Bothrops jararaca venom proteome rearrangement upon neonate to adult transition.

André Zelanis[1,2], Alexandre K. Tashima[1,3], Antônio F. M. Pinto[4], Adriana F. Paes Leme[5], Daniel R. Stuginski[6], Maria F. Furtado[6], Nicholas E. Sherman[7], Paulo L. Ho[2,8], Jay W. Fox[7], Solange M. T. Serrano[1].

[1] Laboratório Especial de Toxinologia Aplicada-CAT/cepid, Instituto Butantan, Brazil.
[2] Instituto de Química, Departamento de Bioquímica, Universidade de São Paulo, Brazil.
[3] Departamento de Ciências Exatas e da Terra, Universidade Federal de São Paulo, Brazil.
[4] Laboratório de Bioquímica Farmacológica, Centro de Biotecnologia (UFRGS), Porto Alegre, Brazil.
[5] Laboratório Nacional de Biociências, LNBio, Campinas, Brazil.
[6] Laboratório de Herpetologia, Instituto Butantan, Brazil.
[7] Department of Microbiology, University of Virginia, Charlottesville, USA.
[8] Centro de Biotecnologia, Instituto Butantan, Brazil.

Corresponding author:

S. M. T. Serrano
Laboratório Especial de Toxinologia Aplicada-CAT/cepid
Instituto Butantan
Av. Vital Brasil 1500, 05503-900, São Paulo, Brazil
Tel./Fax: +55 11 3726-1024
E-mail: solangeserrano@butantan.gov.br

Abbreviations: BPP, bradykinin potentiating peptide; CTL, C-type lectin; CRISP, cysteine-rich secretory protein; LAAO, L-amino acid oxidase; PLA2, phospholipase A2; SVMP, snake venom metalloproteinase; SVSP, snake venom serine proteinase.

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Abstract

The pharmacological activities displayed by *Bothrops jararaca* venom undergo a significant ontogenetic shift. Likewise, the diet of this species changes from ectothermic prey in early life to endothermic prey in adulthood. In this study we used large and representative newborn and adult venom samples consisted of pools from 694 and 110 specimens, respectively, and demonstrate a significant ontogenetic shift in the venom proteome complexity of *B. jararaca*. 2-DE coupled to mass spectrometric protein identification showed a clear rearrangement of the toxin arsenal both in terms of the total proteome, as of the glycoproteome. *N*-glycosylation seems plays a key role in venom protein variability between newborn and adult specimens. Upon the snake development, the subproteome of metalloproteinases undergoes a shift from a P-III-rich to a P-I-rich profile while the serine proteinase profile does not vary significantly. We also used isobaric tag labeling (iTRAQ) of venom tryptic peptides for the first time to examine the quantitative changes in the venom toxins of *B. jararaca* upon neonate to adult transition. The iTRAQ analysis showed changes in various toxin classes especially the proteinases. Our study expands the in-depth understanding of venom complexity variation particularly with regard to toxin families that have been associated with envenomation pathogenesis.

1. Introduction

The variability found in venom proteomes is an intriguing aspect of the evolution of venomous snakes. Although there is not a general pattern, the causes of variability are often related to the snake diet in addition to geographical and biological factors, such as time of isolation of a given species, inbreeding process, sexual dimorphism, and ontogenetic changes in diet habits [1-7]. The relationship between diet and ontogenic shift was showed by Andrade and Abe [8] with two species of the *Bothrops* genus which display quite different dietary habits: *B. alternatus*, a *strictu sensu* generalist, which feeds only on rodents during its whole life span, and *B. jararaca*, a generalist which feeds on ectothermic prey (mainly arthropods, lizards, and amphibians) through juvenile phase and on endothermic animals (mainly small mammals) during adult life [8]. These authors have found striking differences in LD_{50} values, with *B. alternatus* venom being more lethal to rodents and *B. jararaca* more lethal to amphibians. In another study, the relationship between snake diet and venom composition was also shown by Li et al. [3] with a three finger neurotoxin (3FTx) present in the marbled sea snake *Aipysurus eydouxii* venom. The authors suggested that as a result of a significant change in dietary habits, a dinucleotide deletion in the only 3FTx gene expressed in the *A. eydouxii* venom gland resulted in frame shift and truncation, with an accompanying loss of neurotoxicity likely as a secondary consequence of adaptation to a new diet. In this context, it is interesting to note the key role that diet exerts in snake venom composition, acting mainly as a selective factor. In contrast, it has been recently shown that the venom composition of *Crotalus simus* changes dramatically during development, from a neurotoxic to a hemorrhagic phenotype, whereas the species feeds from its birth primarily on small

rodents and lizards [9]. On the other hand, it should also be considered that changes in venom composition may also represent a neutral feature and would become permanent within a snake population only if the resulted venom change conferred reproductive suitability.

Most studies on snake venom proteomes have been conducted using venoms derived from adult specimens and, therefore, the age-related variability is less understood. Since the accidents with newborn specimens show distinct pathological effects in human victims than those with adult specimens [10-12], the knowledge of the content of venom newborn toxins can help to elucidate the mechanisms of envenoming as well as to improve the management of snake bite treatment and the design of antivenoms.

In the last decade proteomic and transcriptomic approaches have been vastly applied in toxinology studies and contributed to new insights into the venom complex mixtures of proteins and peptides and the formulation of hypotheses integrating biological and biochemical aspects of venom production (for reviews see [13] and [14]). Although a number of venom gland transcriptomes have been described, proteomic approaches are crucial for a thorough understanding of venom composition since the majority of snake venom toxins are post-translationally modified. On the other hand, one of the major difficulties faced in the study of venom proteins by mass spectrometry is the lack of a comprehensive sequence database such as a snake genome. From this perspective, as important as it is to know which classes of toxins are present in a given venom proteome, it is also essential to know their abundance in the venom and the implications for the pathophysiology of the accidents.

In Brazil, *B. jararaca* is one of the most abundant venomous snake species [15]. Two-dimensional electrophoresis (2-DE) coupled with LC-MS/MS has been used for examining *B. jararaca* venom complexity along with other interesting approaches to selectively delineate subpopulations of venom toxins based on particular characteristics of the proteins such as antibody cross-reactivity or enzymatic activities [16, 17]. The analysis of *B. jararaca* venom by in-solution digestion with trypsin and ion-currents of tryptic peptides with FT-ICR LC-MS/MS revealed 42 individual proteins representing 12 venom protein classes [18]. Furthermore, the analysis of the venom peptidome of *B. jararaca* sibling and non-sibling specimens showed sex-based differences among the bradykinin potentiating peptides (BPPs) [19].

