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Comparative venomics of the Prairie Rattlesnake (*Crotalus viridis viridis*) from Colorado: Identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom CroFab®

Anthony J. Saviola[a,b], *Davinia Pla*[b], *Libia Sanz*[b], *Todd A. Castoe*[c], *Juan J. Calvete*[b,*], *Stephen P. Mackessy*[a,**]

[a]*School of Biological Sciences, University of Northern Colorado, 501 20th Street, CB 92, Greeley, CO, 80639, USA*
[b]*Instituto de Biomedicina de Valencia, C.S.I.C., Jaime Roig 11, 46010 Valencia, Spain*
[c]*Department of Biology, University of Texas at Arlington, Arlington, TX 76010, USA*

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ABSTRACT

Here we describe and compare the venomic and antivenomic characteristics of both neonate and adult Prairie Rattlesnake (*Crotalus viridis viridis*) venoms. Although both neonate and adult venoms contain unique components, similarities among protein family content were seen. Both neonate and adult venoms consisted of myotoxin, bradykinin-potentiating peptide (BPP), phospholipase A_2 (PLA_2), Zn^{2+}-dependent metalloproteinase (SVMP), serine proteinase, L-amino acid oxidase (LAAO), cysteine-rich secretory protein (CRISP) and disintegrin families. Quantitative differences, however, were observed, with venoms of adults containing significantly higher concentrations of the non-enzymatic toxic compounds and venoms of neonates containing higher concentrations of pre-digestive enzymatic proteins such as SVMPs. To assess the relevance of this venom variation in the context of snakebite and snakebite treatment, we tested the efficacy of the common antivenom CroFab® for recognition of both adult and neonate venoms *in vitro*. This comparison revealed that many of the major protein families (SVMPs, CRISP, PLA_2, serine proteases, and LAAO) in both neonate and adult venoms were immunodepleted by the antivenom, whereas myotoxins, one of the major toxic components of *C. v. viridis* venom, in addition to many of the small peptides, were not efficiently depleted by CroFab®. These results therefore provide a comprehensive catalog of the venom compounds present in *C. v. viridis* venom and new molecular insight into the potential efficacy of CroFab® against human envenomations by one of the most widely distributed rattlesnake species in North America.

* *Correspondence to:* J.J. Calvete, Laboratorio de Venómica Estructural y Funcional, Instituto de Biomedicina de Valencia, C.S.I.C., Jaime Roig 11, 46010 Valencia, Spain. Tel.: +34 96 339 1778; fax: +34 96 369 0800.
** *Corresponding author*. Tel.: +1 970 351 2429; fax: +1 970 351 2335.
E-mail addresses: jcalvete@ibv.csic.es (J.J. Calvete), stephen.mackessy@unco.edu (S.P. Mackessy).

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Biological significance
Comparative proteomic analysis of venoms of neonate and adult Prairie Rattlesnake (*Crotalus viridis viridis*) from a discrete population in Colorado revealed a novel pattern of ontogenetic shifts in toxin composition for viperid snakes. The observed stage-dependent decrease of the relative content of disintegrins, catalytically active D49-PLA_2s, L-amino acid oxidase, and SVMPs, and the concomitant increase of the relative abundance of paralytic small basic myotoxins and ohanin-like toxin, and hemostasis-disrupting serine proteinases, may represent an age-dependent strategy for securing prey and avoiding injury as the snake switches from small ectothermic prey and newborn rodents to larger endothermic prey. Such age-dependent shifts in venom composition may be relevant for antivenom efficacy and treatment of snakebite. However, applying a second-generation antivenomics approach, we show that CroFab®, developed against venom of three *Crotalus* and one *Agkistrodon* species, efficiently immunodepleted many, but not all, of the major compounds present in neonate and adult *C. v. viridis* venoms.

1. Introduction

Produced and stored in a pair of highly specialized cephalic gland, snake venoms represent a complex mixture of bioactive proteins and peptides that exhibit diverse biochemical and pharmacological functions [1]. Venoms likely evolved via the co-opting and secondary modification of endogenous proteins with normal physiological functions early in the evolution of advanced snakes [e.g., 2 but see 3,4], enabling the transition from a mechanical (constriction) to a chemical (venom) means of subduing prey [5]. The complexity of venoms, coupled with the fact that many snake species specialize on specific prey, has led to selective pressures resulting in the evolution of advantageous venom phenotypes that may vary based on phylogenetic affinities [1,6,7], geographic localities [8,9], snake age [10–12] and diet [13,14]. It is this variation and complexity that has continuously led researchers to examine snake venoms and the evolution of venom systems. Research into the origin and evolution of snake venoms offers remarkable insights into the biological roles of venom compounds [15,16] and potential avenues for novel drug discovery [17–19], as well as addressing the ever-growing concern for effectively treating human snakebite [20,21]. Proteomic analyses of venoms, termed "venomics", is significantly expanding our knowledge and understanding of these oral secretions [e.g., 22,23], which are not only critical to the foraging success of the snakes, but may also be of potential value or threat to humans.

Within the superfamily Caenophidia, the family Viperidae consists of approximately 260 species within four subfamilies: Azemiopinae, Causinae, Crotalinae and Viperinae. Of these subfamilies, the Crotalinae (pit vipers) is the most speciose, and currently comprises over 200 species distributed among 28 genera. In the Americas, the only viperids are the monophyletic pit vipers, which appear to have dispersed into the New World during the late Oligocene to early Miocene approximately 22-24 mya [24]. Among New World pit vipers, the genus *Crotalus* currently comprises 30-36 species of venomous snakes distributed throughout much of South, Central and North America (http://www.reptile-database.org). The Prairie Rattlesnake (*Crotalus viridis viridis*) is a medium-sized terrestrial pitviper commonly exceeding 100 cm snout-vent length (SVL) [25]. The range of this species spans much of the Great Plains of the central United States, northwestern Mexico and southwestern Canada, making it one of the most widely distributed rattlesnake species in North America (Fig. 1). Due to this wide geographic distribution, and the sometimes large home ranges, *C. v. viridis* may occur in close proximity to housing developments and are often found migrating into human-inhabited areas [26], increasing the possibility of encounters with humans. Terrestrial habitats occupied by *C. v. viridis* range from semi-desert and plains grasslands to pinion-juniper, mountain shrublands and montane woodlands, up to 2740 m in elevation [25,27]. In grasslands habitat, *C. v. viridis* is a frequent inhabitant of prairie dog towns where burrows are commonly used for prey ambush sites, predator avoidance, and hibernation [26]. Like many other rattlesnake species, the diet of *C. v. viridis* shifts with snake age, generally focusing on small ectothermic prey and newborn rodents as neonates, and switching to larger endothermic prey (small mammals and occasionally birds) as adults [10,27].

Viperid venoms contain an abundance of proteins which interfere with homeostasis and with the blood coagulation cascade, ultimately leading to the immobilization, killing and predigestion of prey. Individual venom may contain well over 100 proteins and peptides (including various protein isoforms); these compounds can, however, generally be classified into 10-15 protein families, such as the enzymatic L-amino acid oxidases (LAAOs), metalloproteinases (SVMPs), phospholipases A_2 (PLA_2) and serine proteases, as well as the non-enzymatic peptide myotoxins, C-type lectins, cysteine-rich secretory proteins (CRISPs) and disintegrins, among others [1]. Venom composition, especially in viperid species, can be classified based on enzymatic activity and toxicity, which are generally inversely correlated [7,28]. For species classified as having type I venom, neonate and juvenile snakes have venoms exhibiting increased toxicity with lower SVMP and serine protease activity, whereas adults have lower toxicity (>1.0 μg/g mouse body weight) but higher SVMP activity [29]. Type II venoms, on the other hand, have been suggested to be paedomorphic [7,28,30,31] since neonates, juveniles and adults all exhibit low SVMP activity but are higher in toxicity (<1.0 μg/g mouse body weight), retaining similar venom characteristics throughout the life history of the snake.

Previous studies of the venom of *C. v. viridis* have shown moderate to high activity levels of LAAO, kallikrein, plasmin, and thrombin-like serine proteases, SVMP, PLA_2 and

Fig. 1 – Geographic distribution of *C. v. viridis* throughout the Great Plains region of the United States, northwestern Mexico and southwestern Canada. Venoms from *C. v. viridis* used in the proteomic characterizations reported here were collected from Weld County, Colorado (indicated by the black dot).

phosphodiesterase enzymes [28,32]. Gel electrophoresis and mass spectrometry indicate that myotoxins, CRISPs and disintegrins are also abundant compounds in the venom of *C. v. viridis*. Venom yields from adult *C. v. viridis* may vary from 40 mg to well over 100 mg of dry venom in Colorado populations [28,33], while neonate snakes may yield only 2–4 mg venom. Further, Mackessy [28] reported mouse intravenous LD_{50} values at 1.55 μg/g of mouse body weight, making it one of the more toxic rattlesnake species in the Western rattlesnake complex.

