Toxicon 96 (2015) 74–81

Contents lists available at ScienceDirect

Toxicon

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

Early significant ontogenetic changes in snake venoms

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ARTICLE INFO

Article history:
Received 14 November 2014
Received in revised form
13 January 2015
Accepted 15 January 2015
Available online 16 January 2015

Keywords:
Crotalus adamanteus
Crotalus horridus
Ecdysis
Ontogeny
Snake
Venom

ABSTRACT

Snake venom plays a critical role in food acquisition, digestion, and defense. Venoms are known to change throughout the life of some snake species, but nothing is known about the venom composition of hatchling/neonate snakes prior to and just after their first shedding cycle, despite this being a critical time in the life of the snake. Using a cohort of *Crotalus horridus* and two cohorts of *Crotalus adamanteus*, we showed for the first time that snakes undergo significant changes in venom composition after the postnatal shedding event. The number of changes among cohorts ranged widely and there was wide variation in the direction of protein regulation, which appeared to be on a locus-specific level rather than protein-family level. These significant venom composition changes that take place in the first few weeks of life most likely play critical roles in venom economy and resource conservation and may partially explain the rare, post-birth maternal care found in some venomous species.

1. Introduction

Snake venom plays a critical, lifelong role in the acquisition and digestion of prey. Many species undergo ontogenetic shifts in diet (Mushinsky, 1987), with juveniles consuming small ectothermic prey items (e.g., invertebrates, amphibians, and lizards) and adults consuming large endothermic items (e.g., birds, rats, and rabbits; Klauber, 1972; Mackessy, 1988; Valdujo et al., 2002), and these dietary shifts are often associated with corresponding changes in venom composition (Andrade and Abe, 1999; Mackessy, 1988; Mackessy et al., 2003; Minton, 1967; Reid and Theakston, 1978). In some venomous species, juveniles possess venoms with higher concentration of neurotoxins and lower digestive properties, whereas adults possess venoms with higher levels of digestive components (e.g., *Crotalus oreganus oreganus*, Mackessy, 1988). However, in some cases the neurotoxic properties of juveniles remain into adulthood in what are thought to be examples of paedomorphosis (e.g., *C. o. concolor*, Mackessy et al., 2003; *Crotalus simus*, Calvete et al., 2010), though other aspects of the venom may still shift ontogenetically. Species displaying the former pattern have been referred to as type I species and the latter as type II species (Mackessy, 2008).

Despite significant research into the ontogeny of snake venoms, very little is known about the venom of hatchlings/neonates. The transition between the aqueous environments of the egg/mother into a terrestrial existence is marked by a number of physiological, morphological, and behavioral changes fueled by the shrinking yolk sac (Morafka et al., 2000). This makes the first meal a critical point in the early life of young snakes and, for venomous species, the formation and readiness of the venom is crucial in securing it. The first meal is not usually consumed until after postnatal ecdysis (i.e., first shed), which generally takes place between 7 and 14 days after hatching/birth. During this time, young snakes seek refuge in the form of hides or, in the case of many pitvipers (e.g., rattlesnakes), by staying close to the mother (Greene, 1997). Whether the venom is fully functional or undergoing further development during this period and, if so, what changes are taking place in venom composition, is unknown. We used neonates of *Crotalus adamanteus* and *Crotalus horridus* to test whether changes in venom composition occurred following postnatal ecdysis and examined the nature of these changes. We used the postnatal ecdysis as our benchmark since a number of key physiological changes (e.g., establishment of the skin permeability layer) are known to take place on either side of this point (Morafka et al., 2000; Tu et al., 2002).

The Eastern Diamondback Rattlesnake (*C. adamanteus*) is the largest species of rattlesnake, ranging throughout the Southeastern Coastal Plain of the United States in parts of North Carolina, South Carolina, Georgia, Florida, Alabama, Mississippi, and Louisiana. Based on venom composition and ontogeny, this species is

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http://dx.doi.org/10.1016/j.toxicon.2015.01.010
0041-0101/

classified as type I (Mackessy, 2008), and bites from this species are medically significant. It is known to consume a variety of small mammals (e.g., mice, rats, squirrels, and rabbits; Klauber, 1972). Currently, it is a candidate species for federal listing under the Endangered Species Act.

The Timber Rattlesnake (*C. horridus*) is a large species of rattlesnake that historically ranged over much of the eastern half of the United States and parts of southeastern Canada. Juveniles of this species consume a variety of small ectothermic and endothermic prey items (e.g., frogs and mice), whereas adults consume a variety of endothermic prey (e.g., rats and squirrels; Klauber, 1972). Based on venom composition and ontogeny, two distinct forms are recognized; type I populations occur throughout the majority of the range and type II populations occur in the extreme southeastern and southwestern portions of the range (Glenn et al., 1994). Bites from *C. horridus* are medically significant, particularly among the virulent, neurotoxic type II populations. This species is classified as endangered in six states, threatened in another five, and considered extirpated from Canada.

Table 1
Dates of key events in two litters of *Crotalus adamanteus* (LSG and MS) and one litter of *C. horridus* (Ch). SVL = snout-vent length, TL = total length.

