Article

# Venom Ontogeny in the Mexican Lance-Headed Rattlesnake (Crotalus polystictus)

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distinct differences in SVMP, kallikrein-like, LAAO, and PLA$_2$ activities and disintegrin content. Venomics of pooled adult C. polystictus venom identified many major venom components that are common in rattlesnake venoms, with bradykinin-inhibitory peptide constituting 36% of the venom proteome. Crotalus polystictus shows a pattern of venom ontogeny similar to other rattlesnakes that produce a type I venom.

## 1. Introduction

The rattlesnakes Crotalus and Sistrurus comprise a monophyletic lineage of vipers unique to the Americas, and they are easily recognized by the presence of the caudal rattle. Rattlesnakes

changes in venom composition, sex-related differences in toxin activities, and LC-MS-MS analysis of the venom proteome in this Mexican rattlesnake.


Figure 1. (A) Distribution of Crotalus polystictus (red) in México (after Campbell and Lamar [4]). The approximate location of México City is shown by the black dot. (B) Adult C. polystictus, photo by EMD.

## 2. Results

### 2.1. Enzyme Analyses

All C. polystictusvenoms exhibited activities of six enzymes commonly found in rattlesnake venoms (Figure 2). Metalloproteinases activity showed a statistically significant difference with age (Figure 3; $F_{2,40} = 37.94, p < 0.001$), typical of many rattlesnake venoms [21]; average activity of adult venoms was approximately 6.5 that of neonate venoms ($p < 0.001$). Juvenile and neonate venoms were also significantly different for SVMP activity ($p < 0.05$), whereas a comparison of juvenile and adult venoms was not significantly different ($p = 0.065$). Kallikrein-like activity differences were statistically significant ($F_{2,40} = 14.28, p < 0.001$), with neonates also having significantly less activity

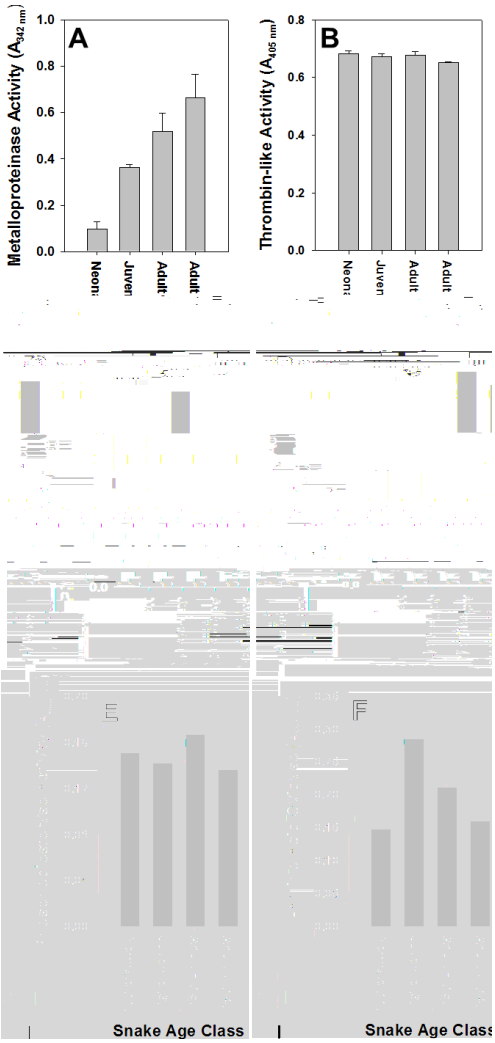


Figure 2. Enzyme activities in *Crotalus polystictus* venoms as a function of age class and sex; sample size for each is given parenthetically. Note that metalloproteinases (SVMP) activity (A) is lowest and PLA$_2$ activity (D)

### 2.2. 1-D SDS-PAGE

Typical of many venoms from within a species, C. polystictus venoms shared most components as revealed by 1D SDS-PAGE (Figure 4

### 2.3. MALDI-TOF-MS Mass Fingerprinting of Venoms

MALDI-TOF mass spectrometry provides more detailed analyses of lower mass components and is complementary to SDS-PAGE (Figures 5 and 6). A $PLA_2$ of 13,905 Da dominates all spectra, and numerous other small peaks are seen between approx. 6.9–11.9 kDa; the prominence of these lower mass components appears muted in neonate venoms, suggesting a lower abundance of these proteins as also observed by SDS-PAGE. Adult venoms showed prominent peaks at approx. 22.6 kDa (likely a P-I SVMP) that were absent from neonate venoms (Figure 6), again consistent with the enzymatic activity assay and SDS-PAGE data. Clusters of several peaks in the 24–30 kDa range are consistent with masses seen for CRiSPs and SVSPs, and these were variable in overall intensity between samples. In general, neonate venoms appeared to contain fewer peaks than adult venoms.

Figure 5. MALDI-TOF-MS analysis of *C. polystictus* venoms; 6–15 kDa mass window.

Figure 6. MALDI-TOF-MS analysis of *C. polystictus* venoms; 10–80 kDa mass window.