Envenomation cases with *B. jararaca* are complex, with local and systemic effects, such as hemorrhage, myonecrosis, bleeding and severe coagulatopathy [11, 20]. Interestingly, the pathophysiological profiles of accidents with newborn and adult *B. jararaca* snakes are distinct, especially regarding the coagulation disorders, which seem to be prominent in accidents with newborn specimens [10, 11]. In a previous study on the variability of *B. jararaca* venom we demonstrated that the adult venom has a slightly higher lethal activity upon mice; however, the newborn venom is extremely more potent to kill chicks [7]. Moreover, the coagulant activity of newborn venom upon human plasma is ten times higher than that of adult venom. These differences in pathological effects were clearly reflected in the venom different electrophoretic, gelatinolytic, immunostaining, peptidomic and glycoproteomic profiles.

In this work we further explored the variability of newborn and adult *B. jararaca* venom proteomes by 2-DE using broad and narrow Immobilized pH Gradients for

first dimension separation. We also used isobaric tag labeling (iTRAQ™ [21] of venom tryptic peptides in order to examine the quantitative changes in the venom toxins of *B. jararaca* upon ontogeny. iTRAQ™ analysis of snake venom proteins has not previously been reported and greatly expanded the in-depth understanding of venom complexity variation particularly with regard to protein families that have been associated with envenomation pathogenesis.

2. Material and methods

2.1 *B. jararaca* venom samples

Pooled venom samples from 694 two-week old newborns (359 male and 335 female specimens) born from snakes bred in captivity at the Herpetology Laboratory of Instituto Butantan, and 110 adults (49 male and 61 female specimens older than 3 years) from São Paulo State (Brazil) was used in this study. The venom was milked, centrifuged for 30 min at 2000 x *g*, 4 °C, to remove any scales or mucus, lyophilized and stored at -20°C until use. Venom protein concentrations were determined using the Bradford reagent [22] (Sigma, St. Louis, MO, USA) and bovine serum albumin (Sigma, St. Louis, USA) as a standard.

2.2 2-D electrophoresis (2-DE)

Venom samples were dissolved in MilliQ® water and mixed with DeStreak rehydration solution containing 1% immobilized pH gradient (IPG) buffer (GE Healthcare, Uppsala, Sweden) to final volume of 450µL (for 24 cm strips) or 125 µL (for 7 cm strips) and sonicated for 10 min at room temperature. First dimension was carried out in an Ettan IPGphor Isoelectric Focusing System (GE Healthcare,

Uppsala, Sweden). Precast IPG strips (24 or 7 cm; pH 3-10 linear or pH 4-7 linear) were employed for first dimension separation at 20°C using a three-phase electrophoresis program for 7 cm strips (500 V for 45 min; 4000 V for 180 min and 5000 V for 60 min) or a seven-phase electrophoresis program for 24 cm strips(30V for 6h, 150V for 2h, 350V for 1h, 500V for 1h, 1000V for 1h, 3000V for 1h and 5000V for 13h). Prior to running the second dimension, IPG strips were placed in a rehydration tray and the protein strips were reduced and alkylated by sequential incubation in the following solutions: 0.05 M Tris- HCl, pH 8.4, 1% SDS; 30% glycerol (equilibration buffer-EB), 20 mg/mL DTT in EB; and then a solution of 30 mg/mL iodoacetamide in EB. Then they were directly applied to 12% SDS-polyacrylamide gels (10 cm x 10 cm x 1.0 mm for 7cm strips or 20 cm x 26 cm x 1.5 mm for 24 cm strips) for second dimension electrophoresis. The gels were stained with silver according to [23].

2.3 *N*-deglycosylation analysis

For protein deglycosylation, venom samples (30 µg) were incubated in 10% SDS for 1 min at 95 °C. After adding 0.02 M sodium phosphate buffer, 0.08% sodium azide, 0.01 M EDTA, 2% Triton X-100, pH 7.0, incubation was prolonged for 2 min at 95 °C. After cooling, 2 U of N-glycosidase F (Roche, Mannheim, Germany) was added, and the mixture was incubated for 18 h at 37°C. The deglycosylation profiles were evaluated by 2-DE using 7 cm, pH 3-10 strips, as described above.

2.4 Mass spectrometric protein identification of 2-DE spots

Protein spots were excised and in-gel trypsin digestion was performed according to Hanna and colleagues [24]. An aliquot (7.5 µL) of the resulting peptide mixture was

separated by in-house C18 column (8 cm x 75 µm) (Phenomenex, Torrence, CA, USA) RP-HPLC coupled with nano-electrospray tandem mass spectrometry on a Thermo Electron LTQ XL ion-trap mass spectrometer at a flow rate of 500 nL/min. The gradient was 2-80% acetonitrile in 0.1 M acetic acid over 45 min. The instrument was operated in the 'top ten' mode, in which one MS spectrum is acquired followed by MS/MS of the top ten most-intense peaks detected. Full dynamic exclusion was used to enhance dynamic range – one spectrum before exclusion for 120 sec . The resulting fragment spectra were processed using Bioworks v3.3.1 (Thermo Scientific, San Jose, CA, USA) and Mascot Generic Files (*MGFs) were created, exported and searched using MASCOT search engine (Matrix Science, UK) against the NCBI NR database restricted to the taxa Serpentes (25211 entries; downloaded in June 20th, 2011) with a parent tolerance of 1.50 Da and fragment tolerance of 1.0 Da. Iodoacetamide derivative of cysteine and oxidation of methionine were specified in MASCOT as fixed and variable modifications, respectively.