Litter	Sample ID	SVL (mm)	TL (mm)	Birth date	First extraction	Postnatal ecdysis	Second extraction
Ch	KW1576	340	370	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1577	335	360	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1578	340	365	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1579	335	360	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1580	350	375	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1581	345	375	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1582	335	360	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1584	340	365	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1585	340	370	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1586	335	365	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1587	360	395	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1588	335	370	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1589	335	360	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1590	345	365	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1591	335	365	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1592	335	365	8/15/2013	8/20/2013	8/26/2013	9/21/2013
Ch	KW1593	335	365	8/15/2013	8/20/2013	8/26/2013	9/21/2013
LSG	KW1725	340	375	8/27/2013	9/1/2013	9/9/2013	10/1/2013
LSG	KW1726	355	390	8/27/2013	9/1/2013	9/9/2013	10/1/2013
LSG	KW1727	380	410	8/27/2013	9/1/2013	9/9/2013	10/1/2013
LSG	KW1728	350	380	8/27/2013	9/1/2013	9/9/2013	10/1/2013
LSG	KW1729	345	370	8/27/2013	9/1/2013	9/9/2013	10/1/2013
LSG	KW1730	350	375	8/27/2013	9/1/2013	9/9/2013	10/1/2013
MS	KW1756	365	384	9/10/2013	9/14/2013	9/22/2013	9/25/2013
MS	KW1759	337	366	9/10/2013	9/14/2013	9/22/2013	9/25/2013
MS	KW1761	336	369	9/10/2013	9/14/2013	9/22/2013	9/25/2013
MS	KW1762	365	388	9/10/2013	9/14/2013	9/22/2013	9/25/2013
MS	KW1763	349	376	9/10/2013	9/14/2013	9/22/2013	9/25/2013
MS	KW1764	350	373	9/10/2013	9/14/2013	9/22/2013	9/25/2013
MS	KW1765	342	376	9/10/2013	9/14/2013	9/22/2013	9/25/2013
MS	KW1767	352	376	9/10/2013	9/14/2013	9/22/2013	9/25/2013

2. Materials and methods

2.1. Specimen and venom sampling

We used two litters of *C. adamanteus* and one litter of *C. horridus*. The first litter (LSG) consisted of seven neonate *C. adamanteus* born on 27 August 2013 to a female measuring 109.5 cm SVL and collected from Little St. George Island, Franklin County, Florida. The second litter (MS) consisted of 13 neonate *C. adamanteus* born on 10 September 2013 to a female measuring 109 cm snout-vent length (SVL) and collected from Camp Shelby Military Base, Forrest County, Mississippi. The final litter (Ch) consisted of 19 neonate *C. horridus* born on 15 August 2013 to a female measuring 117 cm SVL and collected from Baker County, Georgia. All animals were collected and handled under approved Florida State University Institutional Animal Care and Use Committee protocols (#1230, #1333, and #1334).

For each neonate, the SVL and total length was recorded and whole venom was collected (Table 1). We collected venom by coaxing each neonate into an appropriate-sized, clear, plastic tube and then firmly grasping the animal behind the head as it was gently backed out of the tube. The neonate was then presented a sterile collecting receptacle and allowed to bite it. Venom was initially collected at 4 days (MS) or 5 days (LSG and Ch) after birth and then again at 3 days (MS), 22 days (LSG), or 26 days (Ch) after postnatal ecdysis (Table 1). The time differences in post-shed venom extraction were due to the MS cohort needing to be immediately released and the LSG and Ch cohorts being used in other experiments. We attempted to sample all individuals from each litter, however, not all animals would bite and/or deliver venom (even with gentle massaging of venom glands) into the receptacle during one or both extraction sessions. These individuals were excluded from further analysis, leaving $n = 6$ (LSG), $n = 8$ (MS), and $n = 17$ (Ch) individuals for analysis (Table 1). Venom samples were transferred to cryogenic tubes and stored in liquid nitrogen until they could be lyophilized. After lyophilization, samples were resuspended in liquid chromatography/mass spectrometry grade water, followed by centrifugation to remove insoluble materials.

2.2. Reversed-phased high performance liquid chromatography

C. adamanteus venom was analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) as described in Margres et al. (2014). Briefly, approximately 40–60 μg of venom proteins were separated on a Jupiter C18 analytical column (300 Å, 5 micron, 250 × 4.6 mm, Phenomenex) attached to a Beckman System Gold equipped with a model 9725i injector, 125 NM solvent module and 168 NM detector. The column was developed at 1 ml/min with a 5 min isocratic step at 5% B, followed by a 1%/min linear gradient to 25% B, then 0.25%/min to 60% B (solvent A = 0.1%

Table 2
Wilcoxon two-sample test results of pre- and post-shed changes in reversed-phase high performance liquid chromatography peaks from two litters of *Crotalus adamanteus*: Little St. George (LSG; $n = 6$) and Mississippi (MS; $n = 8$). Significant p-values in bold. Note: Peak six was absent from LSG. Toxin protein abbreviations: CTL = C-type lectins, CRISP = cysteine-rich secretory protein, HYAL = hyaluronidase, LAAO = L-amino oxidase, MYO = myotoxin, NUC = nucelotidase, PDE = phosphodiesterase, PLA2 = phospholipase A_2, SVMP = snake venom metalloproteinase (types II and III), SVSP = snake venom serine proteinase. The asterisks in peaks P1b and P3-5 indicate that the SVMPs identified in these fractions are disintegrins, functional domains of SVMPs that are proteolytically cleaved posttranslationally to produce a free disintegrin (see Margres et al., 2014). The LAAO and PDE identified in peak P1b most likely represent degradation products.