## 2.4. MALDI-TOF-MS/MS Identi cation of Speci c Proteins

abundance factor (NSAF), which takes into account both spectral counts and parent protein mass. The peptide TPPAGPDVGPR, which is identical to a bradykinin-inhibitory peptide (P0CJ34) accounts for over 36% of the total venom proteome. Consistent with other type I venoms, C. polystictus venom exhibits moderate SVMP levels, (~19%) representing all three SVMP subfamilies: P-I—2.7%, P-II—8.2%, and P-III—8.6%. Serine proteinases comprise 7.8% of the entire venom proteome, with thrombin-like (5.7%), kallikrein-like (1.9%), and a plasminogen activator (T1D6M5) (0.25%) all being identified by proteomic analysis. Basic (6.0%) and acidic (1.5%) $PLA_2$s, and LAAOs (4.4%) were present at moderate levels. The non-enzymatic disintegrins, C-type lectins, and CRiSPs accounted for approximately 9.0%, 7.8%, and 4.4% of the entire venom proteome, respectively. The remaining seven protein families accounted for a total of approximately 4% of venom proteins.

Figure 8. Venom proteome of adult C. polystictus. Abbreviations: BIP, bradykinin-inhibitory peptide; CRiSP, cysteine-rich secretory protein (helveprin); Dis-Cys Frag, cysteine-containing disintegrin fragment; LAAO, L-amino acid oxidase; NGF, nerve growth factor; PDE, phosphodiesterase (exonuclease); PLA2, phospholipase $A_2$; PLB, phospholipase B; P-I SVMP, class P-I snake venom metalloproteinase; P-II SVMP, class P-II snake venom metalloproteinase; P-III SVMP, class P-III snake venom metalloproteinase.

Table 1. Relative occurrence of the different protein families present in the venom of adult Crotalus polystictus.

| Protein Family | % of Total Venom Proteins |
|---|---|
| L-Amino Acid Oxidase | 4.35 |
| Snake Venom Metalloproteinase (SVMP) | 19.53 |
| P-I SVMP | 2.72 |
| P-II SVMP | 8.23 |
| P-III SVMP | 8.58 |
| Phosphodiesterase (Exonuclease) | <1.0 |

Table 1. Cont.

| Protein Family | % of Total Venom Proteins |
|---|---|
| Glutaminyl Cyclase | <1.0 |
| Hyaluronidase | <1.0 |
| Phospholipase B | <1.0 |
| Bradykinin-Inhibitory Peptide | 36.32 |
| Cysteine-rich Secretory Protein | 4.41 |
| C-type Lectin | 7.80 |
| Vespryn | 1.31 |
| Disintegrin | 9.07 |
| Dis-Cys Fragments | 1.04 |
| Nerve Growth Factor | <1.0 |

### 2.6. Toxicity of Neonate and Adult Venom to Mice

As shown in Figure 9, venom from neonate snakes was somewhat more toxic to NSA mice ($LD_{50}$ = 4.5 g/g) than adult venom ($LD_{50}$ = 5.5 g/g; Figure 9), a pattern typically observed with type I venoms.

Figure 9. Lethal toxicity (24 h post-injection) of *C. polystictus* venoms toward NSA mice.

## 3. Discussion

As trophic adaptations, snake venoms have allowed for the transition from a mechanical (constriction) to a chemical (venom) means of incapacitating and killing prey. Ontogenetic shifts in venom enzymatic and toxic activities often coincide with shifts in prey preference, and the general shift from low SVMP activity and high toxicity in neonates, to high SVMP activity and low toxicity in adult rattlesnakes is frequently observed. The increased SVMP content in adult rattlesnakes likely facilitates tissue degradation of larger, more metabolically favorable prey items, such as rodents. However, the opposite relationship has also been documented in some populations of *C. viridis viridis* [26], in which adult venoms have signi cantly lower SVMP but higher myotoxin a levels than

C. polystictus venom. This compositional pattern appears to be common among rattlesnakes producing type I venoms. In C. o. helleri and C. o. oreganus [21,23], this shift is correlated with a change in diet, from lizards and neonate rodents to larger mammalian prey; venoms from these taxa also show a significant increase in thrombin-like serine protease activity (unpubl. data). However, C. polystictus in this population feeds primarily on small mammals (mammals comprised 87.9% of 545 prey items) throughout their ontogeny, taking juvenile rodents (pygmy mice, Baiomys taylori, and shrews) when neonates and expanding their diet to include larger rodents, especially Microtus mexicanus, as adults (Mociño-Deloya et al., in prep.). This suggests that the main impetus for increases in SVMP activities is utilization of larger, bulkier prey, as has been previously suggested [21], rather than a shift to different taxa of prey. This conclusion is further supported by the fact that adult male C. polystictus consume a greater proportion of larger mammalian prey compared to adult female C. polystictus; venoms from adult males had significantly higher SVMP activity compared to the venoms from adult females, perhaps reflecting the importance of larger rodents and lagomorphs in the diet of adult males compared to females (24.0% vs. 5.1% of prey mass) [35].

