Differential transcript profile of inhibitors with potential anti-venom role in the liver of juvenile and adult Bothrops jararaca snake

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Background. Snakes belonging to the Bothrops genus are vastly distributed in Central and South America and are responsible for most cases of reported snake bites in Latin America. The clinical manifestations of the envenomation caused by this genus are due three major activities – proteolytic, hemorrhagic and coagulant – mediated by metalloproteinases, serine proteinases, phospholipases A₂ and other toxic compounds present in snake venom. Interestingly, it was observed that snakes are resistant to the toxic effects of its own and other snake’s venoms. This natural immunity may occur due the absence of toxin target or the presence of molecules in the snake plasma able to neutralize such toxins. Methods. In order to identify anti-venom molecules, we construct a cDNA library from the liver of B. jararaca snakes. Moreover, we analyzed the expression profile of four molecules – the already known anti-hemorrhagic factor Bj46a, one gamma-phospholipase A₂ inhibitor, one inter-alpha inhibitor and one C1 plasma protease inhibitor – in the liver of juvenile and adult snakes by qPCR. Results. The results revealed a 30-fold increase of gamma-phospholipase A₂ inhibitor and a minor increase of the inter-alpha inhibitor (5-fold) and of the C1 inhibitor (3-fold) in adults. However, the Bj46a factor seems to be equally transcribed between adults and juveniles. Discussion. The results suggest the up-regulation of different inhibitors observed in the adult snakes might be a physiological adaptation to the recurrent contact with their own and even other snake’s venoms throughout its lifespan. This is the first comparative analysis of ontogenetic variation of expression profiles of plasmatic proteins with potential anti-venom activities of...
the venomous snake *B. jararaca*. Furthermore, the present data contributes to the understanding of the natural resistance described in these snakes.
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Abstract

Snakes belonging to the Bothrops genus are vastly distributed in Central and South America and are responsible for most cases of reported snake bites in Latin America. The clinical manifestations of the envenomation caused by this genus are due four major activities – proteolytic, hemorrhagic, coagulant and myonecrotic – mediated by metalloproteinases, serine proteinases, phospholipases A$_2$ and other toxic compounds present in snake venom. Interestingly, it was observed that snakes are resistant to the toxic effects of its own and other snake’s venoms. This natural immunity may occur due the absence of toxin target or the presence of molecules in the snake plasma able to neutralize such toxins. In order to identify anti-venom molecules, we constructed a cDNA library from the liver of B. jararaca snakes. Moreover, we analyzed the expression profile of four molecules – the already known anti-hemorrhagic factor Bj46a, one gamma-phospholipase A$_2$ inhibitor, one inter-alpha inhibitor and one C1 plasma protease inhibitor – in the liver of juvenile and adult snakes by qPCR. The results revealed a 30-fold increase of gamma-phospholipase A$_2$ inhibitor and a minor increase of the inter-alpha inhibitor (5-fold) and of the C1 inhibitor (3-fold) in adults. However, the Bj46a factor seems to be equally transcribed between adults and juveniles. The results suggest the up-regulation of different plasmatic inhibitors in adult snakes B. jararaca snake. The ontogenetic shift in transcript levels related to these inhibitors might (i) be a physiological adaptation to the recurrent contact with their own and even other snake’s venoms throughout its lifespan or (ii) be under genetic control. This is the first comparative analysis of ontogenetic variation of expression profiles of plasmatic proteins with potential anti-venom activities of the venomous snake B. jararaca. Furthermore, the present data contributes to the understanding of the natural resistance described in these snakes.
INTRODUCTION

The genus *Bothrops* is widely distributed in Central and South America, being the most common genus reported in ophidian accidents (Cidade et al. 2006). In Brazil, the species *Bothrops jararaca* (*B. jararaca*) accounts for the majority of the 30,000 cases of envenomation registered annually (Ministério da Saúde 2016), due to its abundance and broad geographical distribution.

