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Neonate-to-adult transition of snake venomomics in the short-tailed pit viper, *Gloydius brevicaudus*

Jian-Fang Gao^a, Yan-Fu Qu^b, Xiu-Qin Zhang^a, Ying He^a, Xiang Ji^{b,*}

^aHangzhou Key Laboratory for Animal Adaptation and Evolution, School of Life Sciences, Hangzhou Normal University, Hangzhou 310036, Zhejiang, China

^bJiangsu Key Laboratory for Biodiversity and Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing 210046, Jiangsu, China

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ABSTRACT

Snake venoms undergo ontogenetic shifts in biochemical and pharmacological activities. This may be related to variation in venom components associated with the ontogenetic shift in diet. We used the short-tailed pit viper *Gloydius brevicaudus* that displays ontogenetic shifts in diet to examine whether the species displays a neonate-to-adult transition of snake venomomics. Venoms from neonates and adults were pooled separately and then analyzed by 2-DE, MALDI-TOF-MS/MS and iTRAQ technologies. The 2-DE profiles showed that the main components in both types of venoms were acidic proteins, and that neonates and adults differed in snake venomomics. The proteins with molecular masses/pI of ~12–39 kDa/4.0–4.6, ~36–57 kDa/5.6–7.0 and ~65–92 kDa/4.5–5.8 were more abundant in the neonate venom, while the proteins with molecular masses/pI of ~12–19 kDa/4.6–6.4, ~23–30 kDa/5.4–6.3 and ~35–62 kDa/4.6–5.4 were more abundant in the adult venom. The iTRAQ analysis showed quantitative changes in various toxin families, including mainly metalloproteinases, serine proteinases, phospholipase A₂s and C-type lectins. The N-deglycosylation analysis demonstrated that glycosylation was an important post-translational modification of snake venom. Our results show a neonate-to-adult transition of snake venomomics in *G. brevicaudus*. Such a transition might be driven by the divergence in dietary habits between neonates and adults.

Biological significance

This study is first to demonstrate a neonate-to-adult transition of snake venomomics in *G. brevicaudus*, and the results will be helpful in predicting and treating clinical pathologic symptoms caused by the snake at different developmental stages.

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1. Introduction

Venom is a key innovation for snakes to subdue, kill and digest prey, and has been demonstrated to vary among and within species [1,2]. At the molecular level, the variability in snake venom is related to the recruitment events of new toxin genes or explosive diversification of existing toxin genes,

which occurred before and across the diversification of the advanced snakes [3,4]. The toxin-coding genes evolve at high rates and present an excess of non-synonymous over synonymous substitutions, suggesting that rapid toxin diversification is propelled predominantly by natural selection [5–7]. The divergence in diet habits has been considered an important selecting force to drive changes in venom composition and

* Corresponding author. Tel.: +86 25 85891597; fax: +86 25 85891526.
E-mail address: xji@mail.hz.zj.cn (X. Ji).

function [8,9]. In two pit vipers (*Calloselasma rhodostoma* [8] and *Trimeresurus stejnegeri* [10]), for example, geographic variation in venom composition is congruent with geographic variation in diet. Similarly, in saw-scaled vipers of the genus *Echis*, the toxicity of venoms to a prey (scorpion) is strongly associated with the percentage of the prey in diet [9]. Another example is the spine-tailed sea snake *Aipysurus eydouxii*. In this species, the loss of the main neurotoxin activity is caused by the dinucleotide deletion of neurotoxin genes, which is the consequence of the snake's shift to fish eggs-only diet [11].

Exploring variation in snake venom is important because symptoms caused by snakebite highly correlate with the venom composition and content [12–17]. Previous studies have mostly focused on differences in venom composition among species, among populations of the same species, and among individuals of the same population using venoms from adults. These studies mainly aim at uncovering the underlying mechanisms of envenoming. Although venom composition has been found to be age-related, few studies address ontogenetic shifts in venom composition except those conducted in America using pit vipers of the genera *Crotalus*, *Bothrops* and *Lachesis* as the model animals [18–26]. Also, some clinical symptoms caused by neonate or young snakes are more severe and thus have a longer recovery time after antivenom treatment than the same symptoms caused by adults. This is illustrated by the hemostatic disturbance induced by *Bothrops jararaca* [27]. A quantitative analysis of age-related variation in snake venom proteome may be helpful in clarifying the potential mechanisms of envenomation caused by neonate and adult snakes. Unfortunately, such analysis has been carried out only in a few American species [16,17,26,28]. To accurately evaluate the severity of envenomings by neonate and adult snakes, clarify the mechanisms of envenoming, and improve the treatment of snakebite and preparation of antivenom, more quantitative studies analyzing age-related variation in venom composition should be conducted.

Snakebites, especially those caused by widely distributed venomous snakes, have long been a serious public health problem in China. There are four venomous snakes (*Naja atra*, *Bungarus multicinctus*, *Deinagkistrodon acutus* and *Gloydus brevicaudus*) that are responsible for the majority of envenomings and deaths in China [30]. The short-tailed pit viper, *G. brevicaudus*, is a medium-sized and genetically diverse snake that ranges from East China to Korea [31,32]. The frequency of envenomings by this snake can be up to 34% in total envenoming cases per year [30]. Neonate *G. brevicaudus* only eat juvenile frogs, ants and centipedes, while adults mainly eat loaches, adult frogs, mice and birds [33,34]. Thus, ontogenetic shifts in diet are evident in the snake. Venomics, which is elucidated by a combination strategy, reveals that the venom of adult *G. brevicaudus* is comprised of cardiotoxins, neurotoxins, hemotoxins, phospholipases A_2s and metalloproteinases [35]. The composition of venoms from neonate *G. brevicaudus* is also diverse. More protein bands revealed by SDS-PAGE were found in neonates, largely because specific protein bands with molecular weights of ~31.1–34.1 kDa can be found only in neonates [36; http://so.med.wanfangdata.com.cn/ViewHTML/DegreePaper_Y1728346.aspx]. However, quantitative differences in venom components between neonate and adult *G. brevicaudus* remain unclear.

