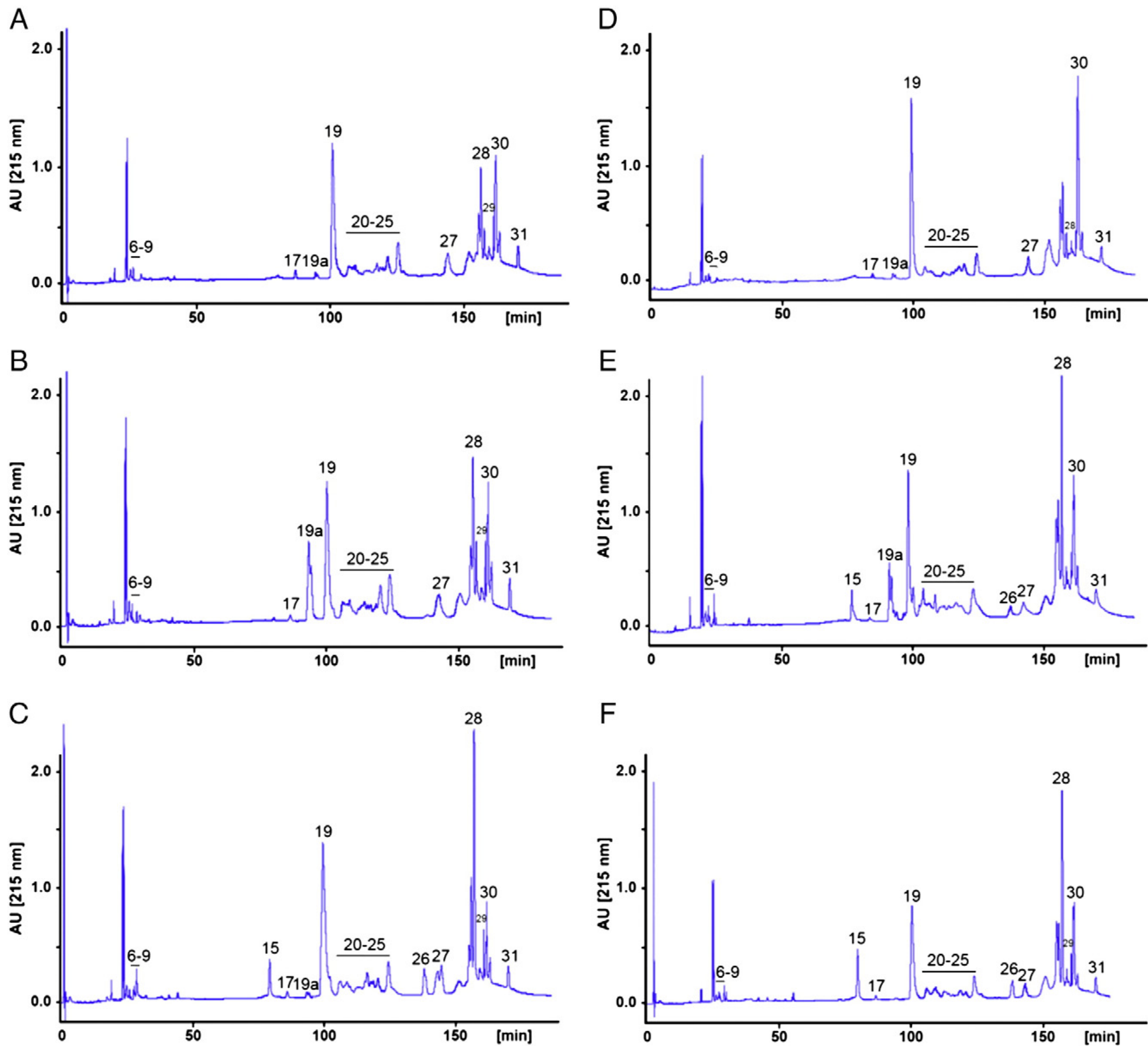

Fig. 2 – Ontogenetic changes in venom samples poooled from *Sistrurus m. barbouri* specimens fed from birth with ectothermic prey. Reverse-phase HPLC whole venom profiles showing ontogenetic changes in pooled venom composition of juvenile pigmy rattlesnakes raised from birth on a diet of lizards (n = 5) (panels A–C) and frogs (n = 1) (panels D–F) (Table 1). Panels illustrate profiles of venom collected at 6 (A and D), 9 (B and E) and 26 (C and F) months after birth. Peak labeling as in Fig. 1., and the relative changes over time are listed in Table 1.

whose relative abundance raised transiently in the venom samples collected from 9-month old specimens in the three diet-treatments, was characterized as a PLA_2 molecule (Fig. 3). Its N-terminal sequence, HLIQFETLIMKIAGRSGVFWYSAYG, MALDI-TOF tryptic peptide mass fingerprint, and isotope-averaged molecular mass (M_{av} = 13999 Da) (Fig. 3C) identified it as a close homologue of the major PLA_2 19 (*mil4*, ABY77929) (M_{av} = 13982 Da, Fig. 3D), and *cat6* (ABY77921 M_{av} calc. = 14025.6 Da) and *cat7* (ABY77922; M_{av} calc. = 13994.6 Da) both amplified from genomic DNA of a *S. c. catenatus* specimen from Killbear Provincial Park (Ontario) [39]. These molecules fall into the D49 subgrouping of PLA_2 venom proteins, which are catalytically active.