2.5 Isobaric tag peptide labeling with iTRAQ™

Venom samples (100 µg) were dissolved in the dissolution buffer of the iTRAQ 4-plex kit™ and the (*in-solution*) trypsin digestion procedure was carried out according to manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The samples were analyzed as follows: the tryptic peptides derived from newborn venom proteins were labeled with 114 and 116 iTRAQ™ reagents while the adult venom peptides were labeled using 115 and 117 iTRAQ™ reagents. The labeled peptide samples (newborn and adult venoms) were mixed and dissolved in Strong Cation Exchange (SCX) buffer A (10 mM phosphoric acid in 25% acetonitrile, pH 2.0) and submitted to SCX chromatography using a MonoS column (GE Healthcare, Uppsala, Sweden).

The labeled peptides were eluted over a gradient of 0-30% of buffer B (1M NaCl in buffer A). Collected fractions were pooled and dried in a SpeedVac concentrator, resuspended in 0.5% trifluoroacetic acid and submitted to desalting using C-18 solid phase extraction cartridges (Sep-Pak light, Waters, Milford, MA, USA). Desalted pools of labeled peptides were dried and resuspended in 20 μL of 0.1% formic acid for LC-MS/MS analysis.

2.6 Mass spectrometric protein identification and quantitation of iTRAQ™ labeled peptides by LC-MS/MS

An aliquot (4.5 μL) of each SCX pool was injected into a trap column packed with C18 (180 *μ*m i.d. × 20 mm) (Waters, Milford, MA, USA) for desalting with 100% solvent A (0.1% formic acid) at 15 *μ*L/min for 12.5 min. Peptides were then eluted onto an analytical C18 column (100 *μ*m i.d. × 100 mm) (Waters, Milford, MA, USA) using a 95 min gradient at a flow rate of 600 nL/min where solvent A was 0.1% formic acid and solvent B was 0.1% formic acid in acetonitrile. The gradient was 2-10% of solvent B in 10 min, 10-40% B in 65 min, 40-80%B in 15 min, 80-90%B in 2 min, then back to 90% A in 3 min. A Q-TOF Ultima mass spectrometer (Waters, Milford, MA, USA) was used to acquire spectra. Spray voltage was set at 2.5 kV and the instrument was operated in data dependent mode, in which one full MS scan was acquired in the m/z range of 100-2000 followed by MS/MS acquisition using collision induced dissociation of the three most intense ions from the MS scan. A dynamic peak exclusion was applied to avoid the same m/z of being selected for the next 120 sec. Raw data files were processed by ProteinLinx 2.2 (Waters, Milford, MA, USA) and converted to *pkl format for searching against an NCBI NR database with the parameters described above using MASCOT search engine, with a parent tolerance

of 0.1 Da and fragment tolerance of 0.1 Da. Methyl methanesulfonate (MMTS) derivative of cysteine and oxidation of methionine were specified in Mascot as fixed and variable modifications, respectively. In addition, chemical derivatization of peptide N-terminus and lysine side chains with iTRAQ^{TM} reagent were specified in Mascot as fixed modification.

Scaffold 3 Q+(version Scaffold_3_00_01, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications and to perform the quantitative analyses between the samples. The ratio values were expressed as mean ± standard deviation (SD) of 4 LC-MS/MS runs. Peptide identifications were accepted if they exceeded specific database search engine thresholds. MASCOT identifications required ion scores greater than both the associated identity scores and 20, 30, 40 and 40 for singly, doubly, triply, and quadruply charged peptides. Protein identifications were accepted if they contained at least 1 identified peptide. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Peptides were quantitated using the centroided reporter ion peak intensity. Multiple isobaric tag samples were normalized by comparing the median protein ratios for the reference channel. Protein quantitative values were derived from only uniquely assigned peptides. The minimum quantitative value for each spectrum was calculated as the 5.0% percent of the highest peak. Protein quantitative ratios were calculated as the median of all peptide ratios. Standard deviations were calculated as the interquartile range around the median. Quantitative ratios were Log_2 normalized for final quantitative testing. Log_2FC was calculated taken into account newborn venom as reference. Differentially expressed proteins were determined using *t*-test analysis. To estimate the False Discovery Rate (FDR)

of the search results a decoy database was used. This database contained the same protein entries in the specified database but with their sequences reverse-oriented. The FDR of all searches was set to <1%.

3. Results and discussion

3.1 Comparison of newborn and adult venoms by 2-DE and mass spectrometric protein identification

Our previous analysis of the newborn and adult *B. jararaca* venoms by SDS-polyacrylamide gel electrophoresis revealed that the venom profiles under reducing conditions are not identical but show various common bands, however, under non-reducing conditions the profiles are rather different in appearance with the adult venom showing various protein bands which are absent in the newborn venom [7]. Here, the 2-DE analysis using a broad range pH gradient in the first dimension separation (3-10 IPG strips) revealed that most *B. jararaca* venom proteins are acidic with p*I* values ranging from 3.5 to 7.0. The second observation was related to a group of smearing spots forming an undefined blur of acidic proteins at p*I* values ranging from 4.0 to ~6.5 that was present in the newborn venom profile. For this reason, in order to achieve a better definition of the individual spots we decided to perform an analysis using linear IPG strips ranging from pH 4 to 7. As observed in Figure 1, as the p*I* range narrows there is an increasing resolution of the proteins within that range with the result of discrimination of additional spots that were not visualized in the broader p*I* range gels of newborn and adult venoms. In fact, the isoelectro focusing on narrower range IPG strips enabled the visualization of subtle

differences in the venom proteomes, as illustrated by a group of spots of p*I* values between 4 and 5.5 and molecular mass of around 50-80 kDa which contained mainly metalloproteinases (Figure 1; Table 1; Supplemental tables 1 and 2). This region of the newborn venom gel showed a higher number of protein spots of higher intensity compared to the same region of the adult venom indicating that metalloproteinases of the P-III class are more abundant in the newborn venom. On the other hand, the adult venom showed a remarkably distinct profile of proteins of molecular mass of ~25 kDa and pI values of 6-7. These spots were much more abundant in the adult venom and were identified as P-I class metalloproteinases (Figure 1; Table 1; Supplemental tables 1 and 2). In our previous comparison of newborn and adult venoms we used an anti-metalloproteinase antibody for immunostaining which recognized similar protein bands in both venoms indicating that the metalloproteinases in these venoms were structurally related and there was no detectable ontogenetic shift in their contents [7]. The distinct profile/content of metalloproteinases detected here by the 2-DE analysis and protein identification clearly indicates a shift from a P-III rich newborn venom to a P-I rich adult venom. Similar results were also reported by [25] on the analysis of the Amazonian viperid species *B. atrox* and by Alape-Gíron et al. [6] for the *B. asper* venom. However, despite of the higher content of P-III SVMPs in newborn venom, our results suggest that newborn P-III metalloproteinases might differ in their degree of post-translation modification. P-III class SVMPs are also abundant in the adult venom, as illustrated by the negative-silver stained spots 1 and 2 in the 2-DE image (Figure 1) which were identified as metalloproteinases (Table 1; Supplemental tables 1 and 2). In accordance to our previous results, the hemorrhagic activity of newborn and adult venoms, a recognized feature of many P-III class metalloproteinases, was found to

