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.

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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 Nα-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 Nα-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,

containing 50 mM CaCl$_2$ and 2% bovine Achilles tendon collagen) was added with 20 μg venom, and centrifuged after a 24h-incubation at 37 °C. Subsequently, the supernatant mixing with ninhydrin reagent was boiled for 10 min. Then, 50% N-propanol was added to the cooled mixture, the absorbance was recorded at 600 nm. The activity was defined as the increase in absorbance per mg protein.

2.7. 5′ nucleotidase activity

The 5′ nucleotidase activity was assayed according to the procedure described by Dhananjaya et al. (2006). Venom was added to the buffer system (50 mM Tris–HCl buffer, pH 7.4, containing 10 mM MgCl$_2$, 50 mM NaCl, 10 mM KCl and 10 mM 5′ AMP) and incubated at 37 °C for 30 min. Adding the solution with 0.42% ammonium molybdate in 1 M sulfuric acid, 10% ascorbic acid stopped the reaction. Then it was left at room temperature for 30 min, and the absorbance was determined at 660 nm. The activity was expressed as nmol of inorganic phosphate released min/mg crude venom.

2.8. Phosphomonoesterase activity

Phosphomonoesterase activity was assayed by the method of Dhananjaya et al. (2006) with some modification. A buffer system (500 mM Tris–HCl, pH 8.5, containing 10 mM MgSO$_4$ and 10 mM p-nitrophenyl phosphate) was incubated with venom at 37 °C for 30 min. The reaction was stopped by 200 mM NaOH, and determined at 405 nm after 20 min standing at room temperature. The activity was expressed as nmol of p-nitrophenyl released min/mg crude venom.

2.9. LAO activity

LAO activity was assayed according to the method of Toyama et al. (2006). Venom was added to the reaction system (50 mM Tris–HCl, pH 8.0, containing 250 mM of L-Leucine, 2 mM o-phenylenediamine, 0.81 U/ml horseradish peroxidase) and incubated at 37 °C for 60 min. This was stopped by adding 2 M H$_2$SO$_4$ and the absorbance was recorded at 490 nm after 10 min at room temperature.

2.10. Phospholipase A$_2$ activity

Phospholipase A$_2$ activity was determined according to the method of Antunes et al. (2010) by a slight modification. Venom was mixed evenly with the test system (100 mM NaCl, 10 mM CaCl$_2$, 7 mM Triton X-100, 0.35% soybean lecithin, 98.8 μM phenol red, pH 7.6), which was immediately measured for the absorbance at 558 nm for 5 min at room temperature. The activity was expressed as a change in absorbance of 0.3 unit min/mg crude venom.

2.11. Hyaluronidasic activity

Hyaluronidasic activity was assayed by the method of Ferrante (1956) with some modification. Snake venom was added to the test system contained 200 mM acetate buffer,

pH 6.0, containing 150 mM NaCl and 200 μg hyaluronic acid, and incubated at 37 °C for 15 min. The reaction was stopped by the addition of 2.5% cetyltrimethyl ammonium bromide reagent in 2% NaOH. After 30min, absorbance of the mixture was determined at 400 nm, and recorded. We used highly purified hyaluronidase as standard. The activity was expressed as National Formulary Units (NFU) min/mg crude venom.

2.12. Fibrinogenolytic activity

Fibrinogenolytic activity was assayed by using human plasma fibrinogen, following the procedure of Menezes et al. (2006) with some modification. Hundred micrograms fibrinogen was incubated with individual venoms (1 μg, solubilized in saline) at 37 °C for 5 min. The reaction was ended by 20 μl denaturing buffer system containing 1 M urea, 4% SDS and 0.4% β-mercaptoethanol. The hydrolytic profile was analysed on 7.5% SDS-polyacrylamide gel.

2.13. Fibrinolytic activity

Fibrinolytic activity was carried out by the fibrin-plate-clearance assay according to the method described by Aguilar et al. (2007) with some modification. The mixture (1 ml saline, containing 0.4% bovine plasminogen, 0.5 U thrombin and 1.25 mM CaCl$_2$) was poured into a 12-well plate, and left at room temperature for 30 min. Then, the venoms were added over the fibrin film, and incubated at 37 °C for 6 h, the diameter of the lysis areas was recorded. The activity was expressed as the square of the clear area formed per microgram of crude venom.