Clinical manifestations of bothropic envenomation are due to the following venom activities: 1) proteolytic, resulting in inflammatory edema at the bite site; 2) hemorrhagic, related to endothelial damage and systemic bleeding; 3) coagulant, responsible for the consumption of coagulation factors and consequent homeostasis disruption; and 4) myonecrotic, related to permanent tissue loss, disability and amputation (Rosenfeld 1971). These activities are mediated by a number of venom components, such as metalloproteinases, serine proteinases, phospholipases A$_2$ (PLA$_2$s), L-amino acid oxidases (LAAOs) and other toxic compounds (Fox et al. 2006; Zelanis et al. 2010). The quantitative and qualitative composition of toxins present in snake venoms may vary according to several factors, such as ontogenetic development (Zelanis et al. 2010), seasonal period (Williams & White 1992), gender (Menezes et al. 2006), diet (Gibbs & Mackessy 2009) and geographical distribution (Alape-Giron et al. 2008).

Another intriguing feature of the physiology of snakes is the “natural immunity” towards the toxicity of their own venom and other snake venoms. This resistance may be a result of a mutation in the gene encoding the target of the venom toxin, rendering the target insensitive (Burden et al. 1975; Ohana et al. 1991) and/or due to the presence of proteins that neutralize venom components in the blood of resistant animals (Clark & Voris 1969; Omori-Satoh 1977;
Omori-Satoh et al. 1972; Straight et al. 1976). This inter- and intra-species resistibility make
snake plasma an interesting and rich source of bioactive compounds, since it can be explored for
the isolation of proteins that can neutralize the toxic components of snake venoms and can
contribute to the development of new approaches for the treatment of ophidic accidents (de
Morais-Zani et al. 2013; Lizano et al. 2003).

It is believed that studies on the natural resistibility of snakes began with Fontana (1781) who
stated that “the venom of the viper is not venomous to its species”, more than 230 years ago.
Eighty years after this pioneer report, Guyon (1861) discovered that the natural immunity is not
species-specific. Since the observations made by Fontana (1781), a number of “plasma factors”
have been identified, isolated and characterized, not only from venomous and non-venomous
snakes (Thwin et al. 2000) but also from different animals (Fortes-Dias 2002; Omori-Satoh et al.
2000; Thwin & Gopalakrishnakone 1998).

In this context, Nahas et al. (1973) were the first to identify the presence of a natural inhibitor in
the plasma of B. jararaca in 1973. Later, Nahas et al. (1983) have also described the
“inactivating effect” of B. jararaca plasma upon the coagulant activity of venom from 27
different snake species. Several inhibitors have already been identified in B. jararaca plasma and
serum. The first molecule isolated from the plasma of this species, to our knowledge, was
described by Tanizaki et al. (1991) and has the ability to inhibit the hemorrhagic and caseinolytic
activity of B. jararaca whole venom. Further, this molecule was reported to also inhibit the
venom pro-coagulant activity and lethality (de Oliveira & Tanizaki 1992). Besides, an anti-
hemorrhagic factor, Bj46a, a potent inhibitor of metalloproteinases and venom hemorrhagic
activity, was also purified from B. jararaca serum (Valente et al. 2001). In addition, some PLA₂s
inhibitors (PLIs) are identified in B. jararaca plasma through proteomic analysis (2D SDS-
PAGE and mass spectrometry) (de Morais-Zani et al. 2013). Interestingly, a comparative study of the plasma composition of juvenile and adult *B. jararaca* snakes showed that the inhibitors aforementioned (Bj46a and PLIs) might be present at different levels during ontogenetic development and that this variability can be related to the ontogenetic shift described in its venom (de Morais-Zani et al. 2013).

Although there is an increasing interest in the natural resistance of snakes against venom toxins, the knowledge about snake plasma constitution is still sparse. Therefore, we constructed a liver cDNA library from *B. jararaca* adults and compared the expression profile of possible anti-venom molecules between adults and juvenile snakes. The results described herein can open perspectives to the design of new molecules for therapeutic and biotechnological purposes and to the development of new strategies to the management of snake envenomation.

**METHODS**

**Ethics statement**

Experimental protocols using animals have been conducted in agreement with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethical Committee for Animal Research of Butantan Institute (CEUAIB) under registry No. 794/11 and No. 931/12.