Peak	Protein(s)	LSG	LSG	W	p-Value	MS	MS	W	p-Value
		Pre-shed median	Post-shed median			Pre-shed median	Post-shed median		
P1a	Unidentified	−0.152	0.832	36	**0.002**	−1.868	−1.885	34	0.879
P1b*	LAAO, SVMPII-2b, MYO-1a/b, SVMPIII-5, PLA2-1a, PDE, SVMPII-1b/d, SVMPIII-2b	−1.208	−1.335	17	0.937	−1.090	−0.948	45	0.195
P2	MYO-1b	3.489	3.997	31	**0.041**	4.321	4.592	50	0.065
P3*	SVMPIII-2b	−0.128	−0.353	10	0.240	−0.229	−1.389	0	>**0.001**
P4*	SVMPIII-2b	−1.027	−0.366	31	**0.041**	−2.967	−1.820	46	0.161
P5*	SVMPIII-2b	−1.125	−1.006	17	0.937	−4.042	−4.097	30	0.879
P6	Unidentified	–	–	–	–	1.452	2.083	55	**0.015**
P7	CRISP	0.532	0.901	24	0.393	0.696	0.758	35	0.798
P8	SVSP-5, CRISP	−1.631	−1.013	25	0.310	−0.488	−0.552	32	1.000
P9	None	−0.808	0.048	33	**0.015**	−0.425	0.444	63	>**0.001**
P10	PLA2-1a/b	3.572	4.089	32	**0.026**	3.476	3.454	35	0.798
P11	SVSP-2, SVSP-5	−2.211	−2.539	18	1.000	−1.264	−0.937	33	0.960
P12	SVSP-4	1.178	2.581	36	**0.002**	1.676	2.210	58	**0.005**
P13	SVSP-7, SVSP-4	0.655	1.987	36	**0.002**	1.129	1.498	49	0.083
P14	CTL-10, SVSP-4, CTL-11a/b/c	0.435	−2.138	3	**0.015**	1.164	0.819	29	0.798
P15a	CTL-10, CTL-9, SVSP-4	0.052	0.182	21	0.699	−1.046	−2.743	26	0.574
P15b	CTL-10, CTL-13f, SVSP-7, LAAO, SVSP-4, CTL-11a/b	−0.010	−0.015	20	0.818	−0.273	−0.238	33	0.959
P16	LAAO, SVSP-7, SVSP-4, CTL-11a/b/c	−0.978	0.059	26	0.240	−1.059	0.204	62	>**0.001**
P17	SVSP-4, SVSP-7, NUC, HYAL	−1.578	−1.691	18	1.000	−1.301	−4.196	13	**0.050**
P18	SVMPIII-4d/e	1.739	2.116	36	**0.002**	1.739	1.988	39	0.505
P19	SVMPIII-3, CTL-11a	0.985	1.570	31	**0.041**	−0.328	−0.238	35	0.798
P20a	SVMPIII-2b, SVMPII-2c/d, SVMPII-2b	2.719	2.967	30	0.065	2.446	2.406	31	0.959
P20b	SVMPII-2b, LAAO, CTL-10, CTL-11a/b, SVMPIII-2b, CTL-13f, SVSP-4	−0.250	−4.570	6	0.065	−0.361	−1.203	27	0.645
P20c	LAAO, SVMPII-2b, CTL-11a/b, SVMPII-1b/d, CTL-10	−1.108	−4.407	1	**0.004**	−2.650	−4.097	24	0.441
P21	SVMPII-1a/b/c/d	2.556	3.125	34	**0.009**	2.599	2.722	48	0.105

trifluoroacetic acid [TFA] in water, solvent B = 0.075% TFA in acetonitrile). For *C. horridus*, approximately 20 μg of venom proteins were analyzed on a Jupiter C18 narrowbore column (300 Å, 5 micron, 250 × 2 mm). The RP-HPLC system was run at 0.2 ml/min with a 5 min isocratic step at 5% B, followed by a 1%/min linear gradient to 25% B, then 0.25%/min to 55% B, followed by 2%/min to 75% B. The *C. adamanteus* samples were separated on the analytical column to maintain consistency with a larger number of *C. adamanteus* samples previously analyzed. The narrowbore column is preferable when sample quantity is small and was therefore used for the *C. horridus* samples. Column effluent was monitored at 220 nm and peak areas were determined using Beckman 32 Karat software v. 8.

2.3. Statistical analyses

The RP-HPLC data gives the percentage of the total absorbance attributable to each peak, resulting in a form of compositional data (Aitchison, 1986; Pawlowsky-Glahn and Buccianti, 2011), which must be analyzed within a multivariate framework accounting for both its sum and positivity constraints. These data exist within a simplex and must be transformed to the real space prior to the application of standard multivariate statistical approaches (Aitchison and Egozcue, 2005; Egozcue et al., 2003). For visualization purposes, we used the centered log ratio (clr) transformation. If the normalized data are $\mathbf{x} = (x_1, \ldots, x_n)$ such that $x_i = 1$, then

$$\mathrm{clr}(\mathbf{x}) = \left(\ln\frac{x_1}{g(\mathbf{x})}, \ldots, \ln\frac{x_n}{g(\mathbf{x})}\right)$$

where $g(\mathbf{x}) = \sqrt[n]{x_1 \ldots x_n}$ is the geometric mean. This transformation takes the data from the simplex to real space and retains the individual identities of the peaks. The transformed data, however, still suffer from a sum constraint; the components must add to zero. This constraint remains because an n-component composition really only has $n - 1$ free dimensions. The clr-transformed data is therefore useful for visualization because it partially ameliorates the issues with percentage data, while retaining peak identities. To test for differences among sets of RP-HLPC profiles, we transformed the n-dimensional composition data on the simplex to the $n - 1$ real space by means of the isometric log ratio (ilr) transformation (Egozcue et al., 2003), which projects vectors in the simplex space into the real space using an orthonormal basis for the simplex and maintains all metric properties in the process. Both transformations are implemented in the R statistical package "robCompositions" (R Developmental Core Team, 2013).