2.14. Coagulant activity

Fibrinogen clotting time was carried out with human plasma fibrinogen according to the method of Komori et al. (1987) by slight modification. Aliquots of 50 μl reaction system contained 100 μg fibrinogen in saline were incubated at 37 °C for 5 min, and then 10 μl snake venom was added. The mixture was incubated at 37 °C by gently intermixing until the first coagulum strand appeared, and the clotting time was recorded. The clotting activity was expressed as: 1 unit = 1/clotting time/mg protein.

2.15. Gelatinolytic activity

Gelatinolytic activity was carried out by zymography according to Menezes et al. (2006) with some modification. Gelatin was copolymerized with 12% polyacrylamide gels at a final concentration of 1 mg/ml. Venoms were separated under non-reducing condition. After electrophoresis, the gels were washed with 2.5% Triton X-100 on a rotary shaker at 37 °C for 30 min to remove SDS, and rinsed with deionized water to remove Triton X-100. Then, the gels were incubated in incubation buffer (50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl and 10 mM CaCl$_2$) at 37 °C for 24 h. Gels were stained with Coomassie blue R250 and destained, the lysis activity was indicated by the clear zones.

2.16. Statistical analysis

We used one-way ANOVA, one-way ANCOVA and linear regression analysis to analyze the corresponding data. Prior to parametric analyses, data were tested for normality using Kolmogorov–Smirnov test, and for homogeneity of variances using Bartlett's test. All values are presented as mean ± 1 standard error, and the significance level is set at $\alpha = 0.05$.

3. Results and discussion

3.1. Body size and venom yield

Female and male hatchlings did not differ in either mean SVL or mean body mass (Table 1). Hatchlings of the two sexes did not differ in liquid venom mass, lyophilized venom mass and total protein content when accounting for their differences in body mass (Table 1). Data pooled for both sexes showed that: (1) positive correlations of liquid venom mass and total protein content with body mass were marginally significant; (2) lyophilized venom mass was not correlated with body mass; and (3) none of the three yield-related parameters was correlated with SVL (Table 2).

It is widespread among venomous snakes that venom yield is positively correlated with body size (Furtado et al., 1991; de Roodt et al., 1998; López-Lozano et al., 2002; Huang et al., 2004; Mackessy et al., 2006; McCleary and Heard, 2010; Travaglia-Cardoso et al., 2010). In species where venom yields differ between sexes (de Roodt et al., 1998; Furtado et al., 2006), sexual dimorphism in venom yield disappears in adults when body size is factored out of the analysis (Mirtschin et al., 2002). In our study, venom yields did not differ between the sexes (Table 1), and this could be due to the fact that female and male hatchlings did not differ in body size. Our finding that venom yield is not correlated with SVL could be explained by the reason proposed for newborn *Crotalus durissus terrificus* where the narrow dispersion of body size eclipses the correlations (de Roodt et al., 1998). Liquid venom mass and total protein content both were marginally correlated with body mass, presumably because body mass varied over a wider range than did SVL (Table 1).

3.2. Venom composition

Using SDS-PAGE under non-reducing conditions, we found that the 17 hatchlings presented two high content areas (~ 22–24.5 and ~ 36.6–52.2 kDa), two bands with high molecular mass (~ 79.7 and ~ 120.5 kDa) and three

Table 2
The regression coefficients of venom yield and total protein content on the size (snout-vent length and body mass) of *D. acutus* hatchlings.

Venoms	Snout-vent length	Body mass
Liquid mass	$r^2 = 0.10$, $F_{1,15} = 1.64$, $P = 0.220$	$r^2 = 0.24$, $F_{1,15} = 4.78$, $P = 0.045$
Lyophilized mass	$r^2 = 0.05$, $F_{1,15} = 0.83$, $P = 0.375$	$r^2 = 0.14$, $F_{1,15} = 2.40$, $P = 0.142$
Total protein content	$r^2 = 0.12$, $F_{1,15} = 2.08$, $P = 0.164$	$r^2 = 0.24$, $F_{1,15} = 4.68$, $P = 0.046$