be similar between newborn and adult venoms [7]. However, other findings related to the abundance of metalloproteinases in the newborn and adult venoms are important to explain the higher coagulant activity displayed by the newborn venom. Functional studies showed that the prominent coagulant activity of the newborn venom upon human plasma and its higher activation of Factors II and X of the blood coagulation cascade are due to the activity of metalloproteinases [7]. The lack of data on the characterization of toxins present in newborn snake venoms leads to the poor knowledge of the biochemical/biophysical properties of these toxins as well as their potential to interfere with biological systems of prey and/or human victims. Metalloproteinases of the P-III class may show a range of molecular masses depending on their post-translational modifications such as glycosylation, dimerization or the addition of lectin-like domains [26]. The abundance of metalloproteinases of high molecular mass in the newborn venom suggests they are implicated in the outstanding coagulant activity that characterizes the envenomation profile. Moreover, the proteinases are expected to undergo post-translational modifications that are important for the interaction with Factors II and X of the coagulation cascade [27-30].Other regions of the gels were less distinct in terms of number and intensity of spots, however, some clear differences were detected in terms of the class of toxins that populate each specific region. For instance, the profile of spots of p*I* 4.5-5.5 and molecular mass of ~14 kDa were similar between the venoms; nevertheless, protein identification revealed the presence of phospholipases A2 in the newborn venom and C-type lectins in the adult venom (Figure 1; Table 1; Supplemental tables 1 and 2). Taken together, these results suggest that although the venoms do share many homologous proteins, the relative amounts of the homologues may vary greatly between the venoms.

3.2. *N*-deglycosylation analysis of newborn and adult venoms

The glycoproteome comparison between newborn and adult venoms using glycosidases and SDS-PAGE analysis showed a significant shift in the molecular masses of proteins of both newborn and adult venoms after removal of *N*-linked carbohydrate chains [7]. On the other hand, *O*-glycosylation did not seem to be a post-translational modification in most newborn and adult venom proteins as no significant change in the pattern of protein migration was observed after incubation of venoms with *O*-glycosidase [7].

Figure 2 shows that the distinct 2-DE profiles of newborn and adult venoms are rendered rather similar after deglycosylation with *N*-glycosidase. Despite the closer resemblance between the venoms after deglycosylation, the region of the newborn venom gel located at ~50 kDa with p*I*s between 4.5 and 5.5 still contains a large smeared spot that is absent in the adult venom gel (Figure 2). Moreover, *N*-deglycosylation did not significantly affect the molecular mass and p*I* of most protein spots found below 15 kDa indicating that glycosylation is not a post-translational modification of these proteins and this feature is common for both venoms. In addition, the complexity of the gel region below 15 kDa seemed to increase likely as a result of the loss of stability of glycoproteins after carbohydrate removal. Another interesting observation stemming from the N-deglycosylation experiments is that the large group of spots of acidic, high molecular mass proteins present in both newborn and adult gel images contains mainly glycoproteins since these spots nearly disappeared from the gels after deglycosylation. The fact that various metalloproteinases were identified as present in these spots (Table 1) points out to

the importance of glycosylation as a post-translational modification of metalloproteinases, as illustrated by the fact that the newborn venom was shown to contain metalloproteinases involved in the activation of coagulation factors II and X which are virtually absent or much less abundant in the adult venom [7]. Glycosylation of snake venom proteins is thought to act in distinct ways, both increasing the stability of glycoprotein scaffolds and preventing the interaction of venom glycoproteins with physiological inhibitors of the prey. In this regard, is would be interesting to explore whether the difference in the *N*-glycosylation level between the venoms is quantitative, qualitative or both (*e.g.* distinct composition of glycan structures attached to Asn side chains, different levels of glycosylation motif occupancy, or both). Whichever is the case, it is clear that *N*-glycosylation is one of the main factors related to the differences in the venom proteomes of newborn and adult specimens of *B. jararaca*.

3.3 Quantitative comparison of newborn and adult *B. jararaca* venom proteomes

In order to further explore the variability of the newborn and adult *B. jararaca* venom proteomes we carried out for the first time a quantitative analysis of venom proteins using iTRAQ™ tags. For this purpose, the newborn and adult venoms were separately submitted to trypsin digestion and then the iTRAQ™ tags were attached to the peptides via free amines at the peptide N-terminus and on the side chain of Lys residues. The labeled samples were then pooled and analyzed simultaneously by LC-MS/MS using a Q-TOF Ultima mass spectrometer. To diminish risk of reporting false positive identifications and to assess the quality of obtained data, a

false discovery rate analysis was performed using a decoy database composed of the protein entries of the NCBI NR database of the taxa Serpentes with reverse-oriented sequences. After processing the resulting MS/MS spectra and bioinformatic analysis, we were able to identify and quantify 29 venom proteins (Table 2). The efficient labeling of newborn and venom tryptic peptides with iTRAQ™ tags is illustrated in Figure 3 which shows a representative spectrum of peptide LDSPVK (*m/z* 473.78^{2+}) derived from the serine proteinase KN-BJ (gi|13959622|).