3.2. *Changes in venom composition of adults*

Fig. 4 shows HPLC profiles for venom samples for adult females fed different prey. Table 2 gives the relative abundance of toxins pooled into broad classes and in terms of certain specific molecules for these profiles. Shifts in venom composition occurred in females in all diet treatments with the magnitude of those changes strongly related to diet. Specifically, mouse-fed females showed substantial (≥50%) changes in the relative abundance of all five major classes of toxins, except SVMPs, and these changes appear to be driven by specific toxins in each class. In general, the venom of mouse-fed females became more enriched for disintegrins,

Table 1 – Ontogenetic changes in the relative occurrence of toxins in the pooled venoms of *Sistrurus m. barbouri* fed from birth on different prey. Values represent the relative percent of total venom made up by specific toxins in a pooled sample of venoms in each diet treatment from the first and last sample collected and the % change in this value between these samples. Note that to allow direct comparisons of proportional decreases and increases, increases of more than 100% are shown as > 100% even though the actual value may be higher. Mean change is the average of the absolute value of % change across all toxin classes and are minimum estimates because values of > 100% are scored as 100%.

Toxin Class	Venom Sample - mouse diet (n = 9)			Venom Sample - lizard diet (n = 2)			Venom Sample - frog diet (n = 1)		
	March 2009	November 2010	% Change	March 2009	November 2010	% Change	March 2009	November 2010	% Change
Disintegrin	4.5	4.1	−9	1.2	1.3	8	1.3	2.8	> 100
PLA_2	25.4	23.5	−7	23.1	24.0	4	24.5	23.3	−5
15	1.8	4.4	> 100	0.1	2.5	> 100	0.1	5.6	> 100
19a	0.8	0.7	−13	0.7	0.6	−14	0.7	0.7	0
19	16.8	18.4	10	22.3	20.8	−7	23.4	17.7	−24
CRISP	0.6	0.3	−50	0.7	0.4	−43	0.4	0.2	−50
Serine Proteinase	10.7	11.5	7	21.2	18.8	−11	15.2	13.4	−12
SVMPs	47.6	50.4	6	50.0	46.4	−7	45.9	54.6	19
PI	4.9	11.8	> 100	5.2	8.0	54	3.1	5.8	87
PIII	42.7	38.6	−10	44.8	38.4	−14	42.8	48.8	14
28a	11.1	6.6	−41	12.7	9.9	−22	13.1	10.8	−18
28	3.5	17.6	> 100	7.5	15.5	> 100	2.5	18.9	>100
29	1.9	1.9	0	1.5	1.3	−13	2.6	1.2	−54
30	4.2	2.1	−50	5.2	4.2	−19	18.2	4.0	−78
Mean change (± SE)			36 ± 3			30 ± 3			47 ± 3

PLA_2s (particularly PLA_2 15), serine proteinases, and PI-SVMPs, and less abundant for CRISP and PIII SVMPs. In contrast, changes were much less pronounced in the lizard- and frog-fed females with substantial changes only occurring in overall disintegrin abundance and the PLA_2 15 peak in the lizard-female and in two specific peaks (15a and PLA_2 15) in the frog-fed female (Fig. 4, Table 2). These patterns are mirrored in the mean values for absolute changes in abundance across all toxin categories for each type of female: the value for mouse-fed females (60%) is more than twice as large as the values for the lizard- (29%) and frog-fed (20%) females.

Peak 15a was characterized as D49-PLA_2 *mil1* (ABY77926) (M_{av} calc: 13892.9 Da) [39] by N-terminal sequencing, MALDI-TOF mass fingerprinting, and molecular mass determination by ESI-MS (Fig. 3E). PLA_2 15a expression was only observed in females fed with frogs (Fig. 4; Table 2). Its relative abundance in venom samples exhibited steady increment from 0.4% in August 2008 to 1.5% in October 2009, and then a slight decrease to 1.1% over the next 12 months of the experiment. This PLA_2 molecule was not identified in the venom proteome of adult pigmy rattlesnakes [29] conducted on venom pooled from an undetermined number of *S. m. barbouri* specimens raised in captivity on a mouse diet at the Latoxan Serpentarium (Rosans, France), although a ~2.3 kb type IIA loci (4 exons and 3 introns) containing the entire coding region was PCR-amplified from genomic DNA from a *S. m. barbouri* specimen from Volusia County (Florida) [39].