bands with low molecular mass (~ 13.3, ~ 15.6 and ~ 17.5 kDa) (Fig. 1Aa, b). Under reducing conditions, all hatchlings had two high content areas (~ 25–26.7 and ~ 45–49.1 kDa) and two low content areas (~ 13.8–19.0 and ~ 34.5–40.5 kDa) (Fig. 1Ba, b). Obviously, some protein bands with high molecular mass (e.g. ~ 79.7 and ~ 120.5 kDa) detected only under non-reducing conditions, consist of oligomers; and bands with low molecular mass (e.g. ~ 26.7 kDa) detected only under reducing conditions are derived from polymers. Venom compositions kept almost constant among 17 hatchlings under reducing conditions, but varied remarkably among individuals under non-reducing conditions (Fig. 1A and B). The most variable components were located in region of ~ 29.7–39.6 kDa. Furthermore, components of ~ 22.0–24.5 kDa could be detected in all samples except the component of ~ 22.0 kDa which was absent in m3 and stained weakly in f2, f7, m6 and m9. Neither under reducing conditions (Fig. 1Bc) nor under non-reducing conditions (Fig. 1Ac) did pooled female and male venoms differ in composition.

3.3. Biochemical activities

Previous studies of *D. acutus* show that the metalloproteinases, which are mainly distributed in ~ 13.8–52.2 kDa region, can hydrolyze the casein, haemoglobin and collagen (Ouyang and Huang, 1976; Xu et al., 1981; Mori et al., 1984; Nikai et al., 1991; Zhu et al., 1997; Huang et al., 1999; Wang et al., 2004). In our study, higher proteolytic and collagenolytic activities in male venoms indicate that the male venoms are more active in hydrolytic activity of metalloproteinases than female venoms; male/female ratios for these two activities were 1.1 and 1.4, respectively (Table 3). This difference could be due to the fact that intensities in ~ 33.0–52.2 kDa region are higher in male venoms than in female venoms (Fig. 1A). The higher activities of metalloproteinases in male venoms are also validated by gelatin zymography, which was presented strongly cleaved area in ~ 35.0–45.0 kDa, and

Table 1
Descriptive statistics for body size (snout-vent length and body mass) and venom yield (liquid mass, lyophilized mass and total protein content) of *D. acutus* hatchlings.

	Females ($N = 8$)	Males ($N = 9$)	Results of statistical analyses
Snout-vent length (cm)	27.4 ± 1.2 (24.8–28.5)	27.7 ± 0.9 (26.2–28.6)	$F_{1, 15} = 0.47$, $P = 0.503$
Body mass (g)	14.4 ± 2.4 (8.8–16.6)	15.1 ± 0.9 (14.1–16.6)	$F_{1, 15} = 0.76$, $P = 0.398$
Liquid mass (mg)	18.4 ± 5.9 (9.1–27.8)	21.0 ± 5.2 (14.7–28.7)	$F_{1, 14} = 0.33$, $P = 0.573$
Lyophilized mass (mg)	3.8 ± 1.2 (1.8–5.1)	4.3 ± 1.3 (2.3–6.5)	$F_{1, 14} = 0.13$, $P = 0.725$
Total protein content (mg)	2.8 ± 0.8 (1.3–3.8)	2.9 ± 0.9 (1.6–3.9)	$F_{1, 14} = 0.002$, $P = 0.888$