One common characteristic of the proteomic analysis of snake venoms is the low number of proteins that is identified by mass spectrometric analysis due to the lack of venom sequence databases for analysis of MS data [13]. There are various possible explanations for the low number of matches in the iTRAQ™ experiment, such as differential post-translational modification, differential post-translational proteolytic precursor processes with consequent change in tryptic peptide production, the presence of innumerous isoforms of proteins in snake venoms, incomplete tryptic digestions, etc. As is the case of various shotgun methodologies, some drawbacks were noticed in the quantitative comparative analysis of newborn and adult venoms. Ideally, isobaric labeling of tryptic peptides for quantitative purposes has the aim of analyzing similar samples under different experimental conditions (*e.g.* control cell culture *versus* stimulated cell culture). Our results indicated that the proteomes of newborn and adult *B. jararaca* venoms are significantly distinct, primarily due to post-translational modifications. Therefore, the presence of unknown sequences in one or both samples would make it difficult to assign a given peptide to a specific protein. Another technical issue is related to presence of C-terminal Pro residues in venom proteins and peptides. Virtually a C-terminal Pro will result in a MS/MS spectrum in which a y_1 ion of *m/z* 116 will be

present and, usually, with high intensity. Unless high resolution mass spectrometers are employed for the analysis, the quantification of the iTRAQ™ 116 reporter ion may be biased. Most viperid snake venoms contain large amounts of a toxin family that shows a high content of Pro residues, mainly at C-terminus: the Bradykinin Potentiating Peptides (BPPs). Using electrospray ionization the BPP spectra are often less informative with respect to the *b* and *y* ion series, because of the ion suppression caused by protonation of Pro residues. We have shown that BPP spectra have usually high signals of y_1 and y_2 ions (*m/z* of 116 and 213), corresponding to the terminal Pro residues (P and P-P, respectively) [7]. Therefore, caution must be taken when analyzing iTRAQ™ labeled samples where these peptides are present. Despite these experimental drawbacks, significant differences were detected between the samples. In agreement with our analysis by 2-DE and protein identification (Figure 1; Table 1), the metalloproteinases were clearly detected as more abundant in the newborn venom, however, it was not possible to discriminate among P-III, P-II and P-I class metalloproteinases in the iTRAQ™ experiment because all identified and quantified peptides derived from the catalytic domain of these proteinases (Table 2; Supplemental table 3) [26]. In contrast, serine proteinases were found as more expressed in the adult venom (Table 2). Cysteine-rich secretory proteins and VEGF were more abundant in the newborn venom, while the L-amino acid oxidases were detected in higher amount in the adult venom (Table 2). In the case of the phospholipases A2 no clear differential expression was detected between the newborn and adult venoms. We would suggest that the iTRAQ™ approach is a reasonable method to obtain a relatively good picture of a given venom proteome in different biological situations (*e.g.* life phases), both in terms of what is present (i.e. what toxin classes) as well as the relative amounts of

these toxins. However, unlike the gel-based approaches one gains no information of toxin post-translational modifications, which in many instances are crucial to correctly identify certain venom toxins. The major quantitative changes detected between the newborn and adult venom proteomes suggests a reshuffling of the main components implicated in the pathological effects, i.e. metalloproteinases and serine proteinases, upon transition from early life to adulthood.

4. Concluding remarks

Human accidents caused by newborn and adult *B. jararaca* specimens are characterized by significantly different pathological effects. As demonstrated by Antunes and colleagues [12], *in vitro* and *in vivo* assays showed that the Brazilian commercial antibothropic antivenom, which is produced by immunization with venom from adult specimens, is less effective in neutralizing newborn venom effects. Hence, it would be advisable to incorporate venoms from newborn snakes in the venom pool used for hyperimmunization of horses for antivenom production.

The size (age) change of *B. jararaca* is associated with a clear ontogenetic shift in diet from ectothermic prey in early life to endothermic prey in adulthood. In this study we demonstrated that newborn and adult venoms show notable differences in their 2-DE profiles and the identification of selected differential protein spots between the gels indicated a clear change in the content of metalloproteinases of the P-I and P-III classes. Removal of *N*-linked glycans generated similar 2-DE profiles of newborn and adult venoms indicating that *N*-glycosylation plays a key role in imprinting variability to venom proteomes. The comparative analysis using iTRAQ™ detected quantitative changes in various toxin classes especially among the proteinases. The

metalloproteinases were found as more abundant in the newborn venom and in contrast, serine proteinases were more expressed in the adult venom. The general rearrangement of the proteome profile of newborn and adult *B. jararaca* venom is an interesting biological phenomenon where the body ontogenetic change is combined with a tuning of the venom toxicity to deal with larger prey by adult snakes.

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5. References

[1] Chippaux, J. P., Williams, V., White, J. Snake venom variability: methods of study, results and interpretation. *Toxicon* 1991, *29*, 1279-1303.

[2] Daltry, J.C.; Wüster, W.; Thorpe, R.S. Diet and snake venom evolution. *Nature* 1996, *379*, 537-540.

[3] Li, M., Fry, B.G., Kini, R.M. Eggs-only diet: its implications for the toxin profile changes and ecology of the marbled sea snake (*Aipysurus eydouxii*). *J. Mol. Evol.* 2005, *60*, 81-89.

[4] Mackessy, S.P., Sixberry, N.M., Heyborne, W.H., Fritts, T. Venom of the brown treesnake, *Boiga irregularis*: ontogenetic shifts and taxa-specific toxicity. *Toxicon* 2006, *47*, 537-548.

[5] Zelanis, A., Ventura, J.S., Chudzinski-Tavassi, A.M., Furtado, M.F.D. Variability in expression of *Bothrops insularis* snake venom proteases: An ontogenetic approach. *Comp. Biochem. Physiol. C* 2007, *145*, 601-609.

[6] Alape-Giron, A., Sanz, L., Escolano, J., Flores-Dias, M. et al. Snake venomics of the lancehead *Bothrops asper*. Geographical, individual and ontogenetic variations. *J. Proteome Res*. 2008, *7*, 3556-3571.

[7] Zelanis, A., Tashima, A.K., Rocha, M.M.T., Furtado, M.F., Camargo, A.C.M., Ho, P.L., Serrano, S.M.T. Analysis of the ontogenetic variation in the venom proteome/peptidome of *Bothrops jararaca* reveals different strategies to deal with prey. *J. Proteome Res.* 2010, *9*, 2278-2291.

[8] Andrade, D.V., Abe, A. Relationship of venom ontogeny and diet in *Bothrops*. *Herpetologica* 1999, *55*, 200-204.