As was also observed with C. o. oreganus and C. o. helleri venoms, neonate C. polystictus venoms contained over 2 the levels of $PLA_2$ activity seen in venoms of adult snakes. In Crotalus simus simus, this differential was found manifested as the presence of crotoxin homologs in neonate venoms [41]. However, in C. polystictus, all dominant venom $PLA_2$s showed identical masses of 13,906 Da by MALDI-TOF MS, strongly indicating that a crotoxin homolog is not present in these venoms. Shotgun proteomic analysis also failed to identify crotoxin or Mojave toxin in C. polystictus venom, although several acidic and basic $PLA_2$s are clearly present in the pooled sample. Most notable are peptides that matched a $PLA_2$ (A0A0K8RYR4) from C. horridus, which comprises approximately 5% of the adult C. polystictus venom proteome. The lack of crotoxin is also supported by lethality assays in mice, as rattlesnake venoms containing crotoxin homologs show lethal toxicities below 1.0 g/g; venoms from neonate C. polystictus are considerably less toxic. The slight increase observed with neonate venom toxicity, as compared to adults, may compensate for the significantly smaller venom bolus that can be injected into prey during a predatory strike, and a more toxic venom enables neonate snakes to dispatch prey items more rapidly. However, the similarity in toxicity seen in C. polystictus venoms may also be a reflection of their mammals-only diet; in C. oreganus helleri and C. o. oreganus, juvenile venoms were approximately 2 as toxic as adult venoms, and this species utilizes lizards as a dominant prey of neonate and young snakes [21].

Shotgun proteomic analysis indicates that the venom proteome of adult C. polystictus comprises just over 9% disintegrins, and SDS-PAGE analysis shows a clear distinction in disintegrin band intensity between adult and neonate venoms. Disintegrins are small (4–16 kDa) non-enzymatic proteins that often result from a post-translational processing of the P-II class of SVMPs [42–44]. Therefore, the lack of a disintegrin band in neonate venoms may be correlated with the lower SVMP content and activity observed in these venoms. By blocking integrin $_{IIb}$ $_3$, disintegrins inhibit platelet aggregation and facilitate the circulation of toxins throughout prey [45]. In C. atrox, and likely other species, disintegrins also appear to act as a molecular “tag” by altering the chemical scent of prey and allowing for successful prey recovery by strike-induced chemosensory searching [46]. For adult C. polystictus, the increased disintegrin concentration may assist with successful prey relocation during predatory episodes, as adults frequently release rodent prey following the envenomating strike. Neonates, on the other hand, often strike-and-hold prey, and therefore an abundance of disintegrin(s) in their venom is not necessary. The lack of disintegrins in neonate C. polystictus venom is also likely compensated by the increased toxicity, as venom would more rapidly debilitate prey before it retaliated or could wander far from the attack site. The concentration of disintegrin required for successful prey recovery remains unknown. However, in C. o. concolor, which exhibits a type II venom phenotype [24] and has significantly less disintegrin compared to type I species such as C. atrox, C. o. oreganus/helleri and C. polystictus, successful discrimination between envenomated and non-envenomated prey cues has been documented [47].

reactions and the plate was placed in a Spectramax 190 plate reader at 37 C; absorbance readings (414 nm) were taken every minute for 10 min. Enzyme activity was calculated from the linear portion of reaction rate curves (between 2.0 and 3.0 min), and speci c activity was expressed as mol product formed/min/mg protein.

### 5.4. One-dimensional SDS Gel Electrophoresis

Dithiothreitol (DTT) reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using Nu Page 12% acrylamide gels and Mark 12 standards (Life Technologies, Inc. (Grand Island, NY, USA) as described previously [88]. Twenty-four g of venom was loaded into each lane. Gels were stained in 0.1% Coomassie brilliant blue R-250, destained with 30% methanol/7% acetic acid, and photographed using a Bio-Rad gel imaging system. Protein class was identi ed based on mass and published accounts [15,16].

### 5.5. Mass Spectrometry: MALDI-TOF

Two representatives each of neonate, adult female, and adult male venoms were subjected to mass ngerprinting. Approximately 0.5 g of venom in 1.0 L 50% acetonitrile (ACN) in ddH $_2$O was mixed with 1 L sinapinic acid matrix (10 mg/mL 50% ACN in ddH $_2$O), spotted onto MALDI target plates, allowed to air dry and analyzed using an Ultra ex-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in operating in linear mode using a 25 kV accelerating voltage.

### 5.6. Mass Spectrometry: Orbitrap LC-MS/MS

One pooled venom sample (3 male, 3 female, adults) was subjected to shotgun proteomic analysis (Florida State University College of Medicine Translational Science Laboratory) in order to obtain an overview of the total venom proteome of C. polystictus Samples were digested using the Calbiochem ProteoExtract All-in-one Trypsin Digestion kit (Merck, Darmstadt, Germany) with LC/MS grade solvents according to the manufacturer's instructions. The LC-MS/MS analyses were performed using an LTQ Orbitrap Velos equipped with a Nanospray Flex ion source and interfaced to an Easy nanoLC II HPLC (Thermo Scienti c). Peptide fragments were separated using a vented column con guration consisting of a 0.1 20 mm, 3 mm(3)i250(a)-250(25)-Rd3n535 3592018

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