It is estimated that there are over 9000 venomous snake bites in the United States annually [34], with roughly 99% of these bites from snakes of the family Viperidae [35]. These human envenomations may be characterized by edema, erythema, clotting disorders, hypofibrinogenemia and local tissue necrosis [36,37]. Bites may pose a serious or potentially deadly emergency, and early therapeutic administration of antivenom is necessary if severe envenomation is suspected. In the United States, the antivenom CroFab® (Crotaline Polyvalent Immune Fab (ovine)) is commonly administered during envenomation cases. CroFab® is produced from sheep immunized with one of the following North American snake venoms: *Agkistrodon piscivorus* (Water Moccasin), *Crotalus adamanteus* (Eastern Diamondback rattlesnake), *Crotalus atrox* (Western Diamondback rattlesnake) and *Crotalus scutulatus* (Mojave rattlesnake) [38]; serum collected from hyperimmune animals is affinity purified using columns containing the same immobilized venom, and hyperimmune sera are then mixed to produce a polyvalent antivenom. Surprisingly, in spite of its wide distribution in North America, *C. v. viridis* is not one of the species utilized for CroFab® production. Adequate treatment of snakebite is dependent on the ability of the antivenoms to reverse the pathological symptoms induced by venom by immunologically binding to venom components, facilitating their removal and degradation. Therefore, knowledge on venom composition and inter- and intra-specific venom variability is critical for assessment of antivenom efficacy and treatment of snakebite. The present work was designed to provide a comparative analysis of the venom proteomes of neonate and adult *C. v. viridis*, to determine venom composition and to investigate the immunoreactivity profile of the commercially available antivenom CroFab® against these venoms.

2. Materials and methods

2.1. Venoms and antivenoms

The venoms of fourteen neonate, twelve subadult and twelve adult *C. v. viridis* (equal numbers of female and male snakes) were manually extracted from wild-caught specimens (Weld Co., Colorado, USA). Age classes of snakes were based on snout-vent lengths from a large dataset of mark-recaptures from the same

population (Mackessy, unpub. data); snakes ≤ 300 mm were considered neonates, snakes 500-540 mm were considered subadults and snakes ≥ 800 mm were considered adults. Following extraction, snakes were in captivity for no more than 3 days and were released to the exact location of capture. Venoms were immediately centrifuged at 10,000 x g for 5 min to pellet insoluble material, frozen, lyophilized and stored at -20°C until used. CroFab® was donated by Dr. Robert Palmer of the Rocky Mountain Poison and Drug Center, and anti-myotoxin a antibodies were a gift of Dr. Charlotte Ownby (Oklahoma State University).

2.2. RP-HPLC fractionation

Venom proteins were separated by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Teknokroma Europa C_{18} (250 × 4 mm, 5 μm particle size, 300 Å pore size) column and an ETTAN™ LC HPLC System (GE Healthcare). Two mg of venom from adult (2 samples, one male (specimen 281), one female (specimen 288)) or 1.5 mg neonate (2 samples, one male (specimen 280), one female (specimen 249)) were dissolved in 300 μL of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile, and insoluble material was removed by centrifugation in an Eppendorf centrifuge at 13,000 *g* for 10 min at room temperature. 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 with 0.1% TFA (solution B). Elution was achieved as follows: isocratic at 5% solution B for 5 min, followed by 5–25% B for 10 min, 25–45% B for 60 min, and 45–70% for 10 min. Protein detection was carried out at 215 nm and peaks were collected manually and dried using a Speed-Vac (Savant) for subsequent characterization. These four venom samples were considered the primary samples.

2.3. Characterization of RP-HPLC fractions

Fractions obtained from RP-HPLC (primary samples) were further separated by SDS-PAGE under reduced and non-reduced conditions, using 15% gradient polyacrylamide gels. Chromatographic fractions containing peptides (m/z ≤ 1700) were loaded in a nanospray capillary column and subjected to peptide sequencing using a QTrap™ 2000 mass spectrometer (Applied Biosystems) equipped with a nanospray source (Protana, Denmark). Doubly- or triply-charged ions were selected for collision-induced dissociation (CID) MS/MS analysis. Production spectra were interpreted manually or using the on-line form of the MASCOT program at http://www.matrixscience.com against a private database containing viperid protein sequences deposited in the SwissProt/TrEMBL database plus the protein sequences translated from the species-specific venom gland transcriptome. MS/MS mass tolerance was set to ± 0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were fixed and variable modifications, respectively. Spectra producing positive hits were manually inspected. Good quality spectra that did not match any known protein sequence were interpreted manually to derive *de novo* amino acid sequences. Amino acid sequence similarity searches were performed against the available databanks using the BLAST program [39] implemented in the WU-BLAST2 search engine at http://www.bork.embl-heidelberg.de.

Protein bands of interest were excised from a Coomassie Brilliant Blue-stained SDS-PAGE gel and subjected to in-gel reduction (10 mM dithiothreitol) and alkylation (50 mM iodacetamide), followed by overnight sequencing-grade trypsin digestion (66 ng/μl in 25 mM ammonium bicarbonate, 10% acetonitrile; 0.25 μg/sample) in an automated processor (using a Genomics Solution ProGest Protein Digestion Workstation) following the manufacturer's instructions. Tryptic digests were dried in a vacuum centrifuge (SPD SpeedVac®, ThermoSavant), redissolved in 15 μL of 5% acetonitrile containing 0.1% formic acid, and submitted to LC-MS/MS [40,41]. To this end, tryptic peptides were separated by nano-Acquity UltraPerformance LC® (UPLC®) using a BEH130 C_{18} (100 μm × 100 mm, 1.7μm particle size) column in-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. The flow rate was set to 0.6 μl/min and column was developed with a linear gradient of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B) at 1% B for 1 min, followed by 1–12% B for 1 min, 12–40% B for 15 min, 40–85% B for 2 min. Doubly and triply charged ions were selected for CID MS/MS. Fragmentation spectra were interpreted i) manually (*de novo* sequencing), ii) using the on-line form of the MASCOT program at http://www.matrixscience.com against the NCBI non-redundant database, and iii) using Waters Corporation's ProteinLynx Global SERVER 2013 version 2.5.2. (with Expression version 2.0) against the species-specific venom gland cDNA-derived toxin sequences. MS/MS mass tolerance was set to ± 0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were selected as fixed and variable modifications, respectively.

The relative abundances (expressed as percentage of the total venom proteins) of the different protein families were calculated as the ratio 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 [40,41]. When more than one protein band was present in a reverse-phase fraction, their proportions were estimated by densitometry of Coomassie-stained SDS-polyacrylamide gels using ImageJ version 1.47 (http://rsbweb.nih.gov/ij). Conversely, the relative abundances of different proteins contained in the same SDS-PAGE band were estimated based on the relative ion intensities of the three more abundant peptide ions associated with each protein by MS/MS analysis. Finally, protein family abundances were estimated as the percentages of the total venom proteome.

To evaluate population-level variation in venom composition, and to confirm that trends observed in the primary samples were representative of the population, 34 additional samples (secondary samples) were subjected to RP-HPLC fractionation as above, using a Waters 2485 HPLC system, Empower software and a Phenomenex Jupiter C_{18} (4.0 × 250 mm, 5μm) column. Characterization of these samples was based on the detailed characterizations of the primary samples, and peak identifications were determined by comparison of elution times and visual inspections of chromatograms with the primary samples. These samples consisted of 10 adult, 12 subadult and 12 neonate venom samples for each sex, collected from the same population as the four primary samples. Data from these 34 secondary samples were used to determine protein family abundances as a percent of total

venom proteins, with a particular emphasis on two of the most abundant protein families (SVMPs, peptide myotoxins). Combined samples for each age class were also subjected to RP-HPLC fractionation as above to obtain a population average. One hundred fifty μg from each of 12 individuals (per age class) were combined, fractionated on RP-HPLC and compared to primary samples.