[9] Calvete, J.J., Sanz, l., Cid, P., Torre, P., Flores-Dias, M.F., Santos, M.C., Borges, A.,Bremo, A., Angulo,Y., Lomonte, B., Alape-Girón, A., Gutiérrez, J.M. Snake venomics of the central American rattlesnake *Crotalus simus* and the South american *Crotalus durissus* complex points to neurotixicity as an adaptive

paedomorphic trend along *Crotalus* dispersal in South America. *J. Proteome Res*. 2010, *9*, 528-544.

[10] Ribeiro, L.A., Jorge, M.T. Acidente por serpentes do gênero *Bothrops*: série de 3.139 casos. *Rev.Soc.Bras.Med.Trop.* 1997, *30*, 475-480.

[11] Santoro, M.L., Sano-Martins, I.S., Fan, H.W., Cardoso, J.L.C., Theakston, R.D.G., Warrell, D.A., BIASG. Hematological evaluation of patients bitten by the jararaca, *Bothrops jararaca*, in Brazil. *Toxicon* 2008, *51*, 1440-1448.

[12] Antunes, T.C.,Yamashita, K.M., Barbaro, K.C., Saiki, M., Santoro, M.L. Comparative analysis of newborn and adult *Bothrops jararaca* snake venoms. *Toxicon* 2010, *56*, 1443-1458.

[13] Fox. J.W., Serrano, S.M.T. Exploring snake venom proteomes: multifaceted analyses for complex toxin mixtures. *Proteomics* 2008, *8*, 909-920.

[14] Calvete, J.J., Sanz, L., Angulo, Y., Lomonte, B., Gutiérrez, J.M. Venoms, venomics, antivenomics. *FEBS Lett.* 2009, *583*,1736-1743.

[15] Campbell, J.A, Lamar, W.W. The venomous reptiles of latin america. 6° ed. Ithaca and London, Comstok, 1989**.**

[16] Serrano, S.M.T., Shannon, J.D., Wang, D., Camargo, A.C.M., Fox, J.W. A multifaceted analysis of viperid snake venoms by two-dimensional gel

electrophoresis: An approach to understanding venom proteomics. *Proteomics* 2005, *5*, 501-510.

[17] Paes-Leme, A.F., Kitano, E.S., Furtado, M.F., Valente, R.H., Camargo, A.C., Fox, J.W., Serrano, S.M.T. Analysis of the subproteomes of proteinases and heparin-binding toxins of eight *Bothrops* venoms. *Proteomics*, 2009 *9*, 733-745.

[18] Fox, J.W., Ma, L., Nelson, K., Sherman N.E., Serrano, S.M.T. Comparison of indirect and direct approaches using ion-trap and Fourier transform ion cyclotron resonance mass spectrometry for exploring viperid venom proteomes. *Toxicon* 2006, *47,*700-714.

[19] Pimenta, D.C., Prezoto, B.C., Konno, K., Melo, R.L. et al. Mass spectrometric analysis of the individual variability of *Bothrops jararaca* venom peptide fraction. Evidence for sex-based variation among the bradykinin-potentiating peptides. *Rapid Commun. Mass. Spectrom.* 2007, *21*, 1034-1042.

[20] Rosenfeld, G. Symptomatology, pathology and treatment of snake bites in South America. In: Bucherl, W. Buckley E. (Eds). Venomous Animals and their Venoms, Academic Press, New York, 1971.

[21] Ross, P.L., Huang, Y.N., Marchese, J.N., Williamson, B., Parker, K., Hattan, S, Khainovski, N., Pillai, S., et al. Multiplexed protein quantitation in *Saccharomyces cerevisae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 2004, *3*, 1154-1169.

[22] Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 1976, *72*, 248-54.

[23] Mortz, E., Krogh, T. N., Vorum, H., Gorg, A. Improved silver staining protocols for high sensitivity protein identification using matrix assisted laser desorption/ionization-time of flight analysis. *Proteomics* 2001, *1*, 1359-1363.

[24] Hanna, S.L., Sherman, N.E., Kinter, M.T., Goldberg, J.B., Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography-tandem mass spectrometry. *Microbiology* 2000, *146*, 2495-2508.

[25] Guércio, R.A.P., Shevchenko, A., Shevchenko, A., López-Lozano, J.L., Sousa, M.V., Ricart, C.A.O. Ontogenetic variations in the venom proteome of the Amazonian snake *Bothrops atrox*. *Prot. Sci.* 2006, *4*, 11.

[26] Fox, J.W., Serrano, S.M. Insights into and speculations about snake venom metalloproteinase (SVMP) synthesis, folding and disulfide bond formation and their contribution to venom complexity. *FEBS J.* 2008, *275*, 3016–3030.

[27] Nishida,S., Fujita,T., Kohno, N., Atoda, H., Morita, T., Takeya, H., Kido, I., Paine, M.J.I., Kawabata, S., Iwanaga, S. cDNA cloning and deduced amino acid

sequence of prothrombin activator (ecarin) from Kenyan *Echis carinatus* venom. *Biochemistry* 1995, *34*, 1771-1778.

[28] Kini, R.M. The intriguing world of prothrombin activators from snake venom *Toxicon* 2005, *45*, 1133-1145.

[29] Chen H.S., Chen, J.M., Lin, C.W., Khoo K.H., Tsai, I.H. New insights into the functions and N-glycan structures of factor X activator from Russell's viper venom. *FEBS J.* 2008, *275,* 3944-3958.

[30] Rao, V.S., Swarup, S., Kini, R.M. The nonenzymatic subunit of pseutarin C, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom, shows structural similarity to mammalian coagulation factor V. *Blood* 2003, *102*, 1347-1354.

Legends for figures

Figure 1. Comparison of 2-DE patterns of *B. jararaca* newborn and adult venoms. Venom (350 μg) was applied to IPG strips (24 cm) followed by electrophoresis on 12% SDS-polyacrylamide gels (20 cm x 26 cm). Proteins were stained with silver. The insets at the bottom correspond to magnified sections of the gels showing the differential abundance of proteins of p*I* 6.0-6.5 and 20-25 kDa.

Figure 2. Glycoproteome analysis of newborn and adult *B. jararaca* venoms. 2-DE (7 cm, pH 3-10 strips) profiles of venom proteins (30 μg) incubated in the absence and in the presence of *N*-glycosidase F. Proteins were stained with silver. Electrophoretic

profiles of venoms before (blue) and after (red) *N*-deglycosylation were overlayed (right panel).