4. Discussion

Although limited, our experimental results have implications for understanding the mechanisms that underlie variation in snake venom composition. We stress that variation in venom composition has been demonstrated at practically every level [3], and since variation among snake venoms is adaptive and thus subject to selective forces in the environment that differ among species, this phenomenon may largely follow taxonomic-specific trends. The shifts in the relative abundance of different classes of proteins in both juvenile and adult Dusky Pigmy rattlesnakes confirm that in this species venom composition is at least to some extent plastic. This plasticity has two components: First, ontogenetic changes, which have been well documented in other species (e.g. *Bothrops* [15,16,18,20,40], *Crotalus* [13,19,41], *Boiga* [17], and *Lachesis* [14]) but less so for *Sistrurus*, and rarely using the detailed information available from proteomic-based analyses. The second component is variation in venom composition in adults that may be related to diet. We discuss the significance of both types of plasticity below.

4.1. Ontogenetic changes in venom in juvenile pigmy rattlesnakes

Surprisingly, despite previous suggestions that ontogenetic changes in venom are prey-related [15], juvenile pigmy rattlesnakes raised on different diets showed similar venom compositions by the end of the study at least in terms of overall abundance of different broad categories of proteins. Fine-scale shifts in the relative proportion of specific components within each broad class were due to quantitative changes in the expression of protein already present at the beginning of the experiment, rather than to the differential expression of novel gene products. Retention in the adult of juvenile characters (paedomorphism [42]) has been documented in *Crotalus oreganus concolor* [43], Amazonian *B. atrox* populations [20], insular *B. alcatraz* [44], and South American *C. durissus* subspecies [19,40]. The driving force behind paedomorphism may be dietary shifts caused by interspecific competition or geographic variation in prey species