In this study, we used 2-DE (two-dimensional gel electrophoresis), MALDI-TOF-MS/MS and iTRAQ (isobaric tag for relative and absolute quantification) technologies to explore quantitative changes in the venom components during ontogeny in *G. brevicaudus*. We hope our comparative proteomic analysis can provide an explanation for the potential difference in envenomation caused by neonate and adult *G. brevicaudus*.

2. Materials and methods

2.1. Animals and venoms

A total of 134 adult *G. brevicaudus* (84 females and 50 males) collected in the years of 2008–2011 from Xiaoshan, Zhejiang, East China, and 548 newborns (276 females and 272 males) produced by 54 females in our Hangzhou laboratory were used in this study. Pooled venom sample of 3-week old neonates was milked according to method 1, and that of adults was milked according to method 2 described by Mirtschin et al. [37]. Fresh venoms were centrifuged to remove impurities for 15 min at 10,000 g, 4 °C, and then lyophilized and stored at –80 °C until use. Protein concentrations of venoms were determined according to Bradford [38] using BSA as standard.

2.2. Two-dimensional gel electrophoresis and MS identification of protein spots

Two-dimensional gel electrophoresis was carried out in Protean IEF/Protean II system (Bio-Rad). Venom samples were dissolved in 300 µl rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.5% pharmalyte (pH 3–10) and 0.002% bromophenol blue to a final protein concentration of 1 µg/µl. Precast IPG strips (17 cm, broad range pH 3–10 linear and narrow range pH 4–7 linear) were employed for the protein separation in the first dimension at 20 °C using the following protocol: 50 V for 12 h, 300 V for 1 h, 1000 V for 1 h, 10,000 V for 5 h, and 10,000 V for 60,000 V·h. After reduced and alkylated by sequential incubation with 2% DTT and 2.5% iodoacetamide in equilibration buffer (6 M urea, 20% glycerol, 2% SDS and 2% DTT in 0.375 M Tris–HCl, pH 8.8), each strip was washed in electrophoresis buffer, and applied to 12% SDS-PAGE gels for second dimension separation. The gels were stained in 0.2% Coomassie brilliant blue R250.

Differentially expressed protein spots were excised and in-gel trypsin digested. Each sample was redissolved in 1.5 µl 30% acetonitrile (containing 5 mg/ml α -cyano-4-hydroxycinnamic acid and 0.1% formic acid), then subjected to MS/MS by MALDI-TOF-TOF mass spectrometer (Autoflex speed™, Bruker Dalton). The mass spectrometer was operated under 20 kV accelerating voltage, the MS scan was acquired in the m/z range of 700–3200, and the scanning was processed in a resolution (20,000 FWHM). Raw data files were converted to the *.mgf format for searching against NCBI nr protein (strict to the taxa Serpentes; 34,344 entries; downloaded on 12/1/2013) using Mascot search engine (version 2.3.02). The parent and fragment mass tolerance were set at 50 ppm and 0.5 Da, respectively. The max missed cleavages were set at 1, and the peptide charge was set at 1. Carbamidomethyl (C) was set as fixed modifications, while oxidation (M) was set as variable modifications.

2.3. Trypsin digestion and iTRAQ labeling

Venom samples (100 µg) were digested with trypsin gold with a protein to trypsin ratio of 20:1 at 37 °C for 12 h, and the peptides were dried by vacuum centrifugation. Thereafter, the peptides were reconstituted in 0.5 M triethylammonium bicarbonate and treated with iTRAQ reagent (Applied Biosystems) according to the manufacturer's protocol. Peptides from different samples were labeled with different isobaric tags in same protein group. The iTRAQ-labeled peptides were pooled and dried by vacuum centrifugation, and the mixture was reconstituted with a buffer system (25 mM NaH₂PO₄ in 25% acetonitrile, pH 2.7) and fractionated by SCX chromatography (250 × 4.6 mm, 5 µm particle size, Phenomenex). The peptides were eluted over a gradient of 5–35% 1 M KCl in a buffer system (25 mM NaH₂PO₄ in 25% acetonitrile, pH 2.7). The fractions were desalted, vacuum-dried, and then stored until use.

2.4. Identification and quantification of isobaric labeled peptides by LC-MS/MS

Each fraction was redissolved in 2% acetonitrile (containing 0.1% formic acid), and centrifuged at 20,000 g for 10 min. An aliquot (2.25 µg/9 µl) of supernatant was loaded onto Symmetry C18 column (180 µm × 20 mm, 5 µm particle size, Waters) for online trapping and desalting, the program was carried out at 2 µl/min for 15 min with 99% buffer A (0.1% formic acid in 2% acetonitrile). Then the collected components were loaded onto BEH130 C18 column (100 µm × 100 mm, 1.7 µm particle size, Waters) for analytical separations at a flow rate of 300 nl/min with buffer B (0.1% formic acid in 98% acetonitrile) in buffer A. The system was equilibrated by 5% B for 1 min, then eluted with a linear gradient of 5–35% B for 40 min and maintained for 24 min in 35% B, then 35–80% B for 5 min and maintained for 5 min in 80% B.

