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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

treatment, we tested the effi 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 Ž‚TJ /T1223.32ts915venu.(ith)tfithxnand ouatn-olin-nd

r(al)47ǝ5s(eg(r)ag)[22,23]-4tion)-8(s)472([e.i)-5g.i phosphodiesterase enzymes

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

(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 sarcoplasmTc 2.5f7TJ s reticul(

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 cyclases have also been documented in the proteomes of *C. atrox* [58,67] and *C. d*

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*

Fig. 7 – Western blot and SDS-PAGE analysis of venom and purified myotoxin a (*C. v. viridis* venom). Panel A, venoms and

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

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