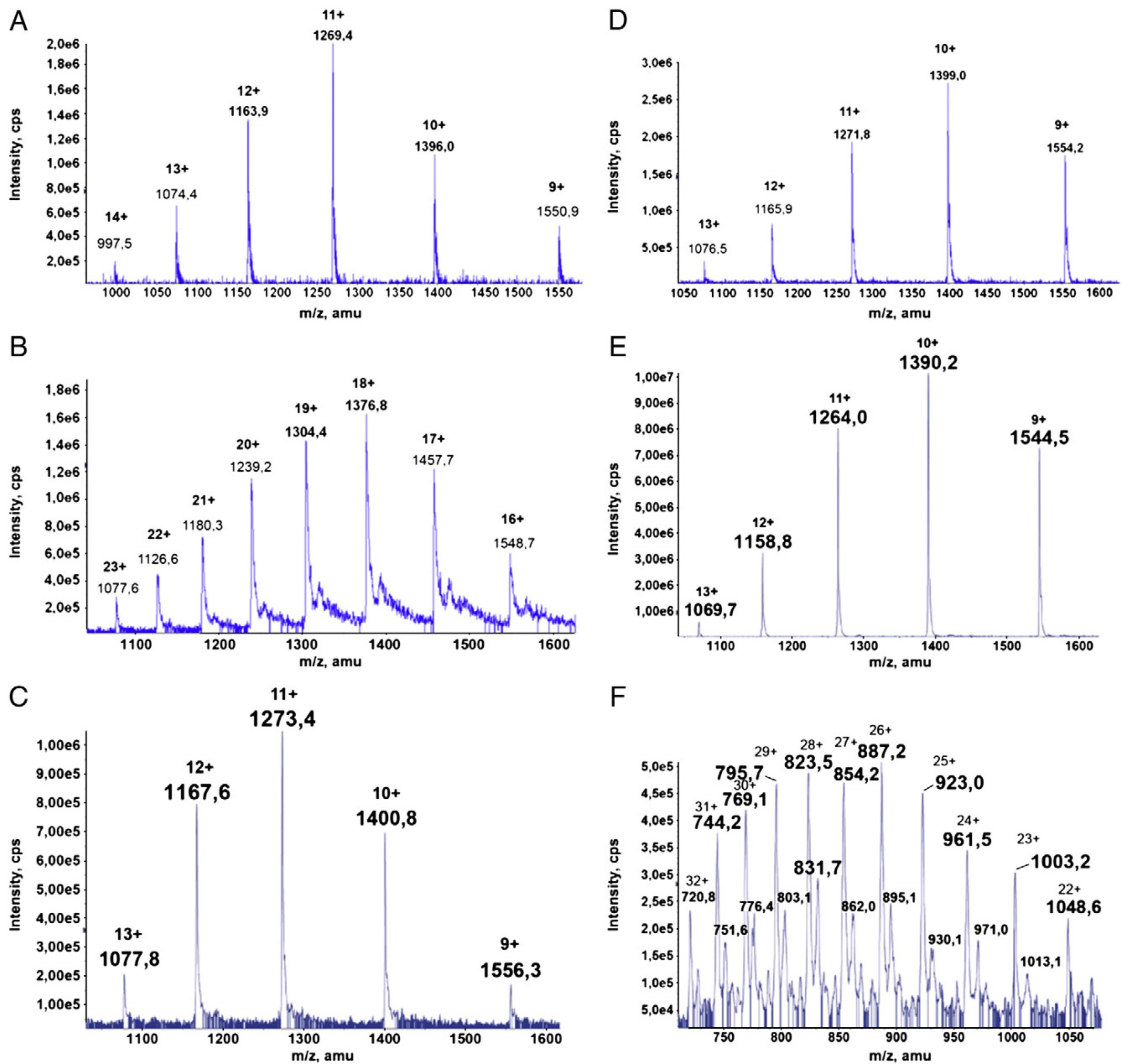

Fig. 3 – Characterization of toxin molecules from venoms of juvenile and adult snakes. Reverse-phase HPLC-separated toxins from the venoms of juvenile and adult specimens subjected to the different prey diets (Figs. 1–3) were analyzed by N-terminal sequential Edman degradation and electrospray-ionization mass spectrometry. Panel A, D49-PLA$_2$ 15 (N-terminal sequence (Nt): NLLQFNKMIKIMTKK; isotope-averaged molecular mass (M$_{av}$): 13952 Da; database accession code (Ac): ABY77927); Panel B, CRISP 17 (Nt: SVNFDSESPRKPEIQ; M$_{av}$: 24764 Da; Ac: ~B0VXV6); Panel C, D49-PLA$_2$ 19a (Nt: HLIQFETLIMKIAGRSGVFWYSAYG; M$_{av}$: 13999 Da; Ac: ~ABY77922); Panel D, D49-PLA$_2$ 19 (Nt: HLIQFETLIMKIAGR; M$_{av}$: 13982 Da; Ac: ABY77929); Panel E, D49-PLA$_2$ 15a (Nt: NLLQFNKMIKIMTKK; M$_{av}$: 13892 Da; Ac: ABY77926); Panel F, PI-SVMP 26 (Nt: NPEHQRYVELFIVVD; M$_{av}$: 23051 Da).

abundance. Retention of juvenile characters in *B. alcatraz* appears to be associated with a diet based on ectotherms (mainly centipedes), owing to the absence of small mammalian prey in the Alcatrazes Archipielago, 35 km off the Atlantic coast of São Paulo, southeastern Brazil, where *B. alcatraz* has been isolated for approximately 10,000 years [45].

Possible reasons for the finding that that juvenile diet does not appear to have a strong influence of the development of venom composition in *Sistrurus m. barbouri*, could be that the use of inappropriate prey not usually encountered in the wild resulted in a lack of response in juveniles in terms of venom development or that similar venom composition reflects a fixed regulatory response due to a limited array of prey items normally encountered over time in the wild. However, at least 1 of the prey items (green tree frogs) is consumed by these snakes in the wild in the area where they originated while the other (brown anole) is congenic with a native lizard (green anole: *A. carolinensis*) which is also a native prey species. Diet breadth and composition of juveniles compared to adults is not known at this point but could be examined using stable