Data were acquired by a TripleTOF 5600™ LC/MS/MS system (AB SCIEX). The ion spray voltage was set at 2.5 kV, the MS scan was acquired in the *m/z* range of 100–3000, and the scanning was processed in a resolution (30,000 FWHM) using 250 ms accumulation per spectrum at a threshold of 120 cps. Dynamic exclusion was set for 1/2 of peak width (18 s). Raw data files were converted to the *.mgf format for searching against NCBI nr protein (strict to the taxa Serpentes; 34,344 entries; downloaded on 12/1/2013) using Mascot search engine (version 2.3.02). The parent and fragment mass tolerance were set at 0.1 and 0.05 Da, respectively, and the max missed cleavages were set at 1. Carbamidomethyl (C), iTRAQ 8-plex (N-term) and iTRAQ 8-plex (K) were set as fixed modifications, while oxidation (M), Gln- > pyro-Glu (N-term Q) and iTRAQ 8-plex (Y) were set as variable modifications.

MS/MS-based peptide, protein identifications and quantifications of samples were validated by scaffold 3 Q+ (Version 3.4.5). Protein identifications were accepted if they contained at least one identified peptide, and false discovery rate (FDR) of all searches was set to <1%. Quantifications were based on the unique peptides, the minimum quantitative peak was calculated as 5% of the highest peak, and the protein quantitative values were presented as median of all peptide values. Log₂ normalized fold changes (calculated with the neonate

venom as the reference) were expressed as mean of four LC-MS/MS and analyzed by permutation test.

2.5. N-deglycosylation analysis

Venom samples (120 µg) were incubated in 5% SDS containing 0.4 M DTT at 90 °C for 15 min. After cooling, aliquots (10 µl) of 120 U PNGase F (New England BioLabs Inc.) in 0.5 M sodium phosphate (pH 7.5) containing 10% NP-40 were added into the samples, which then were incubated at 37 °C for 20 h. Then the mixtures were lyophilized and the deglycosylation profiles were determined by 2-DE with Precast IPG strips (7 cm, pH 3–10 linear) as described above.

2.6. Gelatin zymography

For SDS-PAGE gelatin zymography, deglycosylated and non-deglycosylated venom samples (6 µg), prepared with non-reducing conditions, were separated by 12% gels copolymerized with 2 mg/ml gelatin. After electrophoresis, the gels were incubated at 37 °C for 40 min on a rotary shaker in 0.05 M Tris-HCl, pH 8.0, containing 2.5% Triton X-100 to remove SDS. The gels then were washed several times with deionized water to remove Triton X-100. Finally, the gels were incubated in the buffer system containing 0.05 M Tris-HCl, pH 8.0, 0.15 M NaCl and 0.01 M CaCl₂ at 37 °C for 16 h. The gels were stained in 0.2% Coomassie brilliant blue R250, and the clear zones were recognized as the components with gelatinolytic activity.

3. Results and discussion

3.1. A comparison of neonate and adult venoms by 2-DE

An earlier study on *G. brevicaudus* by SDS-PAGE under non-reducing conditions showed that the neonate venom had more protein bands than the adult venom, suggesting ontogenetic changes in venoms [36]. Ontogenetic changes in venom were also detected in this study by 2-DE profiles (Fig. 1). To optimize 2-DE separation of venom proteins, we used broad and narrow linear strips in first dimension separation. Most venom proteins were acidic components, with pI values ranging from 4.5 to 6.8, and molecular masses from ~12 to 92 kDa; venoms from neonates and adults both had a basic protein area with molecular mass of ~16 kDa (Fig. 1). Some protein spots were blurry in both types of venoms with broad pH range strips, especially those in the area with molecular masses around ~66 kDa. Venom proteins were therefore further separated by narrow 4–7 pH range strips. In the narrow pH experiment, most venom proteins could be discriminated successfully, and the resolution of protein spots was improved. The neonate venom had more spots with higher intensity in the areas with molecular masses/pI of ~12–39 kDa/4.0–4.6, ~36–57 kDa/5.6–7.0 and ~65–92 kDa/4.5–5.8, but fewer spots with lower intensity in the areas with molecular masses/pI of ~12–19 kDa/4.6–6.4, ~23–30 kDa/5.4–6.3 and ~35–62 kDa/4.6–5.4 than the adult venom (Fig. 1). Twenty-three differentially expressed proteins in Fig. 1 were identified by MALDI-TOF-MS/MS (Table 1 and Supplementary Table 1), and the results showed that the components of the region around ~66 kDa were mainly