Zeros in compositional vectors are troublesome when attempting to perform log ratio transforms (Aitchison, 1986). Our primary analyses and visualizations used the multiplicative replacement strategy (Martin-Fernandez et al., 2003) implemented in the R package zCompositions assuming a detection threshold of 0.01% (the smallest measured value among all of the RP-HPLC profiles) and a fraction of 0.5. To assess robustness, we re-

Table 3
Wilcoxon two-sample test results of pre- and post-shed changes in reversed-phase high performance liquid chromatography peaks from a litter of *Crotalus horridus* divided into two groups: individuals without peak 11b ($n = 9$) and those with peak 11b ($n = 8$). Significant p-values in bold. Toxin protein abbreviations: BPP = bradykinin-potentiating peptide, CTL = C-type lectins, CRISP = cysteine-rich secretory protein, LAAO = L-amino oxidase, MYO = myotoxin-A, NGF = nerve growth factor, PDE = phosphodiesterase, PLA2 = phospholipase A_2, SVMP = snake venom metalloproteinase (types II and III), SVSP = snake venom serine proteinase, VESP = vespryn, VEGF = vascular endothelial growth factor. The asterisks in peaks P1–P4b indicate that the SVMPs identified in these fractions are disintegrins, functional domains of SVMPs that are proteolytically cleaved posttranslationally to produce a free disintegrin. The VEGF identified in P1 and the LAAO identified in P3, P4a, P4b most likely represent degradation products.

Peak	Protein(s)	No P11b	No P11b	W	p-Value	P11b	P11b	W	p-Value
		Pre-shed median	Post-shed median			Pre-shed median	Post-shed median		
P1*	SVMPII-3a, SVMPII-4a, SVSP-6, VEGF-1, BPP-1a/c, HistoneH2AZ/H2A	0.335	0.915	72	**0.004**	0.443	0.563	42	0.328
P2*	SVMPIII-4b, SVMPIII-4a, SVMPII-2	0.467	0.476	42	0.931	0.312	0.302	26	0.574
P3*	SVMPIII-4b, SVMPIII-4a, SVMPII-3a, LAAO-1e, LAAO-1b/d	−0.439	−0.103	80	>**0.001**	−0.146	−0.451	40	0.442
P4a*	MYO, SVMPII-4a, LAAO-1b/d, LAAO-1e, SVMPII-3a, SVSP-6	−0.514	−0.581	52	0.34	−0.941	−0.889	37	0.645
P4b*	MYO, SVMPII-4a, LAAO-1b/d, LAAO-1e, SVMPII-3a, SVSP-6	1.193	0.394	2	>**0.001**	0.801	−0.092	8	**0.010**
P5	VEGF-1, NGF-1a	0.637	0.496	8	**0.003**	0.260	0.132	22	0.328
P6	CRISP, NGF-1a, VEGF-1	0.846	0.008	0	>**0.001**	0.623	−0.248	2	>**0.001**
P7	SVSP-2a, SVSP-2b, SVSP-6, PLA2-1d	2.582	2.475	33	0.546	2.421	2.416	29	0.798
P8	SVSP-5, SVSP-6, SVSP-2b, SVSP-2a, NGF-1a, VESP	−1.250	0.128	81	>**0.001**	−1.443	−0.024	56	**0.010**
P9	PLA2-1c, PLA2-1e, PLA2-1d	2.332	2.534	31	0.436	1.088	0.352	18	0.161
P10	SVSP-13b, SVSP-13c, PLA2-1d, SVSP-2b, SVSP-5	0.352	1.179	77	>**0.001**	0.014	0.754	53	**0.028**
P11a	SVSP-13c, SVSP-13b, PLA2-1d, SVSP-2b	0.840	1.282	59	0.114	0.773	0.845	38	0.574
P11b	SVSP-13c, SVSP-13b, PLA2-1d, SVSP-2b	–	–	–	–	1.944	1.755	16	0.105
P12	SVSP-2a, SVSP-2b, SVSP-6, SVSP-11b, SVSP-11d, SVSP-5, CTL-8a, SVSP-1	1.964	1.714	12	**0.011**	1.749	1.494	13	**0.050**
P13	LAAO-1e, PDE, SVSP-6 SVSP-2a, CTL-2	−1.959	−1.084	62	0.063	−1.750	−1.278	43	0.279
P16	SVMPIII-5a, SVMPIII-2a, SVMPIII-4b, SVMPIII-4a, LAAO-1e	0.546	0.048	0	>**0.001**	0.133	−0.507	8	**0.010**
P17	SVMPIII-4a, SVMPIII-4b, SVMPII-3a	−1.908	−3.048	10	**0.006**	−5.910	−4.417	31	0.959

analyzed the data using the log ratio data augmentation method (lrDA; Palarea-Albaladejo et al., 2014) and the log ratio expectation maximization algorithm method (lrEM; Martin-Fernandez et al., 2012) for replacing zeros. Both of these additional methods are also implemented in zCompositions.