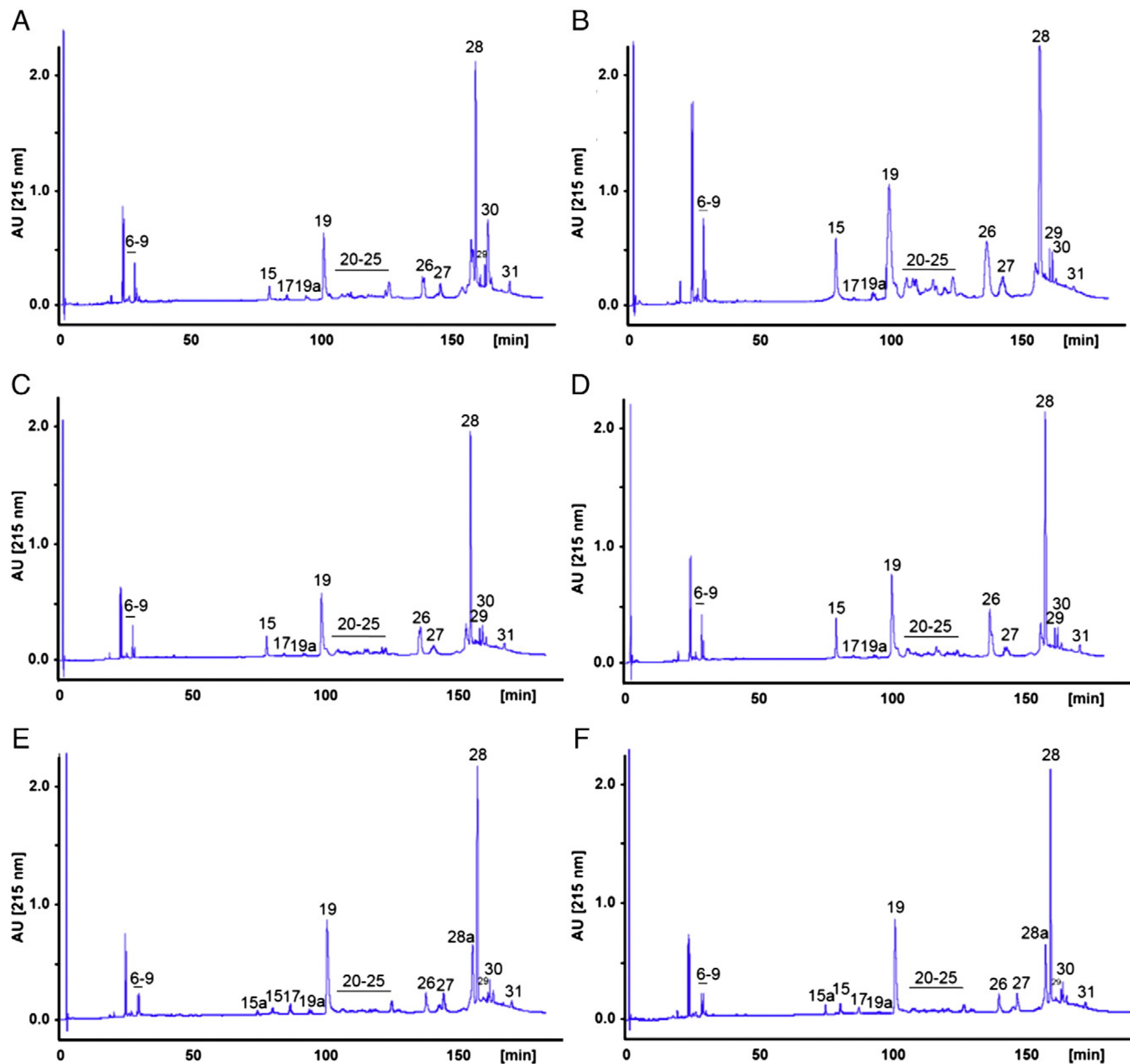

Fig. 4 – Effect of diet on the protein composition of venoms from adult *Sistrurus m. barbouri*. Reverse-phase HPLC whole venom profiles showing changes in pooled venom composition of adult pigmy rattlesnakes raised on diets of mice (n = 3) (panels A and B), lizards (n = 1) (C and D) and frogs (n = 1) (E and F) (Table 1). Panels illustrate profiles of venom collected at 1 month (panels A, C, and E) and 26 months (panels B, D, and F) after the start of the experiment. Peak labeling as in Fig. 1. Relative changes over time are listed in Table 2.

isotope analyses of diet as has been carried out by Pilgrim for pigmy rattlesnakes [46].

4.2. *Diet-related shifts in adult venom*

Limited previous work has suggested that venom profiles of individual snakes are not altered in response to external cues. For example, Gregory-Dwyer et al. [7] found little change in the in isoelectric focusing patterns of venom of adult specimens of *C. viridis helleri*, *C. molossus molossus*, and *C. atrox* housed under controlled light and temperature to simulate seasonal change, fed on the same diet, and milked monthly over a 20 month period. This evidence suggested that intraspecific variation is genetically inherited rather than environmentally induced. Research on venoms has been continuously enhanced by advances in technology. Recently, using the high resolving capability of mass spectrometry, Modahl and colleagues [47] analyzed venoms from two long-term captive-bred Pakistan cobra (*Naja naja*) populations and found that all the venoms consisted of the same protein composition, although the concentration of their proteins differed among the venoms from snakes of two distinct colonies [8]. Our present data suggest that diet is an external cue that can broadly influence the expression of general classes of venom proteins at least in adults. Given that pigmy rattlesnakes fed primarily on ectotherms such as frogs and lizards and rarely mammals ([32], [33])

Table 2 – Changes in the relative occurrence of toxins in the pooled venoms of adult *Sistrurus m. barbouri* fed on different prey. Values represent the relative percent of total venom made up by specific toxins in a pooled sample of venoms in each diet treatment from the first and last sample collected and the % change in this value between these samples. Note that to allow direct comparisons of proportional decreases and increases, increases of more than 100% are shown as >100% even though the actual value may be higher. Mean change is the average of the absolute value of % change across all toxin classes and are minimum estimates because values of >100% are scored as 100.

Toxin Class	Venom Sample - mouse diet (n=3)			Venom Sample - lizard diet (n=1)			Venom Sample - frog diet (n=1)		
	August 2008	November 2010	% Change	August 2008	November 2010	% Change	August 2008	November 2010	% Change
Disintegrin	2.2	3.3	50	0.8	2.5	>100	2.6	2.7	4
PLA_2	12.8	25	95	15.4	19.1	24	23.2	23.7	2
15a							0.4	1.1	>100
15	1.4	6.3	>100	2.5	4.6	84	0.7	1.2	71
19a	0.6	0.8	25	0.1	0.1	0	0.5	0.6	20
19	10.8	17.6	63	12.8	15.1	36	21.6	20.8	−4
CRISP	0.5	0.1	−80	<0.1	<0.1	0	1.6	1.3	−19
Serine Proteinase	5.1	17.6	>100	13.2	10.4	−21	4.4	4.9	11
SVMPs	56.8	38.0	−34	65.0	56.4	−13	65.0	64.7	0
PI	5.6	11.1	98	12.1	13.3	10	6.8	8.1	19
PIII	50.2	29.4	−47	52.9	43.1	−19	58.2	56.6	−3
28a	8.5	3.2	−57	8.4	6.3	−25	16.1	14.7	−9
28	24.3	22.7	−3	28.8	29.9	4	28.4	30.4	7
29	1.6	1.7	6	2.0	2.1	5	1.2	1.2	0
30	12.2	2.5	−80	4.7	1.4	−71	3.1	2.3	−26
Mean change (±SE)			60±2			29±2			20±2