Data are expressed as mean ± 1 standard error (range). *F* values and significance levels of one-way ANOVAs (for snout-vent length and body mass) and one-way ANCOVAs (for the remaining variables with body mass as the covariate) are given in the table.
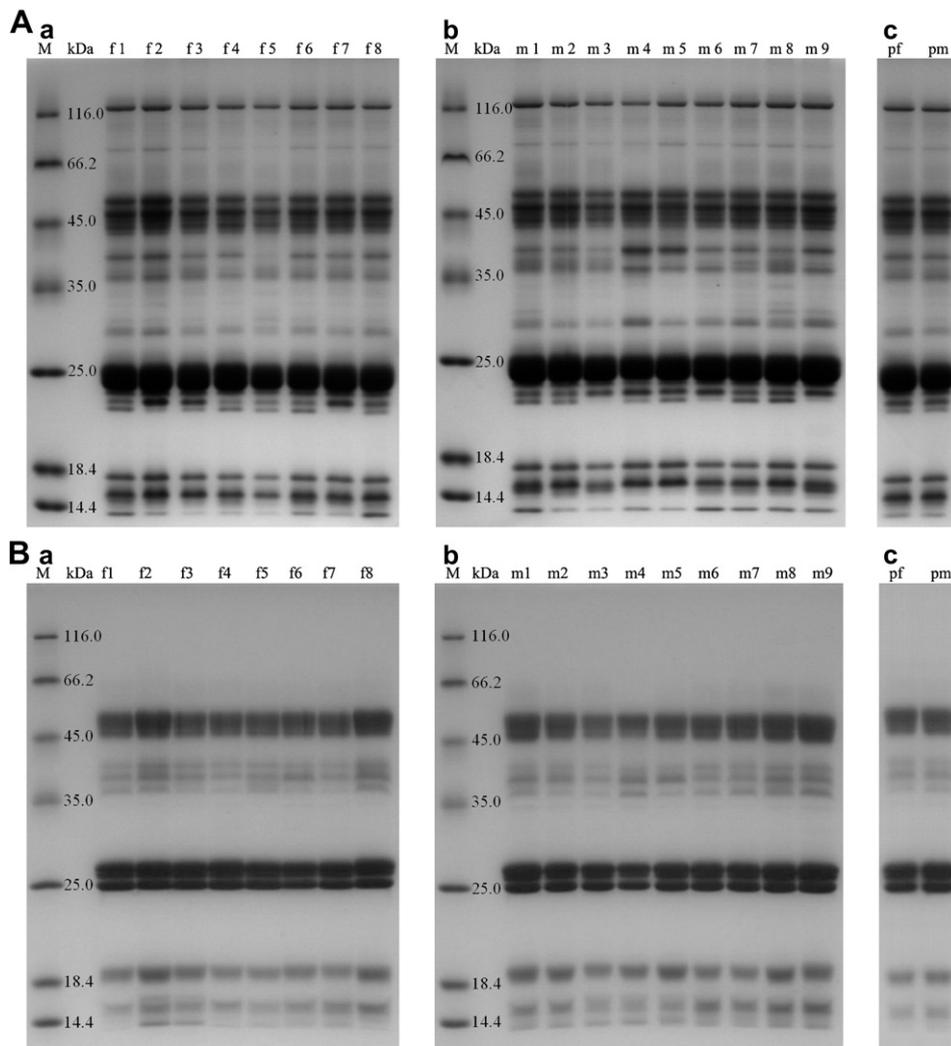
Fig. 1. Electrophoretic profile of individual newborn *D. acutus* venoms from a single clutch. Electrophoretic pattern: under non-reducing conditions (A: a, b, c) and under reducing conditions (B: a, b, c).Venoms: female (f), male (m), pooled female (pf) and pooled male (pm). Numbers at right of the standard protein marker (M) indicate molecular mass.

weakly hydrolyzed area in ∼61.6 kDa, ∼94.9 kDa and 104.7 kDa. Compared with pooled female venom, pooled male venom had a more clearly cleaved region (Fig. 2B). Our pattern of sexual dimorphism in metalloproteinase activities is contrary to that reported for *B. jararaca* where female venoms have higher enzymatic activity and more cleaved areas than male venoms (Furtado et al., 2006). Moreover, gelatinolytic activity differed slightly among the 17 *D. acutus* hatchlings, with components of ∼35.0 kDa and 45.0 kDa having hydrolytic activities in some individuals but not in others (Fig. 2B).

Consistent with the study of *B. jararaca* (Menezes et al., 2006), our data show that female venoms possess higher coagulant (Table 3) and hydrolysis activities on fibrinogen than male venoms, although pooled female venom is only slightly more active than pooled male venom in degrading the Aα chain of fibrinogen (Fig. 2A). This indicates that the

fibrinogenolytic principles (i.e. thrombin-like enzymes) of venoms are more active in females than in males. As male venoms possessed wider cleaved areas on fibrin-plate than females (Table 3), we conclude that the fibrinolytic components of venoms are more active in males than in females. Consistent with studies of *Bothrops* snakes (Menezes et al., 2006; Aguilar et al., 2007; Rodríguez-Acosta et al., 2010), fibrinogenolytic activity of venoms from *D. acutus* hatchings is not correlated with fibrinolytic activity ($r^2 = 0.10$, $F_{1, 15} = 1.67$, $P = 0.22$). In our study, the fibrinogenolytic activity also vary among individuals, with venoms from f1, f2, f5, m1, m6 and m8 degrading Aα chain more strongly than venoms from other individuals (Fig. 2A).