2.4. Antivenomics

A second-generation antivenomics approach [42] was utilized to examine the paraspecific immunoreactivity of commercially available CroFab® against both neonate and adult *C. v. viridis* venom (primary samples). For preparation of the antivenom affinity column, 500 μL of NHS-activated Sepharose 4 Fast Flow (GE Healthcare) matrix was packed in a Pierce centrifuge column and washed extensively with 10 matrix volumes of cold 1 mM HCl followed by two matrix volumes of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) to adjust the pH of the column to 7.0–8.0. Sixty milligrams of CroFab® was then dissolved in 250 μL coupling buffer and incubated with matrix for 4 h at room temperature. The amount of antivenom coupled to the matrix was estimated by measuring the amount of non-bound antivenom by quantitative SDS-PAGE band densitometry (MetaMorph software, MDS Analytical Technologies) of CroFab®, which consists almost entirely of fragment antigen binding antibodies (Fab); the amount remaining in the coupling buffer was subtracted from the starting amount (60 mg), providing an estimate of approximately 16.4 mg (27%) of CroFab® antivenom bound to column matrix. The non-reacted groups were then blocked with 500 μL of 0.1 M Tris-HCl, pH 8.5 at room temperature for 4 h. The column was alternately washed with three 500 μL volumes of 0.1 M acetate containing 0.5 M NaCl, pH 4.0–5.0, and three 500 μL volumes of 0.1 M Tris-HCl, pH 8.5; this was repeated 6 times. The column was then equilibrated with 5 volumes of working buffer solution (20 mM phosphate buffer, 135 mM NaCl, pH 7.4; PBS). For the immunoaffinity assay, 300 μg of neonate (male) or adult (male) *C. v. viridis* venom were dissolved in ½ matrix volumes of PBS and incubated with the affinity matrix for 1 h at room temperature using an orbital shaker. As specificity controls, 500 μL of Sepharose 4 Fast Flow matrix, without or with 16 mg of immobilized control IgGs purified from non-immunized horse serum, were incubated with venom and the columns developed in parallel to the immunoaffinity experiment. Following elution of the non-retained fractions with 500 μL of PBS, the column was washed with 2.5 volumes of PBS, and the immunocaptured proteins were eluted with 5 volumes of elution buffer (0.1 M glycine-HCl, pH 2.0) and neutralized with 500 μL 1 M Tris-HCl, pH 9.0. The non-retained and the immunocaptured venom fractions were fractionated by reverse-phase HPLC using a Discovery® BIO Wide Pore C_{18} (15 cm x 2.1 mm, 3 μm particle size, 300 Å pore size) column and an Agilent LC 1100 High Pressure Gradient System equipped with a DAD detector. The flow rate was set to 0.4 mL/min and the column was developed with a linear gradient of 0.1% TFA in water (solution A) and 0.1% TFA in acetonitrile (solution B): isocratic at 5% solution B for 1 min, followed by 5–25% solution B for 5 min, 25–45% solution B for 35 min, and 45–70% solution B for 5 min. Protein detection was carried out at 215 nm with a reference wavelength of 400 nm.

2.5. Western blot analysis

Venoms (16 μg/lane) were from the four specimens of *C. v. viridis* characterized here, plus venom from one *C. o. helleri* and one *C. s. scutulatus* (both from Los Angeles County, CA, USA), and purified myotoxin a (from this source population of *C.v. viridis* in Colorado; 3 μg/lane); each sample was subjected to Western blot analysis following reducing SDS-PAGE on 12% acrylamide NuPAGE® Bis-Tris precast gels. Proteins were blotted to nitrocellulose (150 mA for 1.5 hr), and the membrane was rinsed in Millipore-filtered water (18.2 MΩ · cm MilliQ™ H_2O) and then blocked in PBS-buffered 3% BSA (Sigma Fraction V) for 1hr at room temperature (RT). The membrane was cut so that one-half of the myotoxin a lane was retained on each part of the membrane. Membranes were rinsed three times in PBS and then incubated with 15 mL primary antibody (CroFab® - 1.0 mg/mL 3% BSA in PBS; or specific anti-myotoxin a antibodies raised in rabbits, 5 μL in 15 mL 3% BSA in PBS) overnight at RT with constant gentle shaking. The membranes were rinsed three times with Tris buffered saline (TBS, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4) and then secondary antibody (5 μL donkey anti-sheep IgG conjugated with alkaline phosphatase for CroFab®; 5 μL goat anti-rabbit IgG conjugated with alkaline phosphatase for anti-myotoxin a) in 15 mL TBS was incubated with the appropriate membrane for 60 min at RT with gentle shaking. Membranes were then washed four times with TBS and alkaline phosphatase substrate (SIGMAFAST™ BCIP®/NBT) in 10 mL of Millipore-filtered water (18.2 MΩ · cm MilliQ™ H_2O) was added. The color reaction was stopped with 20 mM disodium EDTA in PBS after ~5 min. Membranes were washed in MilliQ™ H_2O, dried and photographed. The same venoms (16 μg/lane) and myotoxin a (1, 3 and 5 μg/lane) were also run on a second 12% acrylamide NuPage gel under reducing conditions. This gel was stained with 0.1% Coomassie Brilliant Blue, destained and photographed. The 34 secondary samples were also subjected to electrophoresis using 12% acrylamide NuPage gel under reducing conditions.

2.6. SVMP activity assay

SVMP activity of crude neonate (n = 12), subadult (n = 12), and adult (n = 12) *C. v. viridis* venoms was measured colorimetrically using azocasein as a substrate. Briefly, 2.5 μL of crude *C. v. viridis* venom (4 μg/μL), or 2.5 μL MilliQ H_20 as a control, was added to 247.5 μL of azocasein (2 mg/ml) resuspended in assay buffer (50 mM HEPES, 100 mM NaCl, pH 8.0). The reaction mixture was then incubated at 37 °C for 30 min. The assay was terminated by the addition of 125 μL of 0.5 M trichloroacetic acid, vortexed at room temperature, and centrifuged at 2000 x g for 5 min. Following centrifugation, 100 μL of supernatant was mixed with 100 μL of 0.5 M NaOH and the absorbance was determined at 450 nm using a SpectraMax 190 plate reader. Assays for each sample were performed in triplicate, and activity was reported as ΔA_{450nm}/min/mg protein.

2.7. Statistical analysis

The percent abundance of myotoxin a and SVMP from all RP-HPLC runs was analyzed by Analysis of Variance (ANOVA) followed by Tukey's post-hoc test using R version 2.15.2. Similarly, SVMP activity was also analyzed by ANOVA and Tukey's post-hoc comparison. Comparisons between age classes and between sexes were also analyzed by ANOVA and Tukey's post-hoc comparison and two-tailed t-test. All p values <0.05 were considered as statistically significant.

3. Results and discussion

3.1. The venom proteome of C. v. viridis

In the current study, venoms of both male and female neonate and adult *C. v. viridis*, obtained from snakes from approximately the center of the species' distribution (Fig. 1), were characterized by venomics analysis. These four (primary) venom samples (Fig. 2), as well as the 34 additional (secondary) samples (Fig. 3), exhibited similar chromatographic profiles and toxin family composition (Table 1), but there is apparent variation in concentrations of specific toxins (Supplementary Table 1) and protein families (Table 1). Venoms from all *C. v. viridis* examined shared compounds from 10 protein classes (Table 1; Fig. 3), which are typically abundant in rattlesnake venoms [7]. In addition, some molecules were detected in only a subset of venoms, including an ohanin-like toxin [~*L. muta* Q27J48], PI-SVMP [~*C. atrox* Q90392], phospholipase B [~*C. adamanteus* F8S101], an acidic PLA_2 [P0DJM5], and the tripeptide inhibitors of SVMPs, ZNW and ZQW (Table 1) [43–46]. Both endogenous inhibitors were primarily detected in neonate venoms (peaks 39* and 40* in panels C and D of Fig. 2). Only ZQW was observed in adult female venom (peak 4, Fig. 2B), whereas tripeptide inhibitors were not seen in adult male venom (Table 1). Consistent with previous reports [44], the concentration of endogenous inhibitors correlates with the abundance of SVMPs in the venoms, as overall SVMPs (PI, PII, and PIII classes) were detected in higher percentages in both neonate venoms when compared to adult venoms (Table 1). This observation supports the view that the relatively low affinity endogenous tripeptides