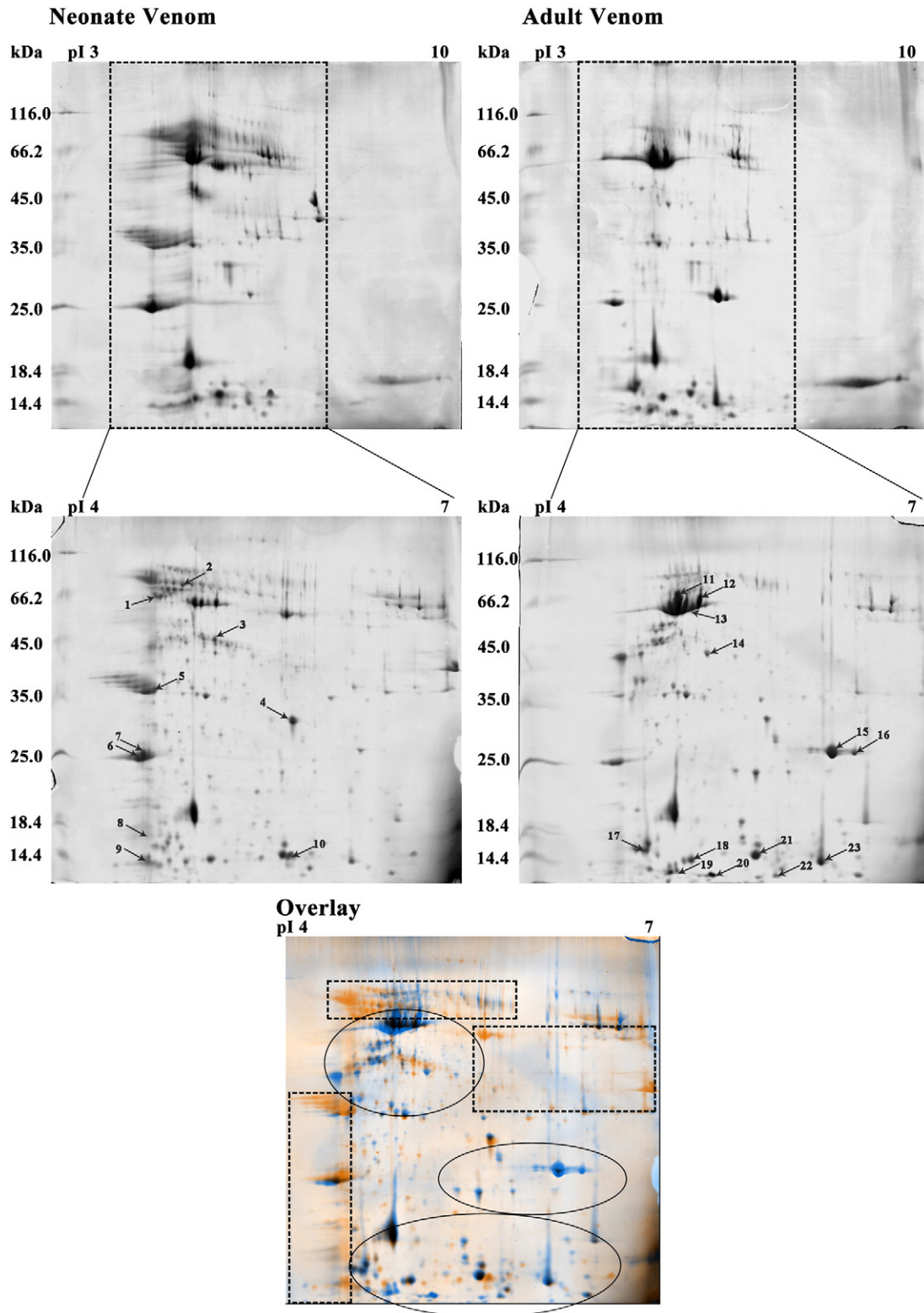


Fig. 1 – A comparison of 2-DE profiles between neonate and adult venoms. Venoms were separated using broad and narrow range precast IPG strips. Colorized images from neonate (orange) and adult (blue) gels were overlapped in PDquest to facilitate comparisons. Black spots: proteins found in both types of venoms; spots in rectangles: proteins expressed higher in the neonate venom; spots in ovals: proteins expressed higher in the adult venom.

Table 1 – Identification of proteins indicated in Fig. 1 by MALDI-TOF-MS/MS.

Spot	Accession number	MW (kDa) ^a	pI ^a	Protein name (family) ^b
<i>Neonate venom</i>				
1	gi 182705250	66	4.64	Zinc metalloproteinase/disintegrin (SVMP)
2	gi 190358877	75	4.84	Zinc metalloproteinase–disintegrin brevilysin-H6 (SVMP)
3	gi 332321851	48	5.10	Zinc metalloproteinase/disintegrin VMP-II (SVMP)
4	gi 48428846	30	5.65	Ablomin (CRISP)
5	gi 4102926	36	4.53	Plasminogen activator precursor (SP)
6	gi 4106007	25	4.38	Metalloprotease (SVMP)
7	gi 4106007	25	4.48	Metalloprotease (SVMP)
8	gi 386364753	16	4.54	CTLs subunit alpha, partial (CTL)
9	gi 82220683	14	4.52	Disintegrin saxatilin (disintegrin)
10	gi 82123640	14	5.63	Zinc metalloproteinase/disintegrin (SVMP)
<i>Adult venom</i>				
11	gi 190358877	62	5.01	Zinc metalloproteinase–disintegrin brevilysin-H6 (SVMP)
12	gi 190358877	62	5.12	Zinc metalloproteinase–disintegrin brevilysin-H6 (SVMP)
13	gi 190358877	62	5.03	Zinc metalloproteinase–disintegrin brevilysin-H6 (SVMP)
14	gi 143681919	44	5.11	Thrombin-like enzyme kangshuanmei (SP)
15	gi 75570154	25	6.15	Zinc metalloproteinase/disintegrin (SVMP)
16	gi 75570154	25	6.29	Zinc metalloproteinase/disintegrin (SVMP)
17	gi 82095086	15	4.67	Coagulation factor IX-binding protein subunit A (CTL)
18	gi 386364751	14	5.06	CTLs subunit alpha, partial (CTL)
19	gi 159162631	13	4.90	Chain A, solution structure of novel disintegrin salmosin (disintegrin)
20	gi 118651	13	5.25	Disintegrin halysin (disintegrin)
21	gi 129437	14	5.57	Neutral phospholipase A ₂ agkistrodotoxin (PLA ₂)
22	gi 118651	13	5.72	Disintegrin halysin (disintegrin)
23	gi 166012647	14	6.06	Phospholipase A ₂ (PLA ₂)

^a Observed molecular weights and pIs.
^b SVMP, snake venom metalloproteinase; CRISP, cysteine-rich secretory protein; SP, serine proteinase; CTL, C-type lectin; PLA₂, Phospholipase A₂.

belonging to P-III metalloproteinases, they were similar to the identification results reported by Li [35]: the components with molecular masses higher than 60 kDa are belonging to P-III class. The components with molecular masses of ~25 kDa were identified as P-II metalloproteinases, and similar to the identified protein spots (spot number: 119,126,129) with molecular masses lower than 30 kDa [35]. The protein with molecular masses of ~35 kDa was identified as serine proteinase, and showed higher abundance in the neonate venom. In the region with low molecular masses, the components were mainly identified as PLA₂, CTL (C-type lectin), disintegrin and P-II metalloproteinases, and the PLA₂ and CTL presented higher abundance in the adult venom. A CRISP (cysteine-rich secretory protein) spot was found in both venoms, and it was more highly expressed in neonates.

