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Within-clutch variation in venoms from hatchlings of *Deinagkistrodon acutus* (Viperidae)

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ABSTRACT

We used 17 hatchling five-paced pit-vipers snakes (*Deinagkistrodon acutus*) to study within-clutch variation in snake venoms. We measured venom yield and total protein content, and examined the correlations between venom yield and hatchling size [snout-vent length (SVL) and body mass]. We also analyzed the electrophoretic profiles and enzymatic activities of venoms from hatchlings. Lyophilized venom mass was not correlated with SVL, nor with body mass. Liquid venom mass and total protein content were not correlated with body mass, but were positively correlated with SVL. Venom composition, as shown in SDS-PAGE chromatograms did vary among individuals but there were biochemical differences in activity which had to be due to subtle venom composition differences between the sexes. Female hatchlings showed higher esterolytic and fibrinogenolytic activities but lower proteolytic, collagenolytic, phosphomonoesterase and fibrinolytic activities than male hatchlings. We did not find sexual differences in 5′ nucleotidase, phospholipase A_2 and hyaluronidase activities, and L-amino acid oxidase activities in either female or male hatchlings. Within-clutch variation in venoms from *D. acutus* hatchlings should be attributed to the individual-based differences in presence or absence, and the relative amount of the protein components, and might have a genetic basis.

1. Introduction

Snake venom, a mixture of enzymes, peptides, metal ions, water and other gradients, varies among species, among populations of the same species, among individuals of the same population, and even at an individual's level due to ontogenetic and seasonal shifts (Chippaux et al., 1991). In the Red Spitting Cobra *Naja pallida*, venoms from consecutive spits differ in the amount and composition (Cascardi et al., 1999).

There have been several studies focusing on among individual variation in snake venoms. Analyzing samples from six Roraima Rattlesnakes (*Crotalus durissus ruruima*) shows that these individuals differ from each other in venom composition and pharmacological properties (Dos-Santos et al., 2005). Willemse (1978) found that venom electrophoresis patterns differ among five individuals in each of six species, including two viperid and four elapid snakes. Among individual variation in venom composition, relative amounts of gradients, and immunological and biochemical activities has been also found in two populations of the African Puff Adder *Bitis arietans* (Currier et al., 2010).

Among individual variation in venoms might have a genetic basis. Mebs and Kornalik (1984) found a basic toxin in venom in two Eastern Diamondback Rattlesnakes (*Crotalus adamanteus*) but not in the other two of the same litter, and the presence or absence of this component was constant. Bothrojaracin isoforms from the Jararaca *Bothrops jararaca* vary among individuals not only in the amount and

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activity but also in amino acid composition and N-terminal sequence, with the observed variation thought to be influenced by genetic factors (Monteiro et al., 1998). The presence or absence, and variation in relative amounts, of major venom protein families, and the effects of advanced gene regulation on venom expression could be the main factors that result in among individual variation in venoms (Gibbs et al., 2009). Most studies on within-clutch variation in venoms supported the idea that such variation results from genetic effects. Efforts have been made to eliminate the proximate influence of environmental factors on venoms by keeping snakes of the same clutch under identical conditions, and it has been found in several species that venoms differ among individuals and between sexes (Chippaux et al., 1982; Furtado et al., 2006; Menezes et al., 2006; Pimenta et al., 2007; Zelanis et al., 2007; Céspedes et al., 2010; Pintor et al., 2011). In most of these studies, venoms were collected from individuals that had been maintained in the laboratory since birth for more than one year. However, factors such as death and escape may result in loss of samples. Moreover, changes in raising conditions may lead to fallibility in conclusions. Using the samples from newborn snakes can avoid these disadvantages, although neonates yield less venom than do adults.