To test for differences among sets of RP-HPLC profiles, we used the adonis function from the vegan package in R with Euclidean distances to perform a permutational or non-parametric MANOVA (McArdle and Anderson, 2001) on the ilr-transformed data, using cohort and shed state as factors. Nonparametric tests do not assume the data is normally distributed as do traditional, parametric tests (McArdle and Anderson, 2001). *P*-values were calculated on the basis of 10,000 permutations. Because samples were paired across individuals (i.e. a pre-shed and post-shed sample from each individual), we stratified permutations across individuals.

We used the conservative, nonparametric Wilcoxon two-sample tests on the clr-transformed data to identify which peaks, if any, were significantly different within each cohort. We then used the genotype–phenotype (GP) maps for *C. adamanteus* (Table 2; Margres et al., 2014) and *C. horridus* (Table 3; Rokyta et al., 2014) to identify the protein(s) represented by each peak.

3. Results

We quantified 25 RP-HPLC peaks for the *C. adamanteus* cohorts (Figs. 1 and 3). The permutational MANOVA showed that the cohorts were significantly different ($R^2 = 0.43$, $P = 0.0009$), the pre- and post-shed samples within cohorts were significantly different ($R^2 = 0.09$, $P = 0.002$), but their interaction was not significant ($R^2 = 0.04$, $P = 0.12$). Independent analysis of each cohort for shed state yielded similar results (LSG: $R^2 = 0.34$, $P = 0.03$; MS: $R^2 = 0.14$, $P = 0.04$). These results were based on multiplicative replacement of zeros. Robustness analyses using both the lrDA and lrEM algorithms gave similar results (not shown).

For *C. horridus*, we quantified 17 RP-HPLC peaks (Figs. 2 and 3). We found a significant difference between the pre- and post-shed samples ($R^2 = 0.05$, $P = < 10^{-4}$), but because of the low R^2 value, we examined the individual venom samples from this cohort and found that eight of the 17 individuals had an additional peak (P11b; consisting of snake venom serine proteases and phospholipase A_2) that was not present in the others. We coded the presence or absence of this peak as a factor and re-ran the analysis with peak P11b presence, shed state, and their interaction. We found that P11b presence/absence ($R^2 = 0.69$, $P = <10^{-4}$) and shed state ($R^2 = 0.05$, $P = <10^{-4}$) were significant, but their interaction was not ($R^2 = 0.008$, $P = 0.21$). Splitting the data into two sets on the basis of the presence/absence of P11b still showed a significant difference associated with shed state for both groups (with P11b: $R^2 = 0.11$, $P = 0.007$; without P11b: $R^2 = 0.35$, $P = 0.004$). Robustness analyses using lrDA and lrEM did not show a substantial