Ontogenetic shifts in venom composition and activity have been documented in some pit vipers of the genus *Bothrops* where the neonate venom is more abundant in metalloproteinases of P-III class but less abundant in metalloproteinases of P-I/P-II class than the adult venom [16,17,28]. The metalloproteinases identified in this study were mainly belonging to P-II and P-III classes (Table 1 and Supplementary Table 1). Although the abundance of each identical spot might be quantitatively different between neonate and adult venoms, the abundance of each class seems not to be quantitatively different (Fig. 1 and Table 1).

Ontogenetic shifts in venom composition and activity may be induced by dietary changes [24,25,28], but whether such shifts are genetically determined is unknown. One of the most important and changeable components is the metalloproteinase family, and it has been found that in some snakes the expression patterns of P-I/PII to P-III classes differ between

neonates and adults [16,26,28,39]. Ontogenetic shifts in venom composition and activity are hypothesized to reflect a trade-off between the metabolic costs of venom production and increment of foraging efficiency [40], or an adaptation to new ecological niches or competition for resources [39,41]. Despite the fact that pedomorphism, which is described as no ontogenetic shifts in venom composition and activity followed by dietary changes, exists in some venomous snakes [22,41], our study on *G. brevicaudus* shows conspicuous ontogenetic shifts in venom composition.

3.2. A comparison of neonate and adult venoms by iTRAQ

Gel-based proteomic quantitative methods have been used to analyze similar proteins of snake venom among and within species. In the past decade, however, these methods have been challenged by LC-based techniques, because of the shortcomings associated with reproducibility and representation of low abundant, highly acidic/basic and hydrophobic components [42]. Furthermore, gel-based techniques cannot work together with mass spectrometer directly, making automatic operation less likely to realize [42]. The above shortcomings can be improved by LC-based methods, especially iTRAQ, an amine-specific tagging technique, by which one can label each peptide successfully, increase the sequencing coverage of peptides, and promote ionization of lysine-containing peptides by a moderately strong basic group (*N*-methylpiperazine). Moreover, highly consistent sample preparation, biological duplication of the samples and bias correction of the data can be helpful in minimizing the potential errors associated with sample handling [42,43].

Table 2 – Identified proteins in the iTRAQ experiment.

Identified proteins	Accession number	Log ₂ FC	p-Value
Metalloproteinases			
Zinc metalloproteinase-disintegrin jerdonitin	gi 48427991	-2.60	8E-04
Dislicrin	gi 91680863	-2.55	3E-05
Metalloprotease	gi 4106007	-2.35	2E-14
Zinc metalloproteinase/disintegrin ussurin	gi 82208020	-1.98	1E-09
Metalloproteinase 8	gi 338855324	-1.65	6E-15
Zinc metalloproteinase-disintegrin agkistin	gi 82211851	-1.50	6E-05
Zinc metalloproteinase/disintegrin	gi 82123643	-1.40	2E-15
Metalloproteinase 4	gi 338855316	-1.05	1E-10
Zinc metalloproteinase-disintegrin stejnihagin-A	gi 123900231	-1.03	3E-15
Zinc metalloprotease-disintegrin halsase	gi 82211845	-0.98	4E-15
Zinc metalloproteinase/disintegrin	gi 52000724	-0.85	0.001
Zinc metalloproteinase/disintegrin	gi 82219563	-0.55	0.001
Metalloproteinase 11	gi 338855330	-0.50	0.64
Metalloproteinase	gi 297594016	-0.48	0.37
Zinc metalloproteinase-disintegrin berythractivase	gi 82216043	-0.30	0.63
Zinc metalloproteinase/disintegrin	gi 50403719	-0.18	0.42
Zinc metalloproteinase/disintegrin	gi 50400453	0.35	0.44
MP_III2 SVMP precursor	gi 308212498	0.50	0.12
Zinc metalloproteinase-disintegrin brevilysin-H6	gi 190358877	0.93	2E-14
Metalloproteinase VMP-III precursor	gi 258618064	1.15	0.004
Zinc metalloproteinase/disintegrin	gi 123912829	2.00	5E-15
Metalloproteinase 3	gi 338855314	4.23	8E-04
Serine proteinases			
Thrombin-like enzyme pallase	gi 158514815	-0.95	3E-15
Thrombin-like enzyme calobin-2	gi 156630857	-0.65	5E-04
Serine protease Haly-2	gi 82249008	-0.35	0.035
Afaacytin chain beta=alpha beta-fibrinogenase	gi 1911436	-0.23	0.56
Thrombin-like enzyme shedaenase	gi 158563968	0.13	0.54
Venom serine proteinase 1	gi 13959617	0.15	0.53
Thrombin-like enzyme ussurase	gi 158563936	0.63	0.001
Brevinase	gi 82117246	0.73	0.018
Serine proteinase isoform 8	gi 109254952	0.75	3E-05
Kinin-releasing enzyme KR-E-1	gi 82094948	0.83	2E-04
Thrombin-like enzyme salmobin	gi 82227939	0.88	0.013
Phospholipase A₂s			
Phospholipase A ₂	gi 166012649	-0.48	9E-10
Phospholipase A ₂ , acidic	gi 47117139	-0.45	1E-14
Acidic phospholipase A ₂	gi 38146948	-0.43	0.025
Phospholipase A ₂ , neutral	gi 129437	-0.23	0.79
N6a basic phospholipase A ₂	gi 38230129	-0.15	0.7
Chain A, structure of cadmium-substituted phospholipase A ₂	gi 22218624	1.80	6E-15
C-type lectins			
Echicetin subunit beta	gi 7993934	-2.58	3E-05
EMS16 subunit B	gi 82131629	-1.53	0.004
Stejaggregin-A subunit beta-1	gi 82129982	-0.83	2E-04
Mamushigin subunit beta	gi 34098771	-0.73	6E-08
Mamushigin subunit alpha	gi 34098769	-0.50	0.043
Salmorin subunit A	gi 82175557	-0.25	0.37
Coagulation factor IX-binding protein subunit A	gi 82095086	1.85	2E-14
Cysteine-rich secretory protein			
Ablomin	gi 48428846	-1.53	7E-15
Hyaluronidase			
Hyaluronidase	gi 113203667	-0.78	3E-04
L-amino-acid oxidase			
L-amino-acid oxidase	gi 82088273	-0.73	5E-15
Nerve growth factor			
Venom nerve growth factor	gi 82217029	-0.90	3E-07
Phospholipase			
Phospholipase B	gi 338855308	-0.35	7E-10
Peptidases			
Aminopeptidase N	gi 211926750	-1.13	0.007
Aminopeptidase A	gi 148367284	0.58	2E-06
Dipeptidylpeptidase 4a	gi 40363634	0.85	0.021
Others			
Glutaminyl-peptide cyclotransferase	gi 82108661	0.35	1E-09
HSP70	gi 63146078	-0.23	0.083
Peroxiredoxin-4	gi 332319804	-0.40	0.25
PDI	gi 63146076	0.18	0.25
Phospholipase A ₂ inhibitor subunit B	gi 7994665	1.10	8E-04