**B. jararaca liver collection**

*B. jararaca* specimens were obtained from Herpetology Laboratory of Butantan Institute (São Paulo – Brazil). Eight females were used, five adults and three juveniles, all from São Paulo State, Brazil. Snakes were euthanized by intracoelomic administration of thiopental (90 mg kg⁻¹)
and lidocaine hydrochloride (5 mg kg\(^{-1}\)). The livers were immediately dissected and stored in liquid nitrogen for cDNA library construction. For qPCR experiments, livers were stored in Trizol (Invitrogen) and kept in -80 °C until use.

**cDNA library construction and sequencing**

The mRNA was isolated from the liver of two adult *B. jararaca* snakes using the RNAeasy Mini Kit (QIAGEN). Thereafter the cDNA library was constructed using the SMART cDNA Library Construction Kit (Clontech) according to manufacturer’s instructions. The *E. coli* BM 25.8 strain was inoculated in 2 mL of LB medium and incubated at 31°C until the OD\(_{600}\) reached 1.3, followed by the addition of MgCl\(_2\) (10 mM) and the amplified cDNA library. Infected bacterial cells were spread on LB plates containing ampicillin and growth overnight at 31°C. Random isolate clones were selected and used for mini plasmid preparation, in which 200 ng of each plasmid were combined with 10 pmol of LD-5’ primer (5’ – CTCGGGAAGCGCGCCATTGTGTTGGT – 3’) and BigDye reagent (Applied Biosystems) in a final volume of 10 µL. Reactions were submitted to 36 cycles of 96°C – 10 sec, 50°C – 10 sec and 60°C – 4 min, followed by precipitation with ethanol and sodium acetate buffer. DNA sequencing was carried on an ABI 3130 sequencer (Applied Biosystems) as described by Buarque et al. (2013).

**Bioinformatics analysis**

Bioinformatics analysis was performed as previously described (Karim et al. 2011). The software was written and provided by Dr. José Marcos Ribeiro (NIAID – NIH) in Visual Basic 6.0 (Microsoft). The functional annotation of CDS was performed through Blastn and Blastx.
(Altschul et al. 1990) against several databases (non-redundant protein, refseq-invertebrate, refseq-protozoa, refseq-vertebrate from NCBI and the custom made LEPIDOSAURIA database). The functionally annotated sequences were plotted in an excel spreadsheet (supplementary data S1).

Quantitative PCR (qPCR)

Quantitative PCR was performed using three biological samples for each group (juveniles and adults). Total RNA was extracted from the liver of adults and juveniles B. jararaca snakes using Trizol (Invitrogen) and quantified using NanoVue equipment (GE Healthcare). Total RNA was treated with one unit of DNase (Fermentas) for 1 h at 37°C. Reactions were stopped by adding EDTA and heating for 10 min at 65°C. cDNA synthesis was performed using the ImProm-II™ Reverse Transcription System (Promega) following the manufacturer’s guidelines and qPCR was performed according Livak and Schmittgen (2001), using the SYBR® Green PCR Master Mix (Applied Biosystems) in a 7500 Real-Time PCR System (Applied Biosystems). The qPCR reaction was performed using: 1 µL of 10X diluted cDNA, 6 µL of SYBR® Green and 150 nM of each specific-primers: Bj46a gene (Bj46a forward and Bj46a reverse), PLI-γ gene (PLI-γ forward and PLI-γ reverse), inter-alpha inhibitor (inter-alpha inhibitor forward and inter-alpha inhibitor reverse) and plasma protease C1 inhibitor-like (C1- forward and C1- reverse), in a 12 µL final volume. Primers sequences are listed in supplementary data S2. β-actin gene was used as the internal control. The PCR program comprised 40 cycles at 94°C (15 sec) and 60°C (1 min), followed by melt curve generation. Melt curves were analyzed to check the specificity of amplification. Reactions were performed in triplicate (for each biological sample) and all values
are represented as the mean ± standard deviation. An unpaired t test was conducted for statistical
analysis, and a significant difference was accepted at p<0.05.