Figure 3. Representative result of iTRAQ™-LC-MS/MS analysis. Example of MS/MS spectrum of a 1.7 fold increased protein (serine proteinase KN-BJ) in the adult venom. The MS/MS spectrum of a peptide (LDSPVK; m/z 473.78^{2+}) related b and y ions is shown to illustrate its lower abundance in the newborn venom (iTRAQ 114 and 116) compared to the adult venom (iTRAQ 115 and 117).

Table 1. Identification of proteins indicated in Fig. 1, by LC/MS/MS.

Spot	MM (kDa)*	p*I* *	gi number#	Identified protein/toxin class	Sequence coverage (%)
Newborn venom					
1	77	4.53	gi|123908731|	Zinc-metalloproteinase-disintegrin bothrojarin (SVMP)	16
2	76	4.41	gi|48427991|	Zinc-metalloproteinase jerdonitin (SVMP)	4
3	62	4.50	gi||82219706|	Zinc metalloproteinase-disintegrin HF3 (SVMP)	8
4	40	4.40	gi|76365442|	Metalloproteinase P-III (SVMP)	5
5	51	4.85	gi|82214993|	Zinc metalloproteinase-disintegrin BITM06A (SVMP)	37
6	40	5.14	gi|82189787|	Zinc metalloproteinase-disintegrin ACLD (SVMP)	3
7	53	5.29	gi|32306927|	Metalloprotease BOJUMET II (SVMP)	8
8	30	4.61	gi|82233395|	Venom serine proteinase HS114 (SVSP)	43
9	26	5.07	gi|82197476|	Zinc metalloproteinase insularinase-A (SVMP)	9
10	15	5.05	gi|166012653|	Phospholipase A2 (PLA2)	23
11	14	5.40	gi|158518414|	Phospholipase A2 (PLA2)	54
12	22	5.65	gi|172044592|	Zinc metalloproteinase BnP2 (SVMP)	42
13	34	6.11	gi|123911605|	Zinc metalloproteinase	28

				(SVMP)	
14	56	6.43	gi|195927838|	L- amino acid oxidase (LAAO)	54
15	28	6.84	gi|48428846|	Ablomin precursor (CRISP)	22
Adult venom					
1	50	4.75	gi|82214993|	Zinc metalloproteinase-disintegrin BITM06A (SVMP)	31
2	50	4.80	gi|231997|	Zinc metalloproteinase-disintegrin (SVMP)	30
3	37	5.07	gi|13959622|	KN-BJ 2 (SVSP)	25
4	50	5.19	gi|82190823|	Zinc metalloproteinase bothropasin (SVMP)	12
5	60	5.56	gi|82127389|	L-amino-acid oxidase (LAAO)	19
6	60	5.67	gi|82127389|	L-amino-acid oxidase (LAAO)	13
7	14	4.88	gi|41353970|	C-type lectin (CTL)	43
8	14	5.01	gi|41353970|	C-type lectin (CTL)	40
9	14	5.08	gi|41353970|	C-type lectin (CTL)	57
10	22	6.13	gi|82219563|	Zinc metalloproteinase/ disintegrin (SVMP)	23
11	22	6.32	gi|82219563|	Zinc metalloproteinase/ disintegrin (SVMP)	23
12	22	6.46	gi|172044592|	Zinc metalloproteinase BnP2 (SVMP)	42
13	23	6.46	gi|461511|	Protein C activator (SVSP)	9
14	28	6.85	gi|48428846|	Ablomin (CRISP)	11

*Observed molecular masses (MM) and p*I*s.

#According to *National Center for Biotechnology Information* (NCBI).

Toxin classes: SVMP = snake venom metalloproteinase; SVSP= snake venom serine proteinase; PLA2= phospholipase A2; LAAO= L- amino acid oxidase; CTL= C-type lectin; CRISP= cystein-rich secretory protein.

Table 2. Identified proteins in the isobaric tag labeling experiment (iTRAQ™).

Identified proteins	NUP#	*gi number**	log_2 FC**	SD	*p*-value***
Metalloproteinases					
Zinc metalloproteinase-disintegrin ACLD	1	gi|82189787|	-2.60	0.14	5.9E-14
Metalloproteinase atrolysin-E	1	gi|462320|	-2.55	0.07	3.6E-11
Zinc metalloproteinase/disintegrin	1	gi|82088458|	-1.70	0.14	0.0022
Zinc metalloproteinase/disintegrin	1	gi|31077169|	-1.20	0.14	0.029
Zinc metalloproteinase/disintegrin	1	gi|50400454|	-0.65	0.07	8.70E-04
Metalloproteinase jararafibrase-2 (Bothrostatin)	2	gi|82219563|	0.85	0.07	0.0477
Metalloproteinase insularinase-A	4	gi|82197476|	1.05	0.07	0.0416
Zinc metalloproteinase-disintegrin bothropasin	4	gi|82190823|	1.20	0.00	0.007
Serine proteinases					
Venom serine proteinase 3	1	gi|13959638|	0.00	0.28	0.62
Venom serine proteinase HS114	4	gi|82233395|	0.30	0.00	0.26
Platelet-aggregating proteinase PA-BJ	3	gi|6093643|	0.85	0.21	0.52
Venom serine protease BthaTL	1	gi|82173559|	1.00	0.00	0.0063
Kinin-releasing and fibrinogen-clotting serine proteinase 2	4	gi|13959622|	1.70	0.00	1.8E-18
Venom serine protease homolog	2	gi|82240434|	2.10	0.00	1.50E-06
Serine proteinase BITS01A	1	gi|82244284|	-0.25	0.15	0.73
C-type lectins					
Bothroinsularin subunit beta	1	gi|229621685|	-0.90	0.00	0.015
Bothroinsularin subunit alpha	1	gi|229621684|	-0.85	0.07	0.0044
Glycoprotein IB-binding protein subunit alpha	2	gi|82116886|	0.90	0.00	9.50E-04
C-type lectin clone 2100755	1	gi|82134956|	2.10	0.14	0.00025