In this study, we used iTRAQ reagents for a more in-depth quantitative analysis of ontogenetic shifts in venom proteomes. Each venom sample was digested with trypsin, and the peptides from different samples were labeled with different isobaric tags. Then the labeled peptides from neonate and adult venoms were pooled and submitted to TripleTOF LC-MS/MS system for identification and quantification. To diminish the risk of reporting false positive identifications and assess the quality of data, a FDR analysis was carried out using a decoy database composed of the NCBI nr protein entries from the taxa *Serpentes*. After bioinformatic analysis, we identified and quantified 59 venom proteins successfully (Table 2, Supplementary Table 2 and Supplementary Table 3). The efficient labeling of neonate and adult tryptic peptides with iTRAQ tags is illustrated in Fig. 2, which shows a representative spectrum of peptide CLYNEPSK (*m/z* 538.29³⁺) derived from the zinc metalloproteinase–disintegrin jerdonitin (gi|48427991).

We identified 90 unique peptides and 22 clusters of metalloproteinases, which accounted for 40% of total unique peptides and 37% of total proteins (Supplementary Table 2). Of the 22 metalloproteinases, 12 were up-regulated in the neonate venom, four were up-regulated in the adult venom, and the remaining six did not differ between neonate and adult venoms in abundance. We identified 26 unique peptides and 11 clusters of serine proteinases, which accounted for 12% and 19% of total unique peptides and proteins, respectively. Of the 11 serine proteinases, three were more abundant in the neonate venom, five were more abundant in the adult venom, and the remaining three did not differ between neonate and adult venoms in abundance. Of the six D49-PLA₂s, three acidic proteins were more abundant in the neonate venom, one basic protein was more abundant in the adult venom, and the remaining two (one basic and one neutral) did not differ between neonate and adult venoms in abundance. Of the seven C-type lectins, five were more abundant in the neonate venom, one was more abundant in the adult venom, and the remaining one did not differ between neonate and adult venoms in abundance.

We detected 13 proteins of low abundance, which accounted for 27% of total unique peptides and 22% of total proteins. Of these proteins, six (cysteine-rich secretory protein, hyaluronidase, L-amino acid oxidase, nerve growth factor, phospholipase and aminopeptidase N) were more abundant in the neonate venom, and three (aminopeptidase A, dipeptidylpeptidase 4a, and phospholipase A₂ inhibitor subunit B) were more abundant in the adult venom. The remaining four have been reported only in a few venomous snakes. The first one is a member of heat shock protein 70 and was first found in *Pseudonaja textilis* venom [44]. In this study, this protein was more abundant in the neonate venom. The second one (peroxiredoxin-4) is a housekeeping protein that may have a key role in maintaining the redox homeostasis, and was first found in *Crotalus atrox* venom [29]. In this study, the abundance of the second one did not differ between

Notes to Table 1:

Up-regulated, down-regulated and unchanged protein expressions in the adult venom are represented with the data shaded in green, red and white, respectively. The significance level is set at $\alpha = 0.05$. Log₂ FC: mean of four LC-MS/MS runs, and was calculated using neonate venom as reference.