RESULTS

Anti-hemorrhagic factor BJ46a

Transcripts encoding to metalloprotease inhibitors were found in our cDNA library of *B. jararaca* liver (suplementary data 1), including the anti-hemorrhagic factor BJ46a, which presents inhibitory activity against venom metalloproteases. Quantitative analyses obtained by qPCR using specific primers for BJ46a showed no significant differences between juvenile and adult *B. jararaca* snakes (figure 1A). The amino acid sequence deduced from our cDNA library (amino acids residues 137 to 345) was aligned against similar proteins described in other snake species (Figure 2) and reinforces the similarity among several related-molecules described in different Viperidae snakes. However, it is important to note that the partial sequence of this transcript contains specific amino acid residues related to BJ46a (an anti-hemorrhagic factor from *B. jararaca*) and HLP-B (a snake fetuin with no anti-hemorrhagic activity from *Gloydius brevicaudus*), as well as exclusive amino acid residues (supplementary data S3). Therefore, the molecule described herein is referred as BJ46a-like.

Gamma phospholipase A₂ inhibitor

Gamma-phospholipase A₂ inhibitor (γ-PLI) expression profile analysis by qPCR reveals an up-regulation around 30-fold in adults in relation to juvenile specimens (figure 1B). From our cDNA library, it was possible to deduce the whole inhibitor amino acid sequence (Figure 3). When aligned to the sequence of a previously reported *B. jararaca* γ-PLI, the two sequences
differ only by four amino acids residues in the positions 48 (G→A), 200 (F→I), 201 (K→R) and 203 (T→A). Note that the amino acid position numbers correspond to the alignment of several γ-PLI displayed in Figure 3, which showed a high degree of similarity. It is interesting to observe the high incidence of amino acid substitutions found in the C-terminal region, not only between the two γ-PLI described in *B. jararaca*, but among the nine inhibitors aligned, described in three different genera of snakes from Viperidae (Bothrops and Protobothrops) and Colubridae families (Elaphe).

**Inter-alpha inhibitor**

Transcripts related to the serine protease inhibitor inter-alpha inhibitor presented a 5-fold up-regulation in the liver of adults *B. jararaca* snakes (Figure 1C). The partial amino acid sequence of inter-alpha inhibitor heavy chain (H3-like) deduced from a nucleotide sequence found in our cDNA library, this is the first description in *B. jararaca*. The inter-alpha inhibitor sequence showed similarity to the protein described in several reptile species, such as non-venomous and venomous snakes (*Python bivittatus* and *Protobothrops mucrosquamatus*, respectively), lizards (*Anolis carolinensis* and *Gekko japonicus*) and turtles (*Pelodiscus sinensis* and *Chrysemys picta bellii*) (Figure 4).

**Plasma protease C1 inhibitor**

Transcripts encoding to plasma protease C1 inhibitor showed a 3-fold increased expression in the liver of adult *B. jararaca* in comparison to juvenile individuals (Figure 1D). This is the first report on the presence of transcripts related to C1-inhibitor in *B. jararaca* liver. The C1-inhibitor C-terminal deduced amino acid sequence showed some degree of similarity to the molecule described in the lizard *Anolis carolinensis*, the alligator *Alligator mississipiensis* and in three
different species of snakes belonging to Pythonidae, Colubridae and Viperidae families (Python bivittatus, Thamnophis sirtalis and Protobothrops mucrosquamatus) (Figure 5). When these sequences were aligned, the high variability in amino acid composition in the C-terminal region of C1-inhibitor among the species above mentioned is remarkable, as shown in figure 5.

DISCUSSION

Although a number of snake venom gland transcriptomes have been characterized and are accessible in databases (for review, see (Brahma et al. 2015)) studies concerning gene expression in other tissues are scarce and only recently became available (Castoe et al. 2011; Schwartz et al. 2010). However, none of these studies focused on the quantitative analysis of inhibitors that might be involved on venom neutralization, with comparison of adult and juvenile profile. This comparative analysis may contribute to the elucidation of the physiology and anti-venom mechanisms described in B. jararaca plasma.