we find it most interesting that the most extreme changes in venom composition in adults (and also in juveniles) were in the mouse-fed females. This is consistent with the idea that when snakes were fed a diet of prey that were likely rarely consumed in nature this induced greater changes in venom composition than diets of prey that were possibly more commonly consumed by the females prior to capture. Our results also suggest further tests which target specific venom proteins (e.g. PLA_2s) to determine if they are more effective at killing and/or digesting small mammals than are other classes of proteins that showed smaller shifts in abundance.

5. Conclusions and perspectives

Our study has a number of weaknesses. Most significantly, although our proteomic survey provides experimental support for venom composition plasticity in both juvenile and adult *S. m. barbouri*, our sample sizes are too small to allow statistically-rigorous analyses of shifts in venom composition across age and diet-related classes of individuals. In addition, for practical reasons we were forced to use lab mice (*Mus musculus*) as our representative mammalian prey instead of a native species.

We also stress that what we have shown is evidence for a correlation of changes in venom composition with diet in snakes raised in a controlled environment. The high level of variation initially observed in adult snakes raises the possibility that individual variation caused by factors unrelated to diet combined with our small simple sizes could provide an alternate explanation for our results. We feel this is unlikely due to the fact that the snakes were maintained in a controlled environment where, to our knowledge, diet was the only factor that varied. However, confirming an impact of diet on venom composition will require 1) additional experiments with larger simple sizes that account for individual variation and 2) experiments in which diets fed to the same animal are varied through time to see if there are consistent and reversible changes in venom composition through time in relation to prey consumed and 3) functional tests to determine if venom components that increase in a particular feeding experiment are more effective on the specific prey being fed to specific snakes.

These issues aside we feel that our study is significant because it illustrates the power of a “snake venomics” approach in revealing detailed information about changes in venom composition in response to age and diet related cues, and it provides the first experimental evidence that venom composition in adult snakes can change in response to diet. If this result is confirmed with additional work then an important implication is that gene regulation can be a significant cause of variation in venom composition as previously suggested for *Sistrurus* rattlesnakes by Gibbs et al. [25], and that this biological feature of individual venom composition needs to be incorporated into adaptive explanations for venom composition [4].

Acknowledgements

We thank Peter May and Liam Kean for help with collecting the snakes, and Mike Jaskot for assistance with snake husbandry. This work was supported by funds from Ohio State University and grants BFU2010-17373 (Ministerio de

Ciencia e Innovación, Madrid, Spain) and PROMETEO/2010/005 (Generalitat Valenciana, Valencia, Spain). The snakes were collected under Florida Fish and Wildlife Conservation Commission License VRC 270 and housed under Ohio State University IACUC Permit 2008A0087.

REFERENCES

[1] Meier J, Stocker KF. Biology and distribution of venomous snakes of medical importance and the composition of snake venoms. In: Meier J, White J, editors. Handbook of clinical toxicology of animal venoms and proteins. Boca Raton, FL: CRC Press; 1995. p. 367–412.

[2] Ohno M, Ménez R, Ogawa T, Danse JM, Shimohigashi Y, Fromen C, Ducancel F, Zinn-Justin S, Le Du MH, Boulain JC, Tamiya T, Ménez A. Molecular evolution of snake toxins: is the functional diversity of snake toxins associated with a mechanism of accelerated evolution? Prog Nucleic Acid Res Mol Biol 1998;59:307–64.

[3] Chippaux J-P, Williams V, White J. Snake venom variability: methods of study, results and interpretation. Toxicon 1991;29: 1279–303.

[4] Daltry JC, Wüster W, Thorpe RS. Diet and snake venom evolution. Nature 1996;379:537–40.

[5] Chippaux J-P, Boche J, Courtois B. Electrophoretic patterns of the venoms from a litter of *Bitis gabonica* snakes. Toxicon 1982;27:1397–9.

[6] Mebs D, Kornalik F. Intraspecific variation in content of a basic venom in eastern diamondback rattlesnake (*Crotalus adamanteus*) venom. Toxicon 1984;22:831–3.

[7] Gregory-Dwyer VM, Egen NB, Bosisio AB, Righetti PG, Russell FE. An isolectric focusing study of seasonal variation in rattlesnake venom proteins. Toxicon 1986;24:995–1000.