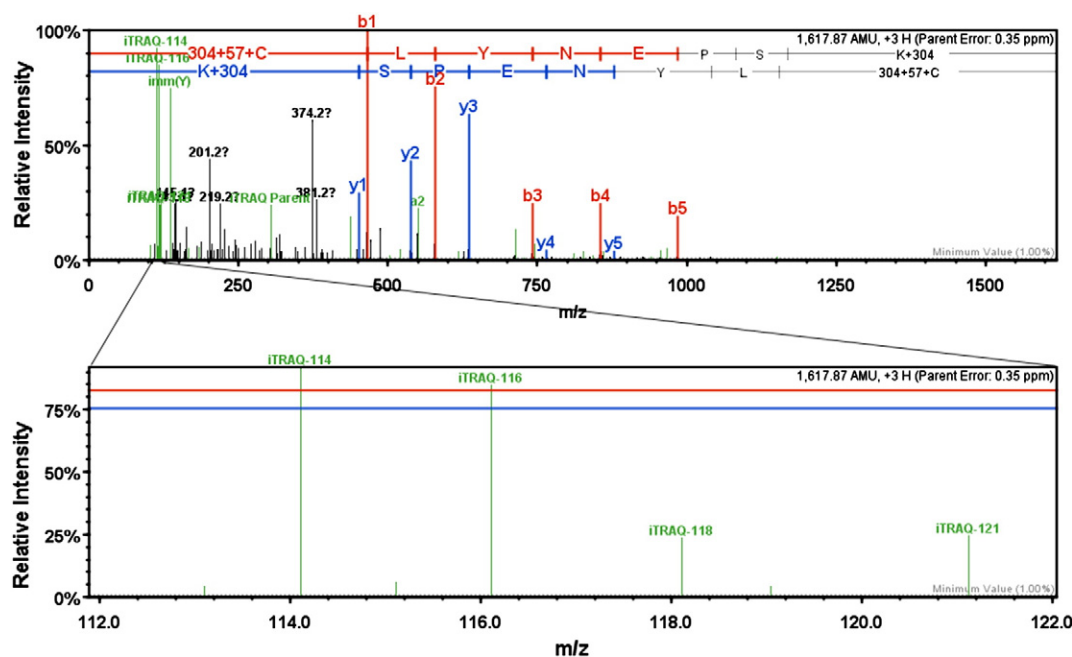


Fig. 2 – Representative result of iTRAQ analysis. The MS/MS spectrum of a peptide (GLYNEPSK, m/z 538.29³⁺) shows a 2.6-fold increased protein (zinc metalloproteinase–disintegrin jerdonitin, gi|48427991) in the neonate venom (iTRAQ 114 and 116) compared to the adult venom (iTRAQ 118 and 121).

neonate and adult venoms. The third one (glutamyl-peptide cyclotransferase) has been found in venoms from *C. atrox*, *B. jararaca* and *Boiga irregularis* [45, NCBI accession number A7ISW1.1], is similar to a conceptual translation of a cDNA sequence isolated from the venom gland of *G. blomhoffi* [NCBI accession number BAB69586.1], and might be responsible for the biosynthesis of pyroglutamyl peptides in venom [29]. We found that in *G. brevicaudus* this protein was up-regulated in the adult venom. The last one was first found in *Pseudonaja nuchalis* venom and is a member of protein disulfide isomerase (PDI) [46]. It is similar to conceptual translations of two cDNA coding sequences from venom glands of *Oxyuranus scutellatus scutellatus* [NCBI accession number AAY33972.1] and *D. acutus* [47]. The last protein may have roles in synthesizing toxic components with disulfide bridges and maintaining their active structures and, in this study, it was expressed no difference in neonate and adult venoms.

Although 224 unique peptides and 59 proteins were identified in this study, information on the differences in proteomes between neonate and adult venoms is still not enough. This should be due to the insufficient sequence databases of venom proteins or the lack of genomic information of venom gland for the analysis of MS data. Also, this might be influenced by different post-translational modifications, innumerable isoforms of venom proteins and incomplete tryptic digestions [17]. Moreover, the potential sample handling errors could be introduced while the parallel samples were digested and labeled separately. These errors might lead to quantitative bias and omission of proteins of low abundance. Thus, for a better understanding of ontogenetic shifts in quantitative venomomics in *G. brevicaudus*, a more powerful and informative database including whole transcriptome sequencing of venom gland should be constructed, and gel-based and MS techniques should

also be used to analyze the venom proteins that cannot be detected in LC-based methods. Twenty-three 2-DE spots were identified by MS/MS in this study. Of these 23 spots, four matched to the proteins identified in iTRAQ experiment. The quantitative differences between neonate and adult venoms could be confirmed mutually by these two methods in these matched spots/proteins (gi|4106007, gi|129437, gi|82095086 and gi|48428846).

3.3. N-deglycosylation analysis of neonate and adult venoms

Glycosylation, which is an important post-translational modification of snake venom, can affect the complexity in snake venomomics and change protein function through its influence on protein folding, oligomerization and stability [48,49]. The influence of N-glycosylation seems to be more prominent than that of O-glycosylation in most snake venom proteins [48].

In this study, we conducted a N-deglycosylation experiment to evaluate the glycosylation levels in neonate and adult venoms. After incubated with PNGase F, venoms were analyzed by 2-DE and gelatin zymography. Although the venom proteins were separated poorly in 2-DE gels while using 7 cm pH 3–10 broad strips than narrow strips, the electrophoretic profiles can still tell us a neonate-to-adult shift in venom proteins after the removal of N-linked carbohydrate chains. In the neonate venom, after N-deglycosylation, proteins with molecular masses/pI of ~61–92 kDa/4.5–7.7, ~45 kDa/5.0–5.4 and ~35 kDa/4.3–7.7 decreased dramatically and even disappeared, while proteins with ~55 kDa/6.6–7.0, ~30 kDa/5.1–7.6, ~25 kDa/4.8 and ~18.5 kDa/4.8–5.1 increased significantly (Fig. 3). In the adult venom, N-deglycosylation resulted in a quantitative rise of low to medium molecular components, including proteins with molecular masses/pI of ~55 kDa/6.6–7.0, ~26 kDa/6.0–6.4, ~25 kDa/4.5 and ~14.4–18.4 kDa/4.5–10.0, and a new protein