Snake venom metalloproteinases (SVMPs) are the most abundant components in adult and juvenile B. jararaca venom proteome and venom gland transcriptome (Zelanis et al. 2012; Zelanis et al. 2016) which displays hemorrhagic activity, as described for jararhagin (Paine et al. 1992), HF3 (Assakura et al. 1986), bothropasin (Queiroz et al. 1985) and jararafibrase (Maruyama et al. 1993). Thus, the presence of inhibitory components in snake plasma may take part in the “accidental envenomation”. This is the case of the anti-hemorrhagic factor BJ46a, a glycoprotein isolated from B. jararaca plasma that inhibits the hemorrhagic activity of its own venom, as well as the activity of isolated metalloproteinases jararhagin and atrolysin C (Valente et al. 2001).
A previous study evaluating the ontogenetic changes in the plasma proteomic profile of *B. jararaca* snakes showed that BJ46a is present in a higher relative abundance in the plasma of adult specimens (de Morais-Zani et al. 2013) suggesting a positive association with the higher hemorrhagic activity described in the venom of adult snakes (Antunes et al. 2010). However, the results presented herein showed no significant differences in BJ46a-like transcript levels between juvenile and adult *B. jararaca* snakes.

A concern that must be pointed out is the high similarity between anti-hemorrhagic factors (HSF from *Protobothrops flavoviridis* and BJ46a included) and other molecules with no inhibitory activity, i.e. HLP from *Protobothrops flavoviridis* and HLP-A and HLP-B from *Gloydius brevicaudus* (Aoki et al. 2009; Yamakawa & Omori-Satoh 1992). These molecules are classified based on their primary structures as members of the fetuin family (Aoki et al. 2009).

The BJ46a-like transcript analyzed herein shows a high degree of similarity to BJ46a and HLP-B. In fact, the partial deduced sequence of this transcript contains specific amino acid residues related to BJ46a and HLP-B, as well as exclusive substitutions (supplementary data S3). These findings indicate that in the present work we described a new molecule, although it is not possible to ensure its anti-hemorrhagic activity based only on its primary (and partial) amino acid sequence.

Aoki et al. (2009) concluded that HLP-B from *Gloydius brevicaudus* corresponds to the ortholog of the mammalian fetuins and that the anti-hemorrhagic factors HSF and HLP-A possibly evolved from this molecule in order to display a protective role against its own venom. In this context, it is possible that the new molecule describe in this work represents this transition in *B. jararaca*. Nevertheless, the mechanisms underlying the regulation of the expression of BJ46a and BJ46a-like molecules in juvenile and adult *B. jararaca* snakes need to be elucidated.
Three structural classes of PLIs have been described in snake plasma: (1) $\alpha$-PLIs, which inhibit specifically acidic PLA$_2$s from group II (found in the venom of Viperidae snakes), (2) $\beta$-PLIs, which inhibit specifically basic PLA$_2$s from group II, and (3) $\gamma$-PLI, which shows inhibitory activity towards group I (from venom of Elapidae, Hydrophiidae and Colubridae snakes) and II PLA$_2$s (Estevao-Costa et al. 2008; Inoue et al. 1997; Kinkawa et al. 2010). Considering the broad spectrum of pharmacological activities displayed by snake venom PLA$_2$s, as neurotoxicity, myotoxicity, edema-inducing and anticoagulant activities, the presence of PLIs in the plasma of these animals is of paramount importance. In addition to the key role of these PLIs for snake physiology, the identification and characterization of PLIs are of great interest for biotechnological purposes, especially those called MIPs (myotoxin inhibitor toxin), which target specifically basic Asp49- and Lys49-PLA$_2$s, responsible for local myonecrosis, one of the most serious consequences of $B. jararaca$ envenoming (Campos et al. 2016; Mora-Obando et al. 2014; Santoro et al. 2008).

In the present work, we analyzed the transcriptional profile of a $\gamma$-PLI in the liver of juvenile and adult $B. jararaca$ snakes. qPCR results showed that the levels of transcripts encoding to $\gamma$-PLI was 30-fold higher in adult than those observed in juvenile specimens. At a first glance, these are unexpected results, since a previous plasma proteomic analysis indicated that $\gamma$-PLI are found in a higher relative abundance in the plasma of juvenile $B. jararaca$ (de Morais-Zani et al. 2013) and the venom of juvenile specimens also displayed higher catalytic PLA$_2$ activity (Antunes et al. 2010). However, a study conducted by Kinkawa et al. (2010) showed that the gene expression of $\alpha$-, $\beta$- and $\gamma$-PLIs was induced by the intramuscular injection of venom in the venomous snake $Gloydius brevicaudus$. Therefore, the higher expression levels of $\gamma$-PLI in adult $B. jararaca$ liver described herein might be the result of the physiological response of the snakes to the repeatedly
contact with their own venom during their development. However, we can not discard the
possibility that this increase is under genetic control, a hypothesis that will be discussed further
in this article.

