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Early significant ontogenetic changes in snake venoms

Kenneth P. Wray*, Mark J. Margres, Margaret Seavy, Darin R. Rokyta

Department of Biological Science, Florida State University, Tallahassee, FL 32306-4295, USA

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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

* Corresponding author. 319 Stadium Drive, Department of Biological Science, Florida State University, Tallahassee, FL 32306-4295, USA.
E-mail address: kwray@bio.fsu.edu (K.P. Wray).

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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%