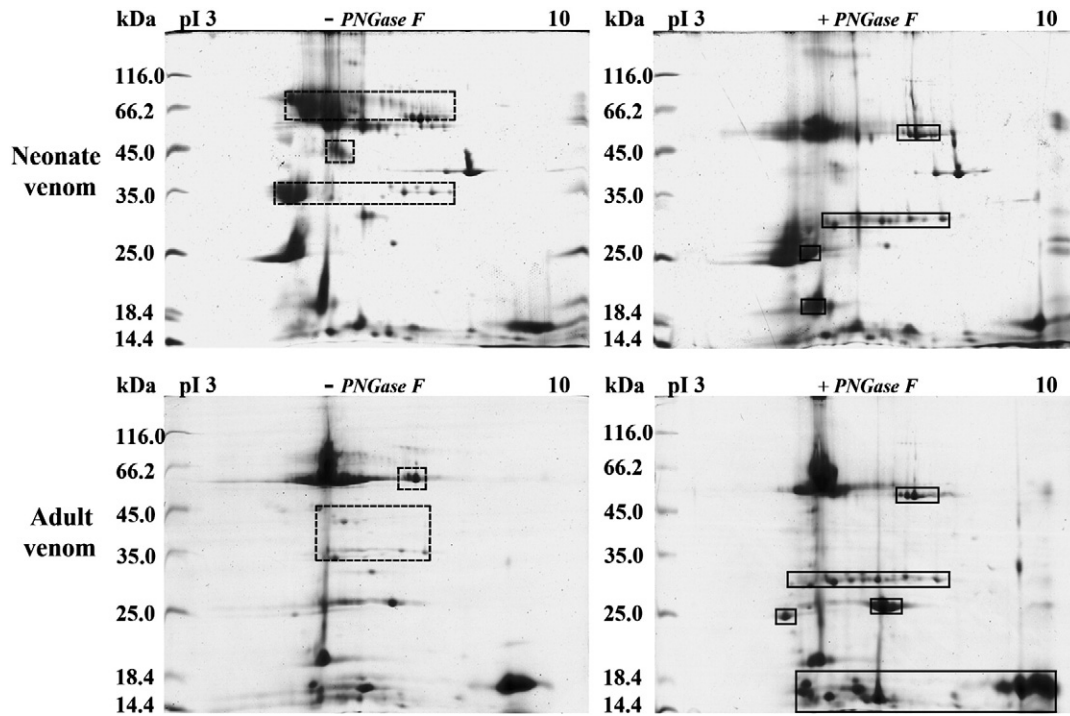


Fig. 3 – Glycoproteome analysis of neonate and adult venoms. Venom proteins incubated in the absence (–PNGase F) or presence (+PNGase F) of N-glycosidase F, and the electrophoretic profiles were carried out by 2-DE (7 cm, pH 3–10 strips). Spots in dash rectangular represent proteins reduced or disappeared after deglycosylation, and the spots in solid rectangular represent proteins increased after deglycosylation.

area with molecular masses/pI of ~30 kDa/4.6–7.6. Proteins with molecular masses/pI ~61 kDa/6.7–6.9 and ~32–45 kDa/5.0–7.0 decreased, even disappeared in the adult venom (Fig. 3). Similar to the glycosylation in *B. jararaca* venoms, the glycosylated proteins in *G. brevicaudus* belonged to different families, mainly including L-amino acid oxidase, metalloproteinase, serine proteinase, PLA₂ and nerve growth factor [50]. The glycosylation site can be predicted by amino acid sequence analysis [35], so further

study of glycoproteins in *G. brevicaudus* venom can be combined with glycosylation site prediction and biochemical experimental validation. In this study, the components around ~66 kDa were mainly identified as P-II and P-III metalloproteinases, and a part of them could be degraded after the treatment of PNGase F. What can be inferred from these results is that at least some P-II and P-III metalloproteinases are glycoproteins.

Our gelatin zymography analysis showed that the components with gelatinolytic activity in the adult venom were in the areas with molecular masses of ~43, 38 and 23 kDa and, in the neonate venom, these components were in the areas with molecular masses of ~46 and 23 kDa. After N-deglycosylation, the gelatinolytic activity was the same in both types of venoms as illustrated by the clear zone at 21 kDa (Fig. 4). This indicates that N-deglycosylation is an important modification that can induce the variability of metalloproteinase and serine proteinase with gelatinolytic activity in snake venom.

Although the results of this study showed that glycosylation could drive the complexity of venom proteins in *G. brevicaudus*, most differences in protein components were still present between neonate and adult venoms after N-deglycosylation. This indicates that N-deglycosylation is not the only post-translational modification that can explain differences between neonate and adult venoms.

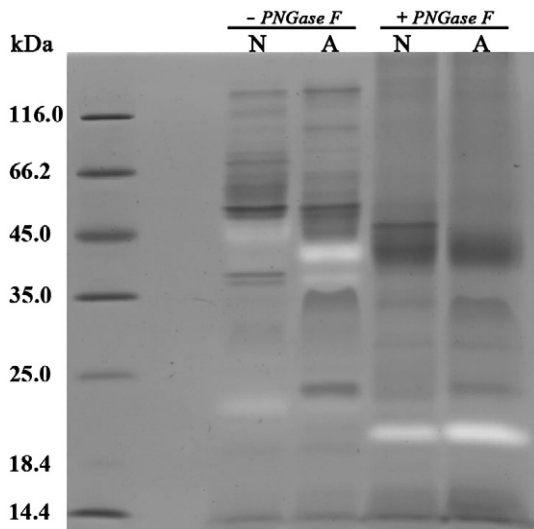


Fig. 4 – Gelatinolytic activity of neonate and adult venoms. Venom proteins incubated in the absence (–PNGase F) or presence (+PNGase F) of N-glycosidase F.

4. Conclusion

We studied the neonate-to-adult transition of snake venomics in *G. brevicaudus*, one of the four venomous snakes that are

responsible for the majority of envenomings and deaths from venomous snakebites in China. We used 2-DE, MALDI-TOF-MS/MS and iTRAQ to analyze the composition, relative quantity and glycosylation levels of venoms from neonates and adults. Metalloproteinases, C-type lectins and acidic D49-PLA₂ were more abundant in the neonate venom, while serine proteinases and basic D49-PLA₂ were more abundant in the adult venom. Quantitative differences between the two types of venoms were evident in several low abundance proteins. The N-glycosylation was an important post-translational modification, but it was not the only modification that could provide an explanation for differences between neonate and adult venoms. The observed neonate-to-adult transition of snake venomomics might be driven by the divergence in dietary habits between neonate and adult *G. brevicaudus*.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.04.003>.

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