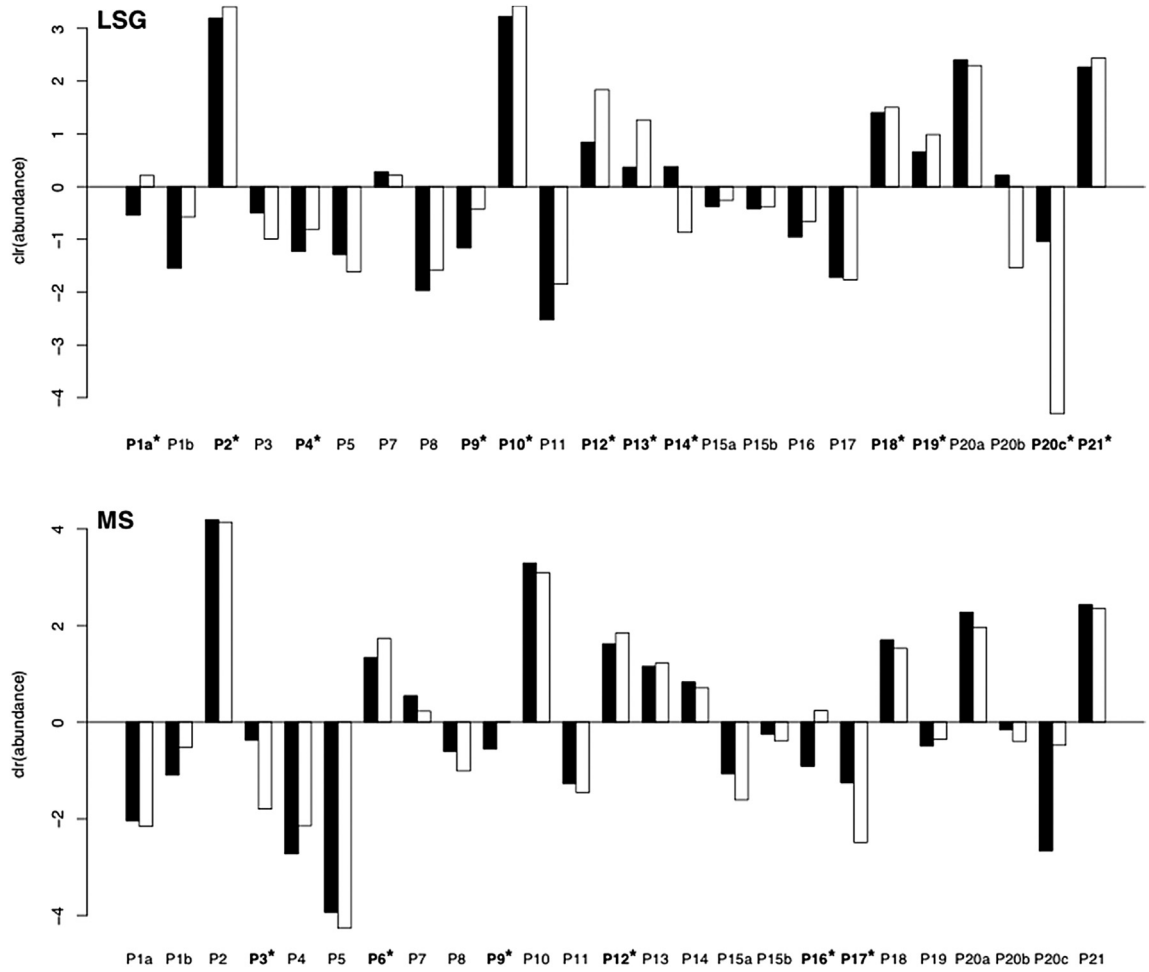

Fig. 1. Mean centered log ratio expression levels from two litters of *Crotalus adamanteus*: Little St. George (LSG; $n = 6$) and Mississippi (MS; $n = 8$). These values represent the clr transformation of the average venom. Peaks that displayed statistically significant changes are in bold and marked with an asterisk (see also Tables 1 and 2). Black bars = before postnatal shed, white bars = after postnatal shed. Note: Peak six was absent in LSG and so was not included.

difference in the results, except that the set without P11b dropped its *P*-value to 0.07 for the shed factor.

In the LSG cohort, we found 12 of 24 peaks (50%) to be significantly different across the postnatal shed. The MS cohort had 6 of 25 peaks (24%) that were significantly different pre- and post-shed. We analyzed the *C. horridus* cohort as two groups based on the presence/absence of peak 11b. Ten out of 16 peaks (63%) were significantly different in the group without peak 11b and six out of 17 peaks (35%) in the group with peak 11b.

4. Discussion

4.1. Patterns and potential causes of variation

We demonstrated that significant changes take place in venom composition of neonate snakes after their postnatal shed. Previous studies have shown differences between neonate and adult snake venoms, but these studies all used pooled venom from numerous litters and from time frames occurring after the postnatal shed (Gao et al., 2013; He et al., 2014; Zelanis et al., 2011). Some snake venoms vary with geography (see Chippaux et al., 1991 for partial review; Margres et al., 2015) and, indeed, we detected geographic variation between the LSG and MS cohorts of *C. adamanteus*. However, despite geographic variation explaining more of the variation between cohorts, we were clearly able to detect significant differences across the narrow time frame of pre- and postnatal ecdysis within each cohort. These differences reflected changes in both number of expression peaks and direction of protein regulation.

The LSG and Ch cohorts had a higher number of differences in venom peaks (14 and 16, respectively) compared to the MS cohort (5). Although cohorts were sampled at nearly the same initial time after birth, the LSG and Ch cohorts were sampled much later after the postnatal shed than the MS cohort. Potentially, these two cohorts could display more differences in the pre- and post-shed venom samples simply because the venom continues to undergo significant changes after the postnatal shed. Though the postnatal shed is known to represent a significant benchmark in the physiology, morphology, and behavior of neonate snakes (e.g., the establishment of the skin permeability layer (Tu et al., 2002) and the end of post-birth maternal care (Greene et al., 2002)), neonate venom may not necessarily be fully developed by this benchmark. Alternatively, the difference in number of observed peak changes may be due to geography, at least in the case of the two *C. adamanteus* cohorts (Margres et al., 2015).

Protein regulation also varied among the three cohorts. In *C. adamanteus*, the majority of peak changes involved upregulation (Fig. 1), whereas in *C. horridus* the majority of peak changes involved downregulation (Fig. 2). These patterns become even more complex when the loci undergoing these regulatory-changes are identified with the GP maps (Tables 2 and 3). For instance, in the