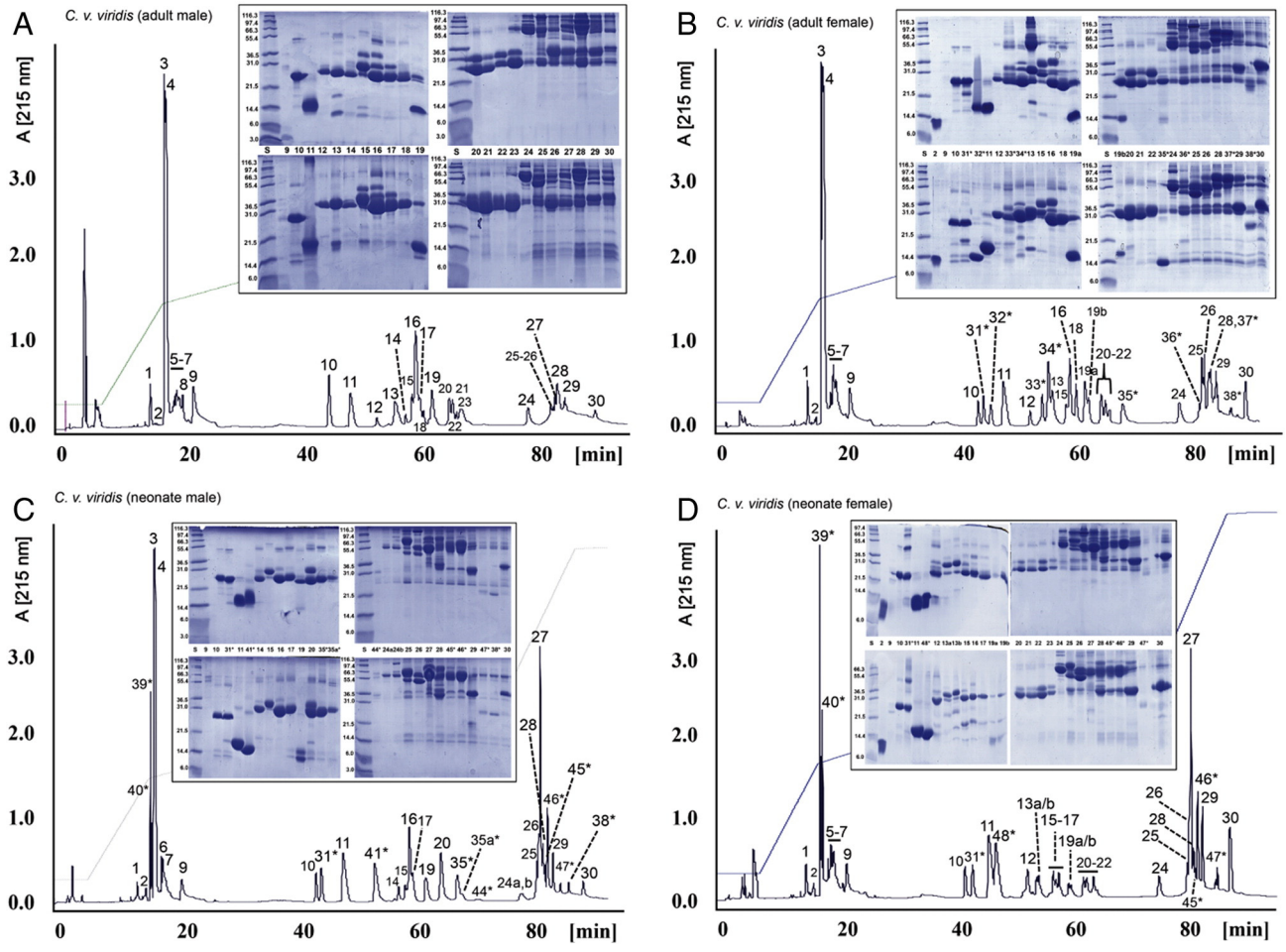

Fig. 2 – Characterization of the venom proteomes of *C. v. viridis*. Panels A-D display reverse-phase HPLC separations of the venom proteins from an adult male, adult female, neonate male and neonate female snake, respectively. Fractions were collected manually and analyzed by SDS-PAGE (insets) under non-reduced (top gel panel) and β-mercaptoethanol-reduced (bottom gel panel) conditions.

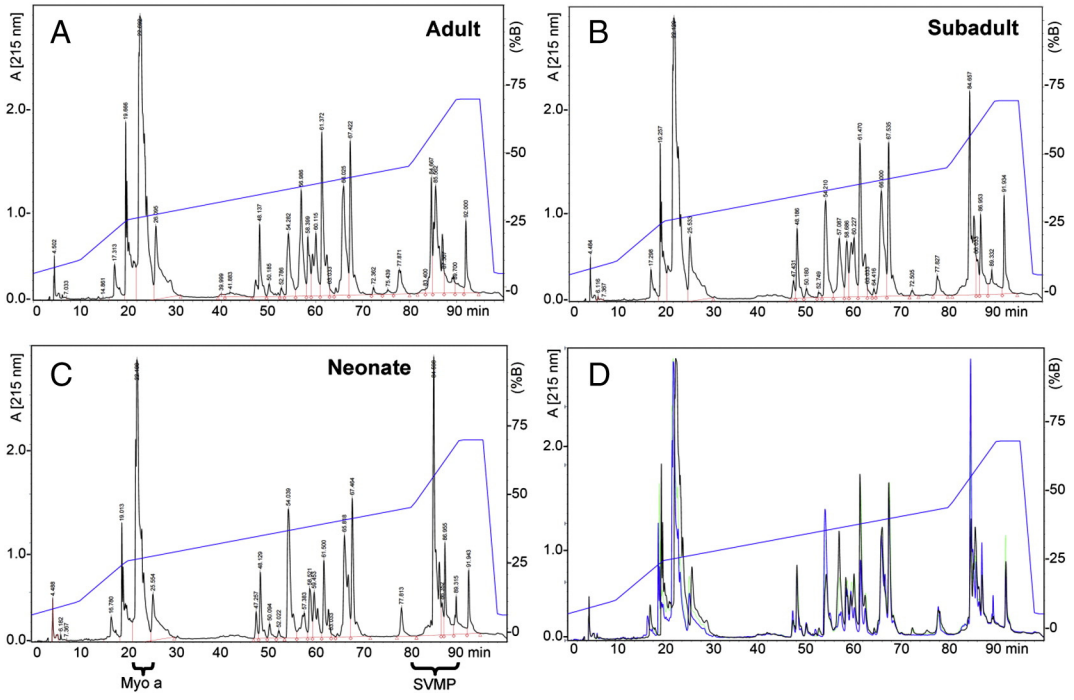

Fig. 3 – Combined samples representing three age classes of *C. v. viridis*. These chromatograms essentially represent a graphical average of 12 individual venoms for each age class. A. Adult venoms. B. Subadult venoms. C. Neonate venoms. D. Overlay of chromatograms A–C; adult – black line; subadult – green; neonate – blue. Note that significant differences exist between adults and neonates, in particular the myotoxin a (myo a) and metalloproteinase (SVMP) peaks.

(Ki = 0.20–0.95 mM) [43] keep SVMPs functionally silent in the venom gland, and disengagement of this control occurs spontaneously at the time of the snakebite.

The major toxins present in both adult and the neonate male venoms were peptide myotoxins (Table 1). There were no statistically significant differences in myotoxin a or SVMP content, or SVMP activity of crude venom, with regards to sex of the snake (all p's > 0.05). However, there was a significant age-related change in myotoxin a content of the venoms, and neonate venoms contain significantly less myotoxin a than adult venoms (Fig. 4; p = 0.05). Further, there was no significant difference between neonate and subadult (p = 0.74) or subadult and adult (p = 0.23) myotoxin a concentration. Both myotoxin a [P01476] and myotoxin 2 [P63175] were detected in adult male *C. v. viridis* venom, whereas only myotoxin a was found in adult female and neonate venoms. Small basic myotoxins represent a Nearctic and Neotropical crotaline innovation of a protein fold acting on the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum [47] and voltage-sensitive Na^{+} channels [12,48–51]. These myonecrotic toxins primarily serve two biological roles: to limit the flight of prey by causing tetanic paralysis of the hind limbs, and to promote death by paralysis of the diaphragm [52,53].

SVMPs are present in the venoms of all families of venomous snakes, and analysis of this activity in all samples of *C. v. viridis* venom showed a significant age-related decrease (Fig. 4A and B). For overall SVMP abundance, ANOVA showed significant differences when comparing neonate to subadult (p = 0.02) and neonate to adult venoms (p = 0.002), yet comparison of subadult to adult venoms was not statistically significant (p = 0.69). SVMP activity assays further support these results with both subadult and adults venoms showing significantly less activity when compared to neonate *C. v. viridis* venoms (both p's < 0.001). There was no difference in SVMP activity between subadult and adult venoms (p = 0.61). Tryptic peptides recovered after in-gel digestion yielded ions matching the highly hemorrhagic PIII atrolysin-A [Q92043], first characterized from the venom of *C. atrox* [54], in the venoms of all four *C. v. viridis* examined here. Adult and neonate male venoms also yielded peptides matching an additional PIII-SVMP [Q9DGB9] from *C. atrox*, and one other PIII-SVMP in the 36 kDa range [C9E1S0] was detected in the venom of the neonate male (supplemental Table 1). Peptides of PI-SVMPs, which are less hemorrhagic than the higher molecular weight PIII-SVMPs [55], were only detected in the adult male and neonate male venoms (Table 1). However, analyses of peak 9 from all four individuals yielded a 3 kDa protein band (see Fig. 2 panel A, protein band 9) that was subjected to tryptic peptide mass fingerprinting, producing the ion YIELVVVADHR that matches a *C. atrox* PI-SVMP [Q90392]. The early HPLC elution of this peptide compared to the other SVMPs, in addition to the low molecular mass of the protein band, suggests possible degradation of these PI-SVMP enzymes, which exhibit an intact mass of 20-24 kDa.