Phospholipases A_2					
Phospholipase A2. acidic	1	gi|129420|	-2.50	0.00	1.30E-07
Phospholipase A2 2	2	gi|158518414|	0.00	0.14	0.37
Phospholipase A2 homolog 1	1	gi|17368325|	3.05	0.07	2.00E-08
Phospholipase A2 homolog 2	3	gi|17865560|	3.40	0.00	7.5E-18
L-amino acid oxidases					
L-amino acid oxidase precursor	11	gi|195927838|	0.30	0.00	0.31
L-amino-acid oxidase	1	gi|123903691|	0.10	0.00	0.78
L-amino-acid oxidase	1	gi|124106294|	3.15	0.07	2.9E-15
Cysteine-Rich Secretory Proteins					
Catrin-1/2	3	gi|48428839|	-1.10	0.00	0.0028
Ablomin	1	gi|48428846|	-0.80	0.00	0.0043
Vascular Endothelium Growth Factor					
Vascular endothelial growth factor toxin	1	gi|48428663|	-1.70	0.14	0.0037

Proteins more abundant in the newborn or in the adult venom are indicated in shades of red and green, respectively. White indicates unchanged protein expression. Color intensity is described by the bar above.
[#]NUP: Number of unique peptides.
*According to *National Center for Biotechnology Information* (NCBI).
****Log$_2$ Fold Change (FC): Values are mean of 4 LC-MS/MS runs. Log$_2$FC was calculated taken into account newborn venom as reference.
*****p*-value obtained after T-test. P-values <0.05 were considered statistically significant.

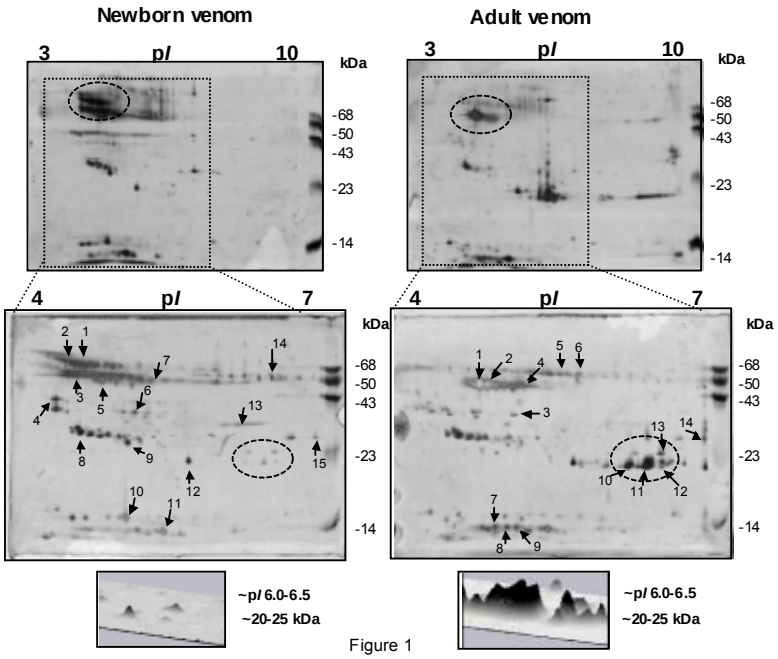

Figure 1

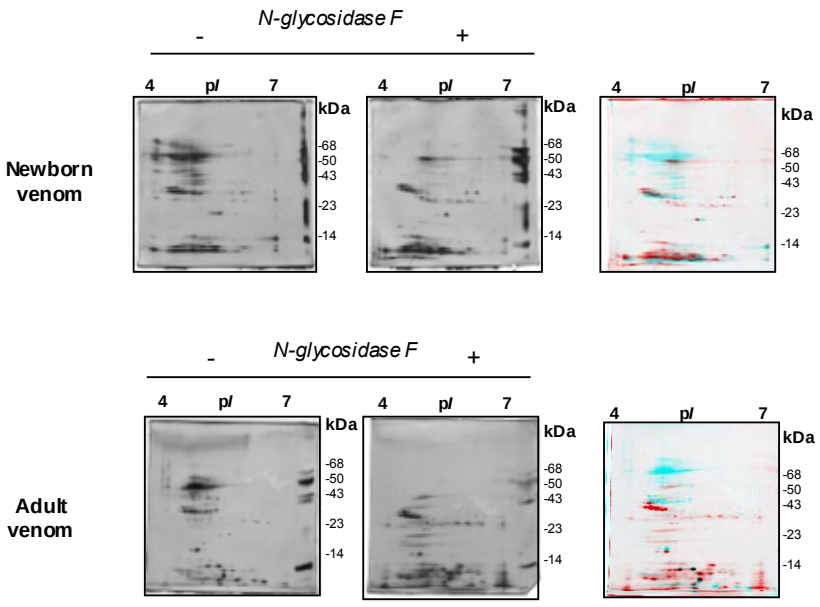

Figure 2

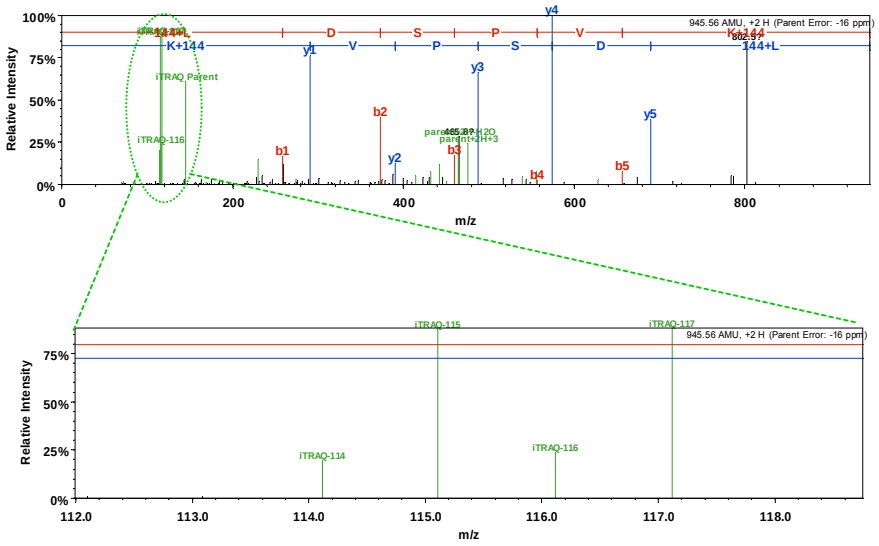

Figure 3