The previously reported *B. jararaca* γ-PLI (Estevao-Costa et al. 2008) and the γ-PLI reported
herein differ only in four of 200 amino acids (including the 19-residue signal peptide), showing
that we described for the first time a new isoform of this inhibitor.

The presence of γ-PLI isoforms was described in *Lachesis muta* (Fortes-Dias et al. 2003) and in
several *Bothrops* species (Estevao-Costa et al. 2008). The variability in amino acid sequence and
in inhibitory activity of these molecules is not surprising, considering that snake venoms that
contains PLA₂ are often composed by a mixture of isoforms of these enzymes (Estevao-Costa et
al. 2008). Therefore, the presence of γ-PLI isoforms in snake plasma may be of great
physiological importance, ensuring the efficiency of these molecules and broadening their range
of inhibition (Estevao-Costa et al. 2008).

Besides a number of amino acid substitutions were present amongst γ-PLI described in species
from *Bothrops* genera, a highly conserved region composed by 15 amino acid residues
(104QFPGLPLSRPGYY118) is present in all of them and it was recognized as the consensus
motif most probably involved in the interaction of γ-PLI with snake venom PLA₂ (Estevao-Costa
et al. 2008).

This 15-residue sequence, as well as the 16 cysteines in the mature protein and the proline-rich
region (residues 100 to 120 in figure 3), which play an important role in maintaining the
conformation of the interaction sites of the molecule (Dunn & Broady 2001; Kini 1998), are
conserved in the molecule described herein, reinforcing its PLA₂ inhibitory activity and
protective role in snake plasma. The four amino acids substitutions observed in the molecule
described in the present work in the positions 48 (A→G), 200 (I→F), 201 (R→K) and 203
(A→T) (the last two also described in γ-PLI of E. climacophora), may exert little or no influence
in its inhibitory activity, since these residues are not identified (to date) as key residues for the
biological activity of the molecule.

Snake venom serine proteinases (SVSPs) are another important group of toxins that play a
central role in the envenomation caused by B. jararaca snake. These enzymes affect mainly the
hemostatic system, acting on the components of the coagulation cascade and on the fibrinolytic
and kallilrein-kinin systems (Serrano 2013). In terms of relative abundance, SVSPs occupy the
second position in the venom proteome of this species (Zelanis et al. 2016). Due to the central
activity displayed by these toxins, we selected two serine proteinase inhibitors to evaluate the
level of their related transcripts in juvenile and adult B. jararaca snakes.

Inter-alpha inhibitors constitute a family of proteins that acts in the regulation of the
inflammatory process and plays a role in wound healing (Kobayashi 2006; Lim 2013). These
molecules broadly inhibit serine proteases, decrease pro-inflammatory and enhance anti-
inflammatory mediators and block complement activation during systemic inflammation (Fries
& Kaczmarczyk 2003; Okroj et al. 2012). These inhibitors can be found in plasma as inter alpha
inhibitor, which is composed by two heavy chains (H1 and H2) and one light chain (LC), or as
pre-alpha inhibitor, which consists of one heavy (H3) and one light chain (LC) (Fries & Blom
2000). In this work, we found that transcripts related to the heavy chain of pre-alpha inhibitor
(H3-like) presented a 5-fold up-regulation in the liver of adult B. jararaca snakes. It seems
plausible that this inhibitor can play a role on the neutralization of the major and minor activities
of SVSP, such as disturbance of hemostasis and induction of inflammatory reactions,
respectively.
Another important plasma serine proteinase inhibitor is C1 inhibitor, a multi-functional molecule that acts inactivating a number of serine proteases in different enzymatic cascades, as complement, coagulation, and fibrinolytic systems (Ghannam et al. 2016). It was hypothesized that this inhibitor could be involved in the neutralization of venom components in case of accidental envenomation. In addition to the impact on blood coagulation, *B. jararaca* SVSP can activate the complement system and, consequently, generate anaphylatoxins that might play a key role in the inflammatory process and also contribute to the spreading of other venom toxins (Pidde-Queiroz et al. 2010). Therefore, we decided to evaluate the levels of transcripts related to C1 inhibitor in the liver of *B. jararaca* snakes. Results described herein showed that transcripts encoding to this plasma inhibitor showed a 3-fold increase in the liver of adult specimens in comparison to juvenile individuals.