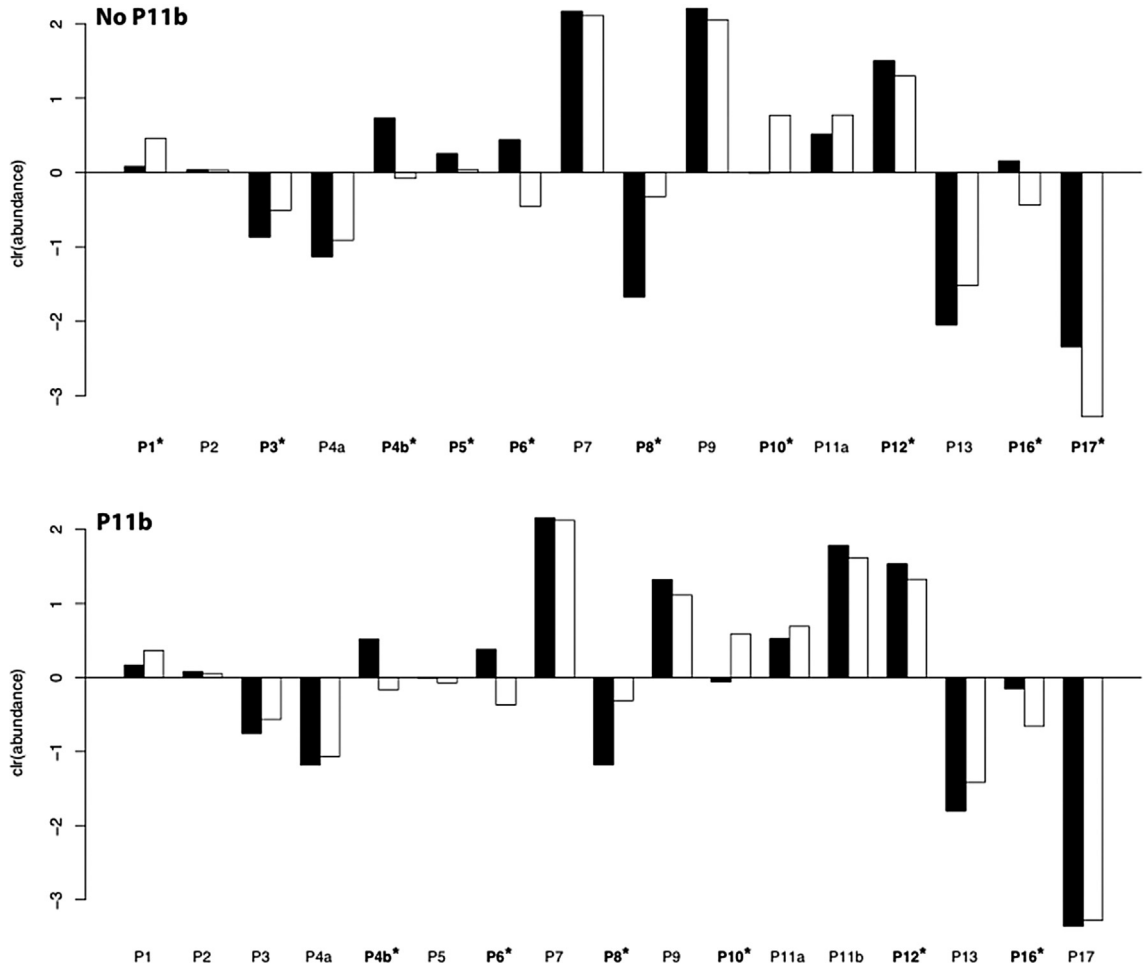

Fig. 2. Mean centered log ratio expression levels from a litter of *Crotalus horridus* divided into two groups: individuals without peak 11b ($n = 9$) and those with peak 11b ($n = 8$). These values represent the clr transformation of the average venom. Peaks that displayed statistically significant changes are in bold and marked with an asterisk (see also Tables 1 and 2). Black bars = before postnatal shed, white bars = after postnatal shed.

LSG *C. adamanteus* cohort, peak 12 (SVSP-4) was upregulated, while peak 13 (partially represented by SVSP-7) was downregulated. Similarly, peaks 20a and 20b are both downregulated and are partially represented by snake venom metalloproteinases type II (SVMPII-2), whereas peak 21 is an upregulated SVMPII (SVMPII-1). Similar patterns are found in the other two cohorts. The significance of these protein regulation changes is unclear, however, it is interesting to note that they occurred in a wide variety of proteins and directions. Our data indicate that these early life changes in venom composition are not taking place on a protein-family level, but rather at the locus-specific level. In order to test whether these changes represent a watershed moment in early venom ontogeny or rather are part of a much longer, continuous process of venom ontogeny, two of the three cohorts (LSG and Ch) are currently being maintained and sampled through adulthood.

4.2. Significance of postnatal ecdysis venom changes

Hatchling/neonate snakes have a limited amount of yolk reserves to fuel a number of physiological, morphological, and behavioral changes early in life (Morafka et al., 2000), making the acquisition of the first meal critical. Our results show that, even when geography plays a role in venom variation, hatchlings/neonates undergo numerous, significant changes in venom composition from birth to shortly after their postnatal ecdysis. Given limited yolk reserves, these young snakes have limited initial resources to produce venom, resulting in limited opportunities to expend venom, and these developmental changes may be critical in young snakes securing future resources.

In a number of species in the family Viperidae, females are known to give birth and remain in the immediate vicinity with their young until after they shed for the first time, presumably to defend them during this vulnerable period (Greene, 1997). Neonate/hatchling snakes are vulnerable prior to their first shed for a variety of reasons. For example, Tu et al. (2002) showed that after postnatal ecdysis, the skin resistance to transepidermal water loss increases almost two fold, likely explaining why young snakes usually stay in the nest site until ecdysis or only move a short distance to a more secure location. Yet, despite this universal vulnerability, very few snake species show post-egg laying/birth maternal care and almost all that do are venomous species. Since venom also functions in defense, maternal care in these species may have evolved because the primary defense of young snakes (venom) is incompletely developed until after postnatal ecdysis.