The Five-paced Pit-viper (*Deinagkistrodon acutus*) is a large sized and highly venomous snake, ranging from the southern provinces of China (including Taiwan) to northern Vietnam (Zhao and Adler, 1993). The venom of *D. acutus* mainly presents proteinase, phospholipase A_2 and esterase activities as well as some subordinate traits including L-amino acid oxidase (LAO), hyaluronidase and collagenase activities (Huang and Qu, 1983; Qin, 1998). Depending on body size, females lay a single clutch of 11–53 eggs per breeding season (Lin et al., 2005). The relatively high fecundity makes *D. acutus* well suited to studying the individual-based variation in venoms within clutch. Previous studies of *D. acutus* show significant geographical and ontogenetic variation in venoms (Huang and Qu, 1983; Komori et al., 1984, 1987; Huang et al., 2004). However, as venoms were pooled for different individuals in all these studies, variation in venoms among individuals remains unknown in this species. In this study, we measured morphological traits, venom yields and biochemical properties of 17 *D. acutus* hatchlings derived from a single clutch of eggs incubated at a constant temperature of 26 °C. We paid particular attention to the individual-based variation in venoms.

2. Materials and methods

2.1. Animals and venoms

We obtained one gravid female [118.0 cm snout-vent length (SVL), and 1105 g post-oviposition body mass] in late June 2006 from a private hatchery in Lishui (Zhejiang, East China), and transported it to our laboratory in Hangzhou. The female laid a clutch of 30 eggs in mid-July. Of the 30 eggs, six were infertile, three were dissected for determination of embryonic stage at oviposition, and 21 were incubated in a Shellab incubator (Sheldon MFG Inc., USA) at 26 ± 0.3 °C. Seventeen eggs hatched in mid-August. All hatchlings were maintained in a 500 × 400 × 300 mm (length × width × height) plastic cage placed in a constant-temperature room at 26 ± 0.5 °C. Venoms milked from hatchlings at the age of 15 days old were lyophilized, weighed and stored at −80 °C until use. To minimise venom wastage, 100 μl plastic pipette tip was placed independently over each fang of the hatching for venom collecting according to Mirtschin et al. (2006). Body mass and SVL were taken for each hatchling on the same day.

2.2. Protein content determination

Protein content was determined according to Bradford (1976), using bovine serum albumin (BSA) as standard.

2.3. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970). Reduced and non-reduced samples were applied to a 3% stacking gel, and electrophoresed into a 12% separation gel. The gels were stained in 0.2% Coomassie brilliant blue R250, and destained with 10% acetic acid in water/methanol ($v/v = 1:1$).

2.4. Proteolytic activity

General proteolytic activity was assayed according to the method modified from Murata et al. (1963). Aliquots (0.1 ml) of venom with 0.4 μg/μl (solubilized in saline) was incubated at 37 °C for 2 h, in the buffer system (0.4 ml 200 mM Tris–HCl, pH 8.5) containing 2% casein as substrate. The reaction was stopped by adding 0.5 ml 440 mM TCA and left to room temperature for 30 min. The mixture was centrifuged at 3000 g for 15 min. Then sodium carbonate (2.0 ml 400 mM) and folin reagent (0.5 ml, water/original regent = 2:1) were added to 0.8 ml of the supernatant, the absorbance was measured at 660 nm. We used L-Tyrosine as standard, and the unit of activity was expressed as nmol of L-Tyrosine released min/mg crude venom. Activity on haemoglobin was also determined in similar conditions.

2.5. Esterolytic activity

Esterolytic activity was assayed using the method modified from Tu et al. (1965). The hydrolytic reaction of arginine esters on *N*α-Benzoyl-L-arginine methyl ester (BAEE) was tested at 25 °C with 1 ml 0.25 mM BAEE in a 66.7 mM PBS system (pH 7.0). And the reaction on *N*α-*p*-Tosyl-L-arginine methyl ester (TAME) was tested at 25 °C with 1 ml 1.5 mM TAME in a 0.1 M Tris–HCl system (pH 8.5). The change in absorbance at 253 nm was recorded after 4 min of reaction. The activity was expressed as nmol of the substrate degraded min/mg crude venom.

2.6. Collagenolytic activity

The collagenolytic activity was assayed following the procedure described by Molina et al. (1990) with some modification. A buffer system (50 mM Tris–HCl, pH 7.8,