Disintegrins are platelet aggregation inhibitors commonly found in viperid venoms as the result of the post-translational proteolytic processing of PII-SVMPs [56]. In *Crotalus*, these non-enzymatic toxins have been shown to range from 0.1% of the venom proteome of *C. tigris* [57] to over 6% of the total venom

Table 1 – Relative occurrence of the different protein families present in the different primary venoms of *C. v. viridis* sampled. –, not detected; M ± SD, mean ± standard deviation.

	Adult			Neonate		
	Male	Female	M (±SD)	Male	Female	M (±SD)
Protein family	% of total venom proteins					
BPP	8.2	6.5	7.4 (0.8)	6.4	11.2	8.8 (2.4)
Disintegrin	0.1	0.1	0.1 (0.0)	0.8	0.7	0.7 (0.1)
CRISP	3.9	2.1	3.0 (0.9)	4.0	4.8	4.4 (0.4)
C-type lectin	1.8	3.3	2.6 (0.7)	7.3	1.9	4.6 (2.7)
PLA_2	7.7	10.6	9.2 (1.4)	10.9	16.3	13.6 (2.7)
• D49 PLA_2	7.7	10.2	9.0 (1.3)	10.9	16.3	13.6 (2.7)
• Acidic PLA_2	–	0.4	0.2 (0.2)	–	–	–
Ohanin-like Toxin	0.5	0.6	0.5 (0.1)	–	0.2	0.1 (0.1)
Myotoxin	38.1	35.6	36.9 (1.2)	25.2	5.7	15.5 (9.7)
• Myotoxin a	37.5	35.6	36.6 (1.0)	25.2	5.7	15.5 (9.7)
• Myotoxin 2	0.6	–	0.3 (0.3)	–	–	–
Serine Proteinase	26.8	26.9	26.8 (0.1)	18.2	20.6	19.4 (1.2)
LAAO	1.9	2.5	2.2 (0.3)	7.6	11.9	9.8 (2.1)
SVMP	11.0	11.4	11.2 (0.2)	14.2	18.0	16.1 (1.9)
• PIII SVMP	3.1	4.9	4.0 (0.9)	8.4	8.8	8.6 (0.2)
• PII SVMP	0.9	3.7	2.3 (1.4)	1.7	6.9	4.3 (2.6)
• PI SVMP	0.2	–	0.1 (0.1)	0.8	–	0.4 (0.4)
• PI SVMP fragments	6.6	2.9	4.8 (1.9)	3.4	2.3	2.9 (0.6)
Glutaminyl cyclase	0.1	0.1	0.1 (0.0)	0.8	0.1	0.5 (0.4)
Phospholipase B	–	0.1	0.1 (0.1)	0.3	0.1	0.2 (0.1)
SVMP Inhibitor	–	< 0.10	0.1 (0.1)	4.5	8.5	6.5 (2.0)
• ZNW	–	–	–	3.0	5.7	4.4 (0.3)
• ZQW	–	< 0.10	0.1 (0.1)	1.5	2.8	2.2 (0.6)

proteome in *C. atrox* [58]. Stage-dependent down-regulation of the precursor metalloproteinases in *C. viridis* may account for the lower abundance of disintegrins in adult compared with neonate venoms.

C-type lectin-like molecules (CTLs), also known as snaclecs (snake venom C-type lectins), are also present in *C. v. viridis* venoms (Table 1). Snaclecs have been reported to bind in a Ca^{2+}-independent manner and via protein-protein interactions with coagulation factors IX/X, X and II, impairing their physiological roles in hemostasis. Snaclecs also reduce platelet function by inhibiting surface receptors such as the von Willebrand receptor, GPIb, and the collagen receptor, integrin $\alpha_2\beta_1$, or by activating platelets via clustering of the collagen receptor GPVI so that they are removed from the circulation, producing thrombocytopenia [59]. Whether this class of toxins participates in age- and gender-dependent prey-securing strategies, and how they participate, deserves further investigation.

Phospholipase A_2 (PLA_2) enzymes are one of the most heavily-studied venom toxin families to date [60] and contribute to local tissue damage due to myonecrosis, edema, and inflammation. However, a single venom may contain numerous PLA_2 isoforms, and each may exhibit varying biological effects. In this respect, protein masses, in addition to tryptic peptides (Supplementary Table S1), indicate the presence of multiple PLA_2 isoenzymes in all four venoms examined. Thus, tryptic peptides matching that of the D49-PLA_2 [Q9I8F8] were found in adult male venom (Fig. 2A, peak 13); D49-PLA_2 [Q800C3] was found in venoms belonging to both adult and neonate male snakes (Fig. 2A and C, peaks labeled 11). Peptides representing another D49-PLA_2 [Q800C4] were seen in the adult male and female venom samples (Fig. 2A and B, peaks 19 and 19a/b, respectively), and ions for D49-PLA_2 [Q71QE8] and acidic PLA_2 [P0DJM5] were present in the adult female venom (Fig. 2B, peak 32*).

Cysteine-rich secretory proteins (CRISPs), which comprise 1.8 to 7.3% of the venom proteome of adult and neonate *C. v. viridis* (Table 1), represent another widely distributed protein family in snake venoms [61,62]. Reported activities of some CRISPs include inhibition of smooth muscle contraction and cyclic nucleotide-gated ion channels; however, their role in envenomation and prey capture has not been established.

L-amino acid oxidases are flavoenzymes that catalyze oxidative deamination of L-amino acids to form corresponding α-keto acids, hydrogen peroxide and ammonia. Due to their wide distribution in snake venom, LAAOs are thought to contribute to the toxicity of the venom due to the production of hydrogen peroxide during the oxidation reaction. In addition, LAAOs have been reported to induce platelet aggregation in platelet-rich plasma [63,64], although the overall functional contribution to the envenoming process remains elusive.

Several somewhat unusual venom constituents, including glutaminyl cyclase (GC) and phospholipase B, were found within the venoms of *C. v. viridis* and deserve further discussion. GCs may contribute indirectly to overall venom toxicity by catalyzing the N-terminal formation of pyroglutamate characteristic of several snake venom toxin families [65,66] and thereby stabilizing them to endogenous scavenging mechanisms. These

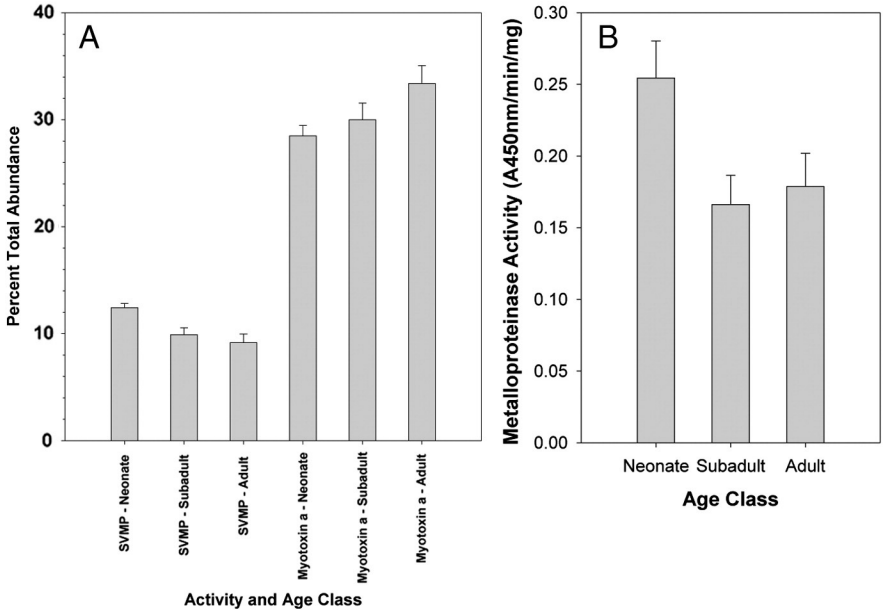

Fig. 4 – Age-related changes in snake venom metalloproteinase (SVMP) and myotoxin a abundance in *C. v. viridis* venoms. A. SVMP and myotoxin a content of all 38 venoms analyzed (12 adult and subadult, 14 neonate) by RP-HPLC. Adult and neonate venoms differ in SVMP ($p = 0.002$) and myotoxin a ($p = 0.05$) content; SVMP content of subadult venoms also significantly differed when compared to neonate venoms ($p = 0.02$), however there was no difference between subadult and adult venoms for myotoxin a or SVMP content (p's = 0.23 and 0.69, respectively). B. SVMP activity toward azocasein substrate. Consistent with the RP-HPLC-based content differences, neonate venom activity levels also differ statistically when compared to subadult and adult venoms ($p < 0.001$). SVMP activity was not significantly different between subadult and adult venoms ($p = 0.61$).