The reactions on synthetical substrates (BAEE and TAME), which specially degraded by serine proteases (Huang and Qu, 1983), strongly validate that the female

Table 3
The biochemical activities of crude venoms from *D. acutus* hatchlings.

Enzymatic activities substrates	Females ($N = 8$)	Males ($N = 9$)	Results of statistical analyses
Proteolytic activity			
Casein (nM/min/mg)	6.2 ± 0.2 (5.6–6.7)	6.6 ± 0.1 (6.3–7.0)	$F_{1, 15} = 5.21$, $P = 0.037$, F < M
Hemoglobin (nM/min/mg)	1.6 ± 0.02 (1.5–1.7)	1.7 ± 0.03 (1.6–1.9)	$F_{1, 15} = 9.53$, $P < 0.01$, F < M
Arginine esterolytic activity			
BAEE (nM/min/mg)	9.5 ± 0.6 (7.4–11.7)	6.3 ± 1.0 (1.6–9.7)	$F_{1, 15} = 7.66$, $P = 0.014$, F > M
TAME (nM/min/mg)	2.1 ± 0.1 (1.6–2.4)	1.5 ± 0.2 (0.6–2.1)	$F_{1, 15} = 10.99$, $P < 0.01$, F > M
Collagenolytic activity			
Collagen (U/mg)	0.28 ± 0.02 (0.20–0.38)	0.38 ± 0.03 (0.28–0.53)	$F_{1, 15} = 7.08$, $P = 0.018$, F < M
5′ nucleotidase activity			
AMP (nM/min/mg)	184.3 ± 27.1 (51.0–299.3)	233.7 ± 15.8 (174.2–302.5)	$F_{1, 15} = 2.62$, $P = 0.126$
Phosphomonoesterase activity			
pNPP-Na (nM/min/mg)	1.5 ± 0.3 (0.4–2.7)	4.6 ± 1.0 (1.2–11.4)	$F_{1, 15} = 7.39$, $P = 0.016$, F < M
Phospholipase A_2 activity			
Soybean lecithin (U/min/mg)	390.7 ± 52.1 (120.7–541.6)	430.7 ± 27.1 (337.6–543.0)	$F_{1, 15} = 0.49$, $P = 0.493$
Hyaluronidasic activity			
Hyaluronic acid (NFU/min/mg)	2.2 ± 0.2 (1.2–3.3)	2.5 ± 0.3 (1.6–3.7)	$F_{1, 15} = 0.73$, $P = 0.407$
Fibrinolytic activity			
Fibrin (mm^2/μg)	25.3 ± 3.1 (6.4–34.3)	33.1 ± 1.9 (27.0–43.3)	$F_{1, 15} = 4.95$, $P = 0.042$, F < M
Coagulant activity			
Fibrinogen (U/mg)	17.9 ± 1.1 (14.7–24.6)	14.5 ± 1.0 (11.0–20.6)	$F_{1, 15} = 5.04$, $P = 0.040$, F > M

Data are expressed as mean $\pm$ 1 standard error (range). F: females; M: males.

venoms are about 1.5 times more active in arginine ester-olytic activity than male venoms (Table 3). Our data also suggest that male venoms are about 3.1 times more active than female venoms in phosphomonoesterase activity (Table 3).