To effectively test whether these hypotheses explain postnatal ecdysis venom changes, more data is required. Though we examined three cohorts from two different species, larger sample sizes within each species should help clarify the patterns we observed. Furthermore, expanding a data set to include additional species, particularly ones with different life histories, would further

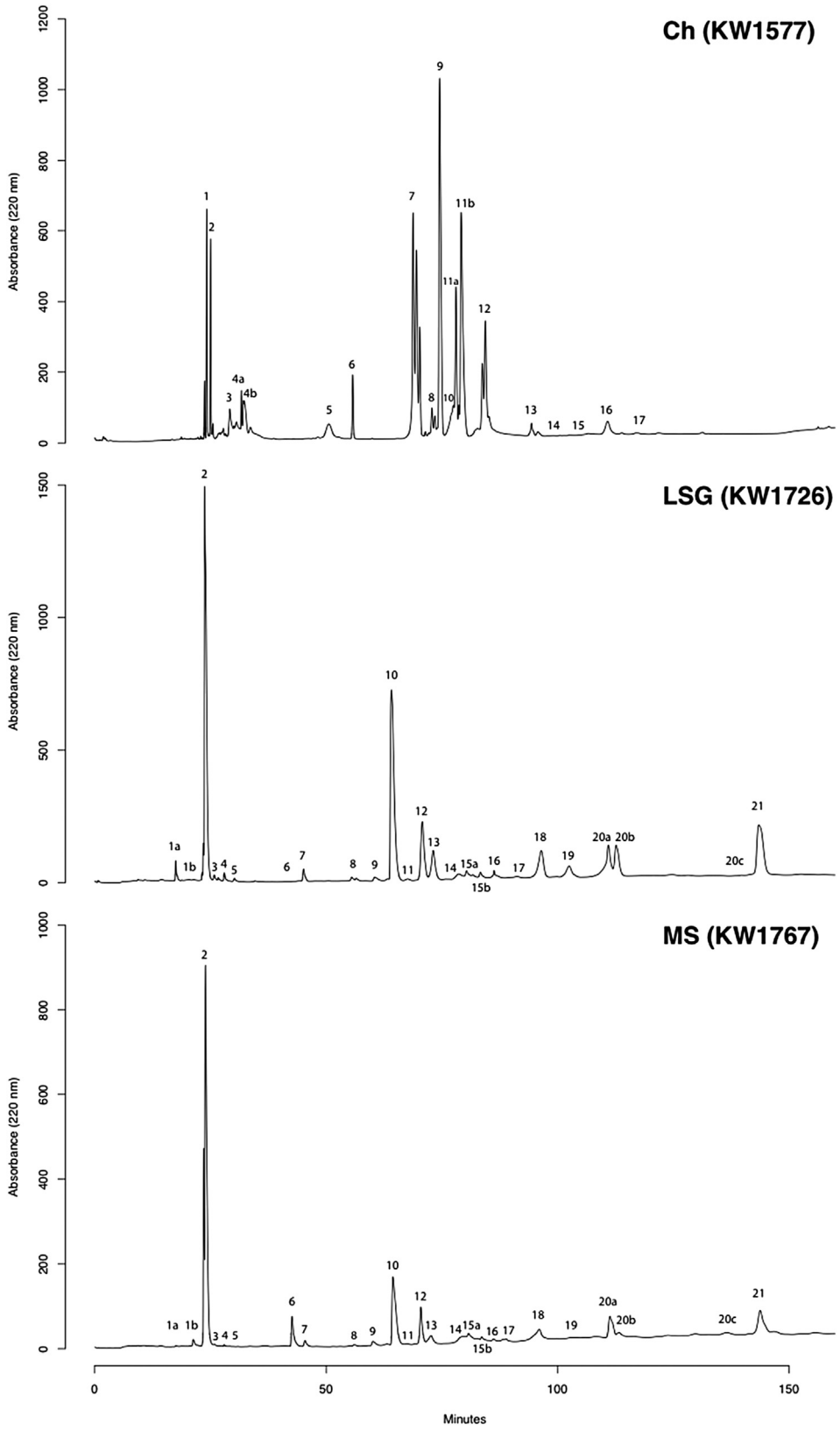

Fig. 3. Representative chromatograms of individuals from each cohort. KW numbers in parentheses refer to the sample identifications in Table 1. Time scale is the same for all three cohorts.

elucidate the role of early venom changes in snakes. Of particular interest would be the inclusion of egg laying species, such as those of the large family Elapidae, and type II species or populations of viperids (e.g., type II *C. horridus* populations), which do not appear

to undergo ontogenetic shifts in juvenile to adult venoms. Such an expansion could test if certain venom types are more developed at hatching/birth than others, whether delayed development is correlated with maternal care, and whether different mechanisms are involved in these changes (e.g., protein family changes vs. locus-specific changes). Functional characterizations, such as LD_{50} tests, using pre- and post-natal shed venoms would help elucidate differences in activity/toxicity. These and a number of other hypotheses and tests would provide further insight into our understanding of the evolution of snake venoms.

Ethical statement

This paper represents research involving the use of animals and was carried out under standard procedures of scientific ethics and with oversight from the Florida State University Institutional Animal Care and Use Committee. All authors have read the manuscript and agree to having adhered to the rules of ethics presented in the Elsevier's Ethical Guidelines for Journal Publication and to its publication in Toxicon.

Conflicts of interest statement

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors would like to thank Jim Lee (The Nature Conservancy at Camp Shelby Military Base), Megan Lamb (Apalachicola National Estuarine Research Reserve), and Jenna Wanat (Apalachicola National Estuarine Research Reserve) for assistance in securing specimens and James McGivern (Florida State University) for assistance in venom extractions. The National Science Foundation (NSF DEB-1145978) provided funding for this work.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2015.01.010.

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