cyclases have also been documented in the proteomes of *C. atrox* [58,67] and *C. d. terrificus* [68], as well as in the transcriptomes of *C. adamanteus* [69], *B. jararaca* [70] and the colubrids *Boiga dendrophila* and *B. irregularis* [65]. Snake venom gland GC is also likely involved in the biosynthesis of pyroglutamyl peptides such as bradykinin-potentiating peptides (BPPs) [71,72] that contribute to symptoms of hypotension experienced by snakebite victims [73], and of endogenous inhibitors of metalloproteinases, ZQW and ZNW, discussed above [44,45]. Although GCs are found in low concentrations in snake venoms, the enzyme may play a significant role in post-translational modifications of functionally important and abundant venom proteins. Thus, mature PIII-SVMPs and other venom proteins, eg. svVEGF (http://www.ncbi.nlm.nih.gov/protein/?term=svVEGF) and colubrid three-finger toxins [19], usually contain an N-terminal pyroglutaminyl residue, indicating that the action of glutaminyl cyclase is downstream of the proteolytic processing of the pre-pro-precursors.

Reverse-phase peak 28 of venom samples from the adult female and both neonate *C. v. viridis* (Fig. 2B-D) yielded numerous ions matching a phospholipase B (PLB) from *C. adamanteus* (F8S101, J3S4V6; supplemental Table 1). The occurrence of PLB in snake venoms was initially reported by Doery and Pearson [74] and was characterized as being responsible for the high direct hemolytic activity of several Australian elapid venoms [75–77]. PLB molecules have been identified in the venom proteome of the *C. adamanteus* [78], *B. atrox*, *B. jararacussu*, *B. jararaca*, *B. neuwiedi*, *B. alternatus*, and *B. cotiara* [79], and *Porthidium lansbergii* [80]. The functional relevance of this class of proteins in envenomation, represents another intriguing topic that requires future detailed study.

3.2. *C. v. viridis exhibits a novel pattern of ontogenetic venom proteome changes*

The ontogenetic compositional shift in *C. v. viridis* venom is characterized by a stage-dependent decrease of the relative content of SVMPs, disintegrins, catalytically active D49-PLA_2s, and L-amino acid oxidase, and the concomitant increase in the relative abundance of small basic myotoxins, serine proteinases and an ohanin-like toxin (Table 1; Figs. 3-5). We focused on SVMPs and myotoxin a levels as these ontogenetic venom shifts may represent an age-dependent "strategy" for effectively securing prey, because the snake prey regime switches with age from newborn rodents and small ectothermic prey to larger endothermic prey.

PIII-SVMPs are often highly hemorrhagic, promoting prey immobilization and tissue necrosis by degradation of the basement membrane surrounding capillary vessels [81]. SVMPs occur in venoms of all families of advanced snakes, suggesting the recruitment and modification of an ADAM (A disintegrin and metalloproteinase)-like gene early in the evolutionary history of venomous snakes [82,83]. Although these enzymes are generally highly expressed in venoms within the Viperidae [84,85], the venom of the Black-speckled Palm Pitviper, *Bothriechis nigroviridis*, a neotropical arboreal pit viper from Costa Rica, does not possess detectable

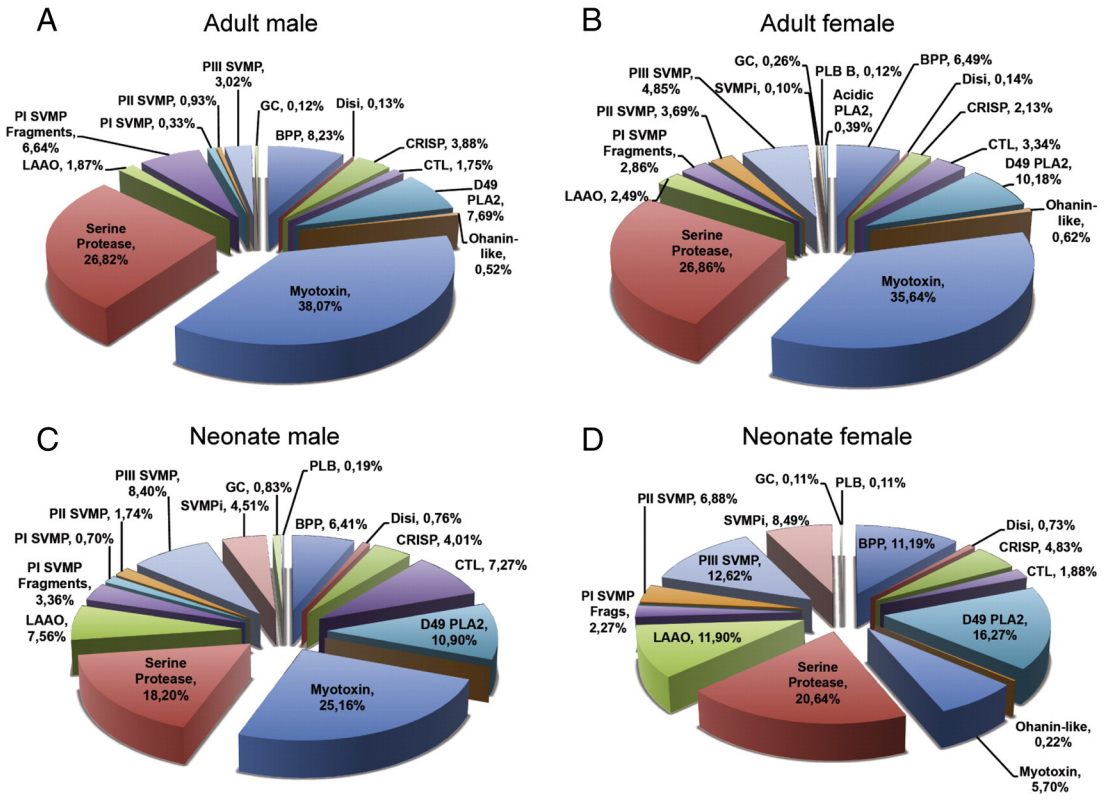

Fig. 5 – Protein family composition of primary *C. v. viridis* venoms (adult male 281; adult female 288; neonate male 280; and neonate female 249). Pie charts represent the relative occurrence of proteins from the different toxin families as identified in the current work. Percentages below protein families represent the percent of the total RP-HPLC-separated components found in *C. v. viridis* venom. BPP, bradykinin-potentiating peptide; Disi, disintegrin; CRISP, cysteine-rich secretory proteins; CTL, C-type lectin-like; PLA_2, phospholipase A2; LAAO, L-amino acid oxidase; SVMP, snake venom metalloproteinase; GC, glutaminyl cyclase; PLB, phospholipase B.

Zn^{2+}-dependent metalloproteinases and is unique among *Bothriechis* species by possessing a high content of neurotoxic PLA_2 and vasoactive peptides [86]. These data suggest that distinct evolutionary solutions have evolved within the arboreal genus *Bothriechis* for the same trophic purpose, and it underscores the versatility of viperid venoms as adaptive traits. The evolutionary justification for the ontogenetic decrease of PIII-SVMP hemorrhagins in *C. v. viridis* is elusive, although it is tempting to hypothesize that their biological role has been successfully replaced by the paralytic action of small basic myotoxins, the locomotion-disrupting and hyperalgesia-inducing ohanin-like protein [87], and the hemostasis-disrupting serine proteinases [88]. These latter enzymes comprise the second most abundant venom protein family in both adult male (26.82%) and female (26.86%) *C. v. viridis* (Table 1).