[8] Daltry JC, Ponnudurai G, Shin CK, Tan NH, Thorpe RS, Wüster W. Electrophoretic profiles and biological activities: intraspecific variation in the venom of the Malayan pit viper (*Calloselasma rhodostoma*). Toxicon 1996;34:67–79.

[9] Chijiwa T, Deshimaru M, Nobuhisha I, Nakai M, Ogawa T, Oda N, Nakashima K, Fukumaki Y, Shimohigashi Y, Hattori S, Ohno M. 2000) Regional evolution of venom-gland phospholipase A_2 isoenzymes of *Trimeresurus flavoviridis* snakes in the southwestern islands of Japan. Biochem J 2000;347:491–9.

[10] Deshimaru M, Ogawa T, Nakashima K, Nobuhisa I, Chijiwa T, Shimohigashi Y, Fukumaki Y, Niwa M, Yamashina I, Hattori S, Ohno M. Accelerated evolution of crotalinae snake venom gland serine proteases. FEBS Lett 1996;397:83–8.

[11] Chang L-S, Lin SR, Wang JJ, Hu WP, Wu B, Huang H. Structure function studies on Taiwan cobra long neurotoxin homolog. Biochim Biophys Acta 2000;219:116–21.

[12] Wooldridge BJ, Pineda G, Banuelas-Ornelas JJ, Dagda RK, Gasanov SE, Rael ED, Lieb CS. Mojave rattlesnakes (*Crotalus scutulatus scutulatus*) lacking the acidic subunit DNA sequence lack Mojave toxin in their venom. Comp Biochem Physiol B 2001;130:169–79.

[13] Mackessy SP. Venom ontogeny in the Pacific rattlesnakes, Crotalus viridis helleri and C. viridis oreganus. Copeia 1988;1988:92–101.

[14] Gutiérrez JM, Avila C, Camancho Z, Lomonte B. Ontogenic changes in venom of the snake *Lachesis muta stenophrys* (Bushmaster) from Costa Rica. Toxicon 1990;28:419–26.

[15] Andrade DV. Abe AS (1999) Relationship of venom ontogeny and diet in *Bothrops*. Herpetologica 1999;55:200–4.

[16] Guércio RAP, Shevchenko A, Shevchenko A, López-Lozano JL, Paba J, Sousa MV, Ricart CAO. Ontogenetic variations in the venom proteome of the Amazonian snake *Bothrops atrox*. Proteome Sci 2006;4:11.

[17] Mackessy SP, Sixberry NM, Heyborne WH, Fritts T. Venom of the Brown Treesnake, *Boiga irregularis*: ontogenetic shifts and taxa-specific toxicity. Toxicon 2006;47:537–48.

[18] Alape-Girón A, Sanz L, Escolano J, Flores-Díaz M, Madrigal M, Sasa M, Calvete JJ. Snake venomics of the lancehead pitviper *Bothrops asper*: Geographic, individual, and ontogenetic variations. J Proteome Res 2008;7:3556–71.

[19] Calvete JJ, Sanz L, Cid P, De La Torre P, Flores-Diaz M, Dos Santos MC, Borges A, Bremo A, Angulo Y, Lomonte B, Alape-Giron A, Gutiérrez JM. Snake venomics of the Central American rattlesnake *Crotalus simus* and the South American *Crotalus durissus* complex points to neurotoxicity as an adaptive paedomorphic trend along Crotalus dispersal in South America. J Proteome Res 2010;9:528–44.

[20] Calvete JJ, Sanz L, Pérez A, Borges A, Vargas AM, Lomonte B, Angulo Y, Gutiérrez JM, Chalkidis HM, Mourão RH, Furtado MF, Moura-Da-Silva AM. Snake population venomics and antivenomics of *Bothrops atrox*: Paedomorphism along its transamazonian dispersal and implications of geographic venom variability on snakebite management. J Proteomics 2011;74:510–27.

[21] Pahari S, Bickford D, Fry BG, Kini RM. Expression pattern of three-finger toxin and phospholipase A_2 genes in the venom glands of two sea snakes, *Lapemis curtus* and *Acalyptophis peronii*: comparison of evolution of these toxins in land snakes, sea kraits and sea snakes. BMC Evol Biol 2007;7:175.

[22] Casewell NR, Harrison RA, Wüster W, Wagstaff SC. Comparative venom gland transcriptome surveys of the saw-scaled vipers (Viperidae: Echis) reveal substantial intra-family gene diversity and novel venom transcripts. BMC Genomics 2009;10:564.

[23] Tsai I-H, Chen YH, Wang YM, Liau MY, Lu PJ. Differential expression and geographic variation of venom phospholipase A_2 *of Callosellasma rhodostoma* and *Trimeresurus muscrosquamatus*. Arch Biochem Biophys 2001;387:257–64.