Venoms from female and male hatchlings have the same hydrolytic activities on 5′ AMP, soybean lecithin and hyaluronic acid, suggesting that the potential blood coagulant, hemolysis activity and venom permeability induced respectively by 5′ nucleotidase, phospholipase A_2 and
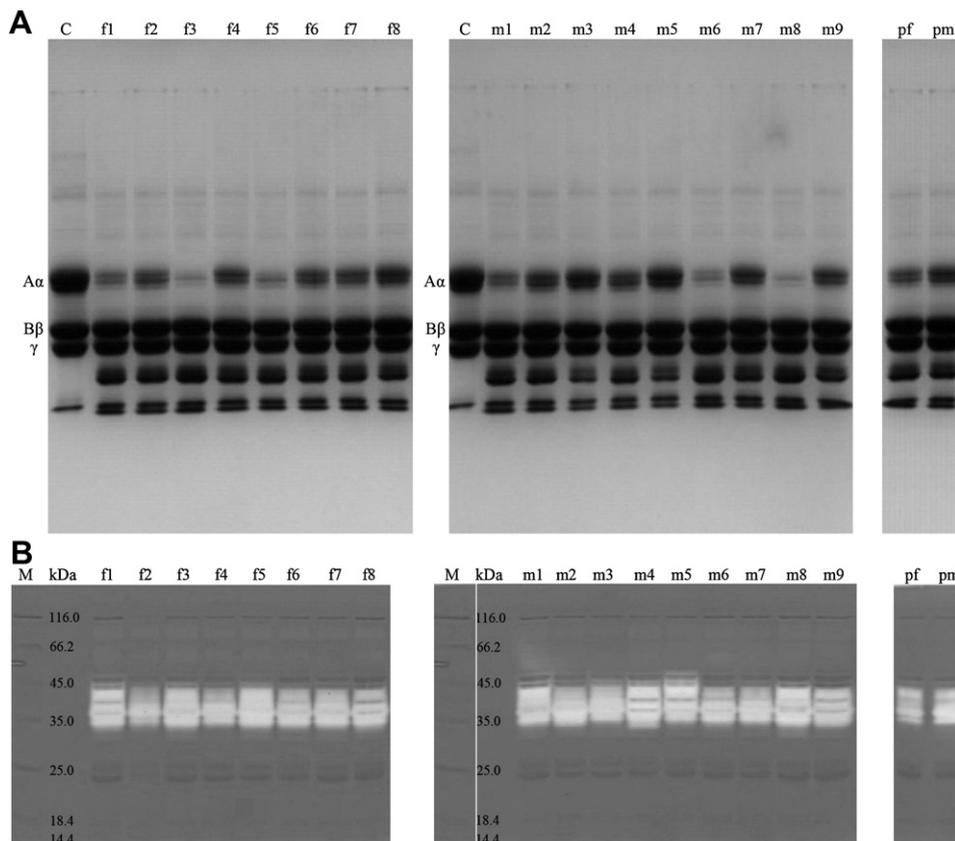

Fig. 2. Fibrinogenolytic and gelatinolytic activities of individual *D. acutus* venoms. Fibrinogenolytic activities (A), gelatine zymography (B). Venoms: female (f), male (m), pooled female (pf) and pooled male (pm). Letters (Aα, Bβ, γ) at left indicate three chains of fibrinogen.

hyaluronidase did not differ between the sexes. LAO is a venom component in adult *D. acutus* (Qin, 1998). However, we did not find any LAO activity in hatchlings.

Variation in venom composition and biochemical properties may be induced by the differences in presence or absence, and the relative amount of the components (Menezes et al., 2006; Pimenta et al., 2007; Gibbs et al., 2009). The diversity of protein structure and content could be affected by the regulation of gene expression (Gibbs et al., 2009), gene duplication (Oguiura et al., 2009) and accelerated evolution (Zha et al., 2006), and hence, the venom composition and biochemical properties may be more variable than expected. Thus, the genetic explanation of variations in venom based on snakes maintained under identical conditions would be more reliable.

In conclusion, female and male hatchlings used in this study did not differ in venom yield, total protein content. While not differing in 5′ nucleotidase, phospholipase A_2 and hyaluronidasic activities, female and male hatchlings showed differences in other enzymatic activities. Venom composition did not differ between the sexes, but varied significantly among individuals. Our study by examination of venoms from hatchlings of the same clutch supports the idea that the individual-based variation in venoms may have a genetic basis. Our observations and conclusions should be restricted to this single clutch, and further work is needed to examine whether they can be extrapolated to all other clutches for *D. acutus*.

Acknowledgment

Conflict of interest

None.

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