Variation in the biochemical composition of venoms from different geographic locations and with age has long been appreciated by herpetologists and toxinologists [10,89–91]. Stage-specific venom proteins differentially expressed during ontogenetic development have been reported in just a few species, and in each taxa investigated a somewhat different pattern of ontogenetic changes has been described. The ontogenetic shifts reported here for *C. v. viridis* represent a novel pattern of age-related venom compositional transitions among viperid species. For example, in *Bothrops asper*, major ontogenetic changes involve a shift from a PIII-SVMP-rich to a PI-SVMP-rich venom and the secretion in adults of a distinct set of PLA_2 molecules than in the neonates [8]; ontogenetic changes in the toxin composition of *L. stenophrys* venom results in the net shift from a vasoactive (bradykinin-potentiating and C-type natriuretic) peptide (BPP/C-NP)-rich and serine proteinase-rich venom in newborns and 2-year-old juveniles to a (PI > PIII) SVMP-rich venom in adults [92]; age-dependent venom changes in *C. simus* involve a shift from a neurotoxic to a hemorrhagic venom phenotype [29]; conversely, *Sistrurus m. barbouri* showed little evidence for an ontogenetic shift in venom composition [93].

Although the environmental and molecular mechanisms that generate this age-dependent venom diversity remain unclear [94], age-dependent changes in the concentration of venom gland microRNAs have recently been shown to influence the translation of venom proteins from genes transcribed in the venom gland [29]. While the generalization of this finding requires additional study in other species, posttranscriptional modulation of the venom transcriptome could conceivably contribute broadly to differential venom

composition without large-scale alterations of the underlying gene expression machinery.

3.3. Assessment of the immunoreactivity of CroFab®

In the United States, human envenomation due to snakebite is relatively rare, and CroFab® is the antivenom administered universally to treat bites. CroFab® is produced utilizing venoms from four different North American viper species, *A. piscivorus*, *C. adamanteus*, *C. atrox* and *C. scutulatus*. Venomic profiles of all four species used in producing CroFab® have been published (*A. piscivorus*: [95]; *Crotalus adamanteus*: [78]; *C. atrox*: [58]; *C. scutulatus*: [49]), and these species collectively show varying relative concentrations of typical viperid venom protein families. For example, in *C. atrox*, the venom proteome consisted of nearly 50% PI and PIII-SVMPs, with approximately 20% serine proteases and 7% PLA_2s [58]; this species lacked small basic myotoxins, which represent approximately 22% of the venom proteome of *C. adamanteus* [78]. In addition to small basic peptide myotoxins, PLA_2s and SVMPs represent a significant proportion (~59%) of the overall venom composition of *C. adamanteus*. Further, venomics analysis of *A. piscivorus* showed that over 75% of venom proteins consisted of PLA_2 (33.6%), SVMP (33.1%), and serine protease (13.2%) [95]. However, *C. scutulatus* shows significant venom compositional diversity, with several distinct venom phenotypes varying in overall composition and toxicity [49]; venoms containing high amounts of the presynaptic neurotoxin Mojave toxin are typically used in the production of CroFab® (pers. comm., SPM: R. Straight).

Our antivenomic assessment of *C. v. viridis* venoms against CroFab® (Fig. 6) showed that significant amounts of the peptides and proteins in early eluting HPLC fractions (1-8 of adult and neonate venoms, and peaks 39* and 40* of neonate samples) were not immunocaptured by CroFab® affinity chromatography (Fig. 6C and F); several additional downstream protein peaks were also not immunodepleted from neonate venom (Fig. 6F). Our venomic analyses indicate that these non-depleted HPLC fractions consist of bradykinin inhibitory peptides, myotoxins a and 2, and SVMP inhibitors. It has recently been shown that the BPP family of venom proteins from *Lachesis* species were also not immunocaptured by antivenoms developed at Instituto Vital Brazil (IVB) and Instituto Clodomiro Picado (ICP). In spite of this, caudal vein injection of BPP proteins in mice failed to demonstrate toxicity or elicit abnormal behavior [96], suggesting that BPPs, even if not recognized by antivenoms, may not contribute to the often severe pathologies seen in viperid envenomations.

The immunoaffinity antivenomics assessment of CroFab® indicated that it exhibits partial immunoreactivity towards small basic myotoxin a (Fig. 6, panels C and F). However, Western blot analysis shows that CroFab® does recognize myotoxin a in the crude venoms of several species as well as the purified toxin from *C. v. viridis* venom, as does a specific anti-myotoxin a antibody (Fig. 7). Myotoxin a produces rapid tetanic contraction of skeletal muscles in prey [97], leading to rapid immobilization of prey, and the poor immunodepletion by the CroFab® affinity column suggests that this should be problematic during human envenomations. However, the amount of CroFab® utilized was relatively small compared

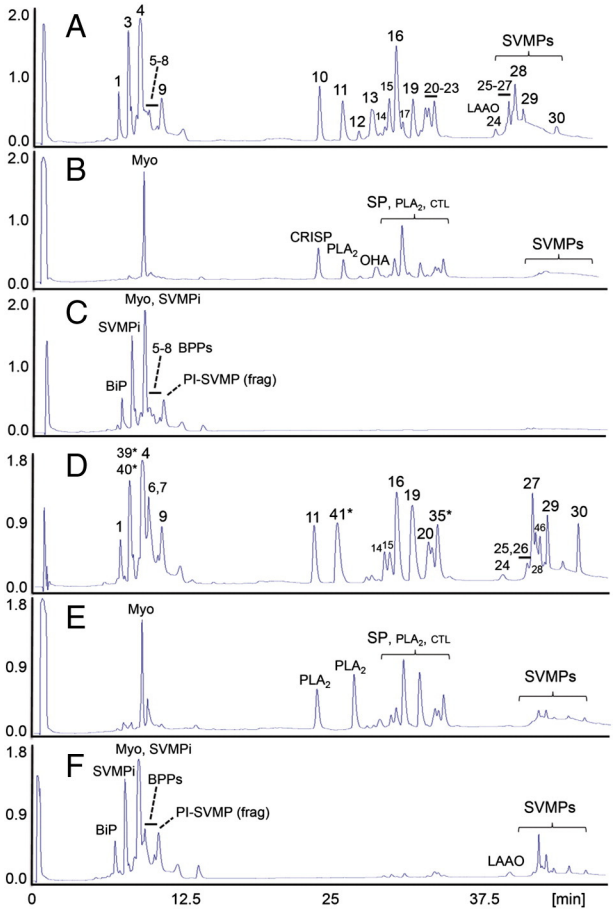

Fig. 6 – Antivenomic analysis on a CroFab® antivenom affinity column. Panels A and D, RP-HPLC separation of the venom proteins of one adult and one neonate male *C. v. viridis*. Panels B and C show, respectively, reverse-phase HPLC separations of the components of adult male *C. v. viridis* recovered in the bound and the flow-through fractions of the affinity column. Panels E and F show the affinity column immunocaptured and non-retained protein fractions of neonate *C. v. viridis* venom, respectively. Protein peaks are labeled as in panels A (adult male) and C (neonate male) of Fig. 2. Supplemental Table S1 lists the proteins found in each chromatographic fraction. BiP, bradykinin inhibitory peptide; OHA, ohanin-like protein. Other acronyms as in the legend of Fig. 3.

to human dosages, and so if anti-myotoxin a antibodies represent only a small percentage of CroFab® antibodies, this deficit may be compensated by high clinical dosages. Further, case log data from the American Association of Poison Centers for rattlesnake bites in Colorado (*C. v. viridis* is the most probable source of bites) over four years (2010-2013) indicated no fatalities (0/175 cases); unfortunately, long-term data for snakebites is generally lacking from all health databases, so chronic effects cannot be evaluated. These data suggest that in spite of minimal immunodepletion, CroFab® did provide sufficient protection for patients. Although quantitative estimates of anti-myotoxin a antibodies