[24] Tsai I-H, Wang Y-M, Chen Y-H, Tu AT. Geographic variations, cloning and functional analyses of the venom acidic phospholipases A_2 of *Crotalus viridis viridis*. Arch Biochem Biophys 2003;411:289–96.

[25] Gibbs HL, Sanz L, Calvete JJ. Snake population venomics: Proteomics-based analyses of individual variation reveals significant gene regulation effects on venom protein expression in *Sistrurus* rattlesnakes. J Mol Evol 2009;68:113–25.

[26] Da Costa G, Lamy E, Capela e Silva F, Potes J, Coelho AV, Baptista ES. Salivary amylase induction by tannin-enriched diets as a possible countermeasure against tannins. J Chem Ecol 2008;34:376–87.

[27] Kurashashi M, Inomata K. Effects of dietary consistency and water content on parotid amylase secretion and gastric starch digestion in rats. Arch Oral Biol 2008;44:1013–9.

[28] Kochva E. The origin of snakes and evolution of the venom apparatus. Toxicon 1987;25:65–106.

[29] Sanz L, Gibbs HL, Mackessy SP, Calvete JJ. Venom proteomes of closely related *Sistrurus* rattlesnakes with divergent diets. J Proteome Res 2006;5:2098–112.

[30] Klauber LM. Rattlesnakes: Their Habitats, Life Histories, and Influence on Mankind. 2nd Edition. Berkeley: University of California Press; 1997.

[31] Kubatko LS, Gibbs HL, Bloomquist EW. Inferring species-level phylogenies and taxonomic distinctiveness using multilocus data in *Sistrurus* rattlesnakes. Syst Biol 2011;60:393–409.

[32] Campbell JA, Lamar WW. The Venomous Reptiles of the Western Hemisphere. Ithaca (NY): Comstock Publishing Associates; 2004.

[33] Gibbs HL, Mackessy SP. Functional basis of a molecular adaptation: Prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. Toxicon 2009;53:672–9.

[34] Deyrup S, Farrell T, Nicholas D. Venom ontogeny in dusky pigmy rattlesnakes (*Sistrurus miliarius barbouri*). Fla Acad Sci 2000;63:23.
[35] Calvete JJ, Juárez P, Sanz L. Snake venomics. Strategy and applications. J Mass Spectrom 2007;42:1405–14.
[36] May PG, Farrell TM, Heulett ST, Pilgrim MA, Bishop LA, Spence DJ, Rabatski AM, Campbell MG, Aycrigg AD, Richardson WE. The seasonal abundance and activity of a rattlesnake (*Sistrurus miliarius barbouri*) in central Florida. Copeia 1996;1996:389–400.
[37] Le Blanc JC, Hager JW, Ilisiu AM, Hunter C, Zhong F, Chu I. Unique scanning capabilities of a new hybrid linear ion trap mass spectrometer (Q-TRAP) used for high sensitivity proteomics applications. Proteomics 2003;3:859–69.
[38] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[39] Gibbs HL, Rossiter W. Rapid evolution by positive selection and gene gain and loss: PLA_2 venom genes in closely related *Sistrurus* rattlesnakes with divergent diets. J Mol Evol 2008;66: 151–66.
[40] Furtado MFD, Maruyama M, Kamiguti AS, Antonio LC. Comparative study of nine Bothrops snake venoms from adult female snakes and their offspring. Toxicon 1991;29: 219–26.
[41] Boldrini-França J, Corrêa-Netto C, Silva MM, Rodrigues RS, De La Torre P, Pérez A, Soares AM, Zingali RB, Nogueira RA, Rodrigues VM, Sanz L, Calvete JJ. Snake venomics and antivenomics of *Crotalus durissus* subspecies from Brazil: assessment of geographic variation and its implication on snakebite management. J Proteomics 2010;73:1758–76.
[42] Garstang W. The theory of recapitulation: a critical re-statement of the biogenetic law. Linn Soc J Zool 1922;XXXV:81–101.
[43] Marques OAV, Martins M, Sazima I. A new insular species of pitviper from Brazil, with comments on evolutionary biology and conservation of the *Bothrops jararaca* group (Serpentes, Viperidae). Herpetologica 2002;58:303–12.
[44] Mackessy SP, Williams K, Ashton K. Characterization of the venom of the midget faded rattlesnake (*Crotalus oreganus concolor*): a case of venom paedomorphosis? Copeia 2003;2003:769–82.
[45] Grazziotin FG, Monzel M, Echeverrigaray S, Bonatto SL. Phylogeography of the *Bothrops jararaca* complex (Serpentes: Viperidae): past fragmentation and island colonization in the Brazilian Atlantic forest. Mol Ecol 2006;15:3969–82.
[46] Pilgrim MA. Expression of maternal isotopes in offspring: implications for interpreting ontogenetic shifts in isotopic composition of consumer tissues. Isotopes Environ Health Stud 2007;43:155–63.
[47] Modahl CM, Doley R, Kini RM. Venom analysis of long-term captive Pakistan cobra (*Naja naja*) populations. Toxicon 2010;55:612–8.