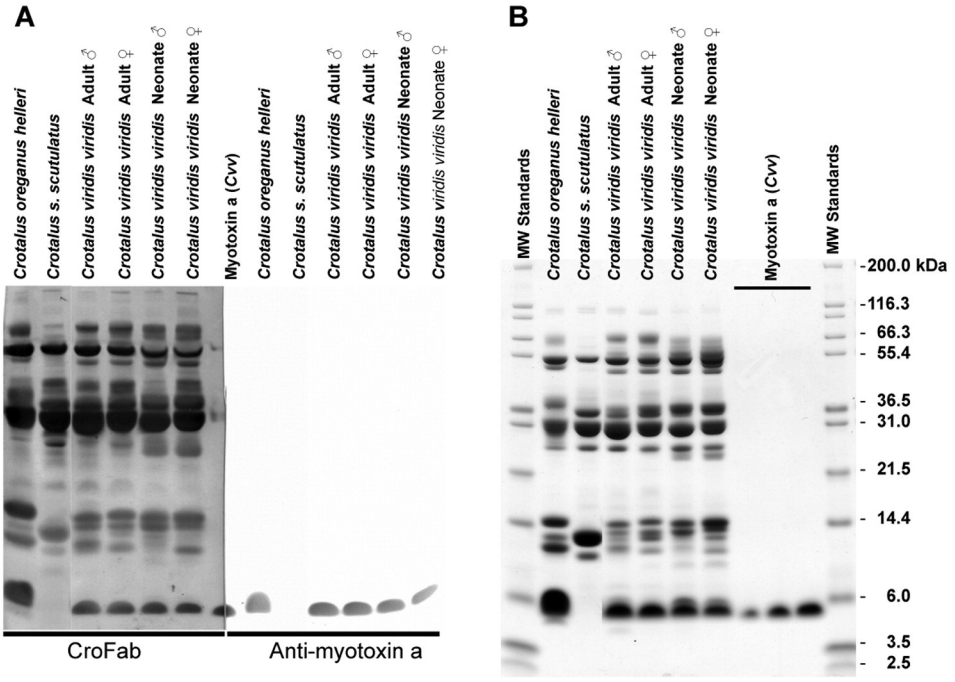

Fig. 7 – Western blot and SDS-PAGE analysis of venom and purified myotoxin a (*C. v. viridis* venom). Panel A, venoms and myotoxin a on nitrocellulose were detected with either CroFab or specific anti-myotoxin a antibodies (rabbit). Note that myotoxin a is detected by both CroFab® and specific anti-myotoxin a antibodies. Panel B, SDS-PAGE analysis of the same venoms (16 μg/lane) and myotoxin a (1, 3 and 5 μg/lane) as in A. For both panels A and B, *C. o. helleri* and *C. s. scutulatus* venoms were included as myotoxin a-positive and negative controls, respectively.

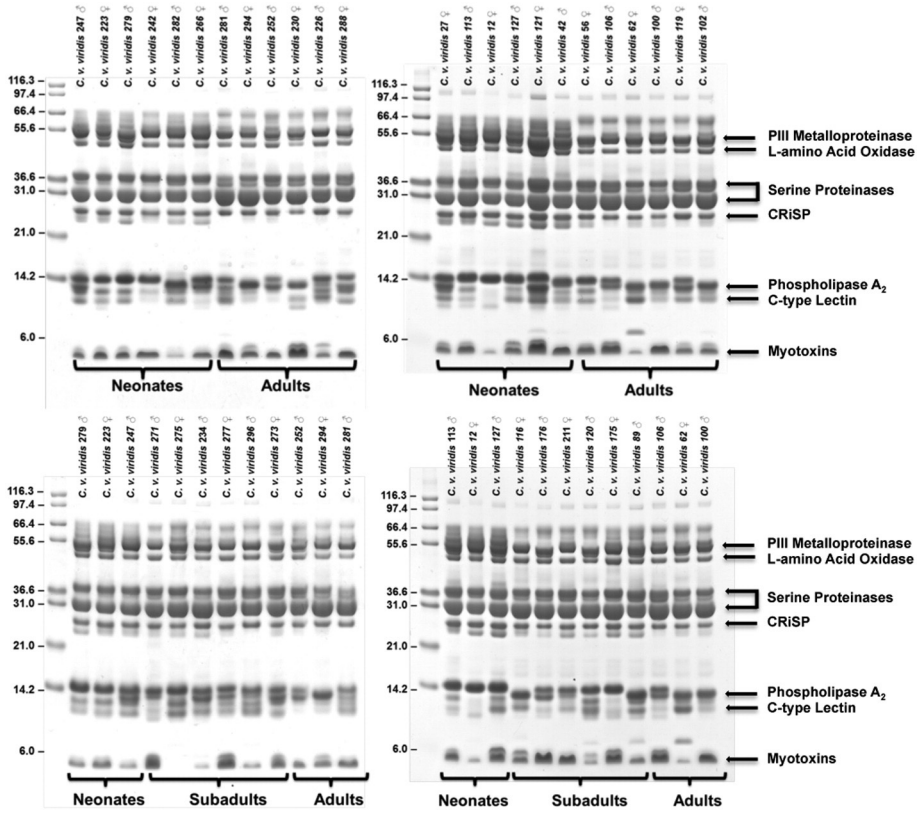

Fig. 8 – Reducing SDS-PAGE of all 34 secondary venom samples – 16 μg/lane. Protein families found in bands of specific masses [1,7] are indicated on the right. Note that although most bands are shared between all individuals, differences in intensities (representing differing concentrations) exist, particularly among P-III metalloproteinases, PLA_2s and myotoxin a bands.

are not yet available for CroFab®, our data show that CroFab® does contain significant amounts of antibody which recognize myotoxin a, whereas the antivenom previously used in the United States (Wyeth polyvalent Crotalidae) was shown to contain very low titers to myotoxin a [98]. The low recovery of SVMPs in the immunocaptured and the non-bound fractions of both adult and neonate venoms contrasts with the clear immunoreactivity towards these components exhibited by CroFab® in Western blot analysis. This indicates that the high binding affinity of the antivenom for SVMPs likely prevents their elution from the column.

The antivenomic analysis also indicated that CroFab® effectively recognizes and depletes other potent and abundant venom components, including PLA_2s, serine proteases, LAAOs and SVMPs, indicating that the similarity in venom protein family representation in *C. v. viridis* venom and venoms of the four species utilized in CroFab® production is reflected in the immunoreactivity of this antivenom. While comparing the levels of immune recognition gathered from antivenomics with the *in vivo* neutralization capacity of an antivenom is not straightforward, since both experiments involve radically different protocols, in our experience, even a moderate immunocapturing capability of ~20%–25% correlates with a satisfactory outcome in the *in vivo* neutralization tests [99]. Consistent with these observations, CroFab® shows high efficacy in treatment of human and domestic animal envenomations by *C. v. viridis*, including snakes from Colorado [100,101], so even partial binding/recognition of myotoxin a by Fabs appears sufficient to ameliorate symptoms effectively.

4. Concluding remarks

In this study we conducted venomic and antivenomic analyses of *C. v. viridis* (Prairie Rattlesnake), one of the most widely distributed rattlesnake species in North America. The previously reported LD_{50} of 1.55 μg/g (inbred mice) for *C. v. viridis*, coupled with the SVMP concentrations detected here, confirms *C. v. viridis* as possessing type I venom as described previously [7]. Ontogenetic variation in prey preference has been reported in *C. viridis* [10,27] and changes in diet are correlated with ontogenetic changes in venom composition in Pacific Rattlesnakes [10]. These age-related changes in venom composition may facilitate prey handling and possibly digestion [10,11]. Although a common ontogenetic trend documented in rattlesnake venoms is a shift from a type II venom composition (high toxicity, low SVMP activity) in neonates to a type I venom in adults (lower toxicity, high SVMP activity), our results clearly indicate the opposite relationship for *C. v. viridis*, with overall SVMP concentrations being lower in venoms from adult snakes, and myotoxin (a and 2) concentrations being higher in adult samples. Further, classic venom paedomorphism [11,12,30] does not occur in this population, as venoms analyzed here do show age-related functional (Fig. 4) and compositional (Figs. 3 and 8) changes. It should be noted, however, that total SVMP activity of venoms from this population of *C. v. viridis* are not particularly high when compared with several type I venoms [7,10].

Our antivenomics results show that CroFab®, developed against venom of three *Crotalus* and one *Agkistrodon* species, efficiently immunodepleted many of the major compounds present in *C. v. viridis* venom. Our antivenomics results show that CroFab®, developed against venom of three *Crotalus* and one *Agkistrodon* species, efficiently immunodepleted many of the major compounds present in *C. v. viridis* venom. Myotoxin a, abundant in both adult and neonate *C. v. viridis* venoms, did not appear to be efficiently immunocaptured during the antivenomics experiment, but Western blot analysis indicated that it is recognized by CroFab® as well as by the specific myotoxin a antibody. Considering the high efficacy of CroFab® in treating *C. v. viridis* snakebites, it appears that the relatively low immunoreactivity of CroFab® to myotoxin a is indeed sufficient for effective treatment of snakebite. The current study defines the venom proteome of a discrete population of *C. v. viridis* from Colorado, but a more detailed population venomics study evaluating venom composition, and antivenom reactivity, of this species throughout its entire range (spanning 22° of latitude) may demonstrate distinct regional differences in venom protein family distribution, concentration, and immunoreactivity against existing antivenoms.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.03.015.

Transparency Document

The Transparency Document associated with this article can be found, in the online version.

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