The analysis of ontogenetic variation in venom activities of *B. jararaca* showed that the activity of serine proteinases is slightly higher in adult individuals, which could justify the higher expression of serine proteinase inhibitors, as inter-alpha inhibitor and C1 inhibitor, found in the liver of adult snakes. Besides the possible role in neutralization of venom components, plasmatic serine proteinase inhibitors play a fundamental role in hemostasis maintenance in reptiles. Reptiles have armored skin, low blood pressure, slow circulation time, and are commonly lethargic in their movements. Therefore, their arterial blood moves in conditions more like those of the mammal venous side, which would favor the occurrence of intravascular clotting (Tanaka-Azevedo et al. 2003). Nevertheless, blood coagulation in reptiles is slower than in mammals, due to the absence or low concentration of some coagulation factors (such as VIII and IX) (Didisheim et al. 1959) and the
presence of specific inhibitors (Morais et al. 2009; Tanaka-Azevedo et al. 2003; Tanaka-Azevedo et al. 2004).

However, it is noteworthy that 3 out of 4 mRNA related to the plasmatic proteins studied in the present work are more expressed in the liver of adult snakes. Bearing in mind the results described by Kinkawa et al. (2010), regarding the control of PLIs expression, and taken together the results presented in herein, it is tempting to suggest that the higher expression levels of γ-PLI, inter-alpha inhibitor and C1-inhibitor observed in adult snakes might be a natural physiological response of the snakes to the recurrent contact with their own venom throughout the life.

However, venom neutralizing factors are identified in the serum of neonates of the ophiophagus snake Clelia clelia, before any contact with venom during feeding on venomous snakes. This observation shows that, at least for this species, the contact with venoms is not the driving factor for the expression of plasmatic molecules with antivenom properties, being under genetic control. In this context, another possibility to be considered is that the expression of transcripts related to protease inhibitors in B. jararaca is under a genetically programmed control, being predetermined to be highly expressed in the adult stage. Indeed, bites between snakes are more prone to happen among adult individuals, especially during the period of intercourse, when the encounter and contact between snakes are more frequent (Sávio Stefanini Sant’Anna, personal communication). Nevertheless, these conclusions are speculative and it is important to emphasize that complementary studies are necessary to support these hypotheses.

In summary, this work provides the first comparative analysis of ontogenetic variation of expression profiles of plasmatic proteins with potential anti-venom activities of the venomous snake B. jararaca. Our data contributes to the understanding of the natural resistance against
“self-envenomation” described in these snakes and provide new target molecules with biotechnological potential that can be useful for the development of new approaches for the treatment of ophidic accidents.

ACKNOWLEDGEMENTS

We are grateful to Ms. Jacilene Barbosa da Silva Monteiro, for technical assistance in DNA sequencing and Real Time PCR experiments (INFAR, Escola Paulista de Medicina, Universidade Federal de São Paulo, Brazil).

REFERENCES

Alape-Giron A, Sanz L, Escolano J, Flores-Diaz M, Madrigal M, Sasa M, and Calvete JJ. 2008. Snake venomics of the lancehead pitviper Bothrops asper: geographic, individual, and ontogenetic variations. J Proteome Res 7:3556-3571. 10.1021/pr080332p

Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403-410. 10.1016/S0022-2836(05)80360-2

Antunes TC, Yamashita KM, Barbaro KC, Saiki M, and Santoro ML. 2010. Comparative analysis of newborn and adult Bothrops jararaca snake venoms. Toxicon 56:1443-1458. S0041-0101(10)00329-6 [pii] 10.1016/j.toxicon.2010.08.011

Aoki N, Deshimaru M, Kihara K, and Terada S. 2009. Snake fetuin: isolation and structural analysis of new fetuin family proteins from the sera of venomous snakes. Toxicon 54:481-490. 10.1016/j.toxicon.2009.05.018 S0041-0101(09)00268-2 [pii]
Assakura MT, Reichl AP, and Mandelbaum FR. 1986. Comparison of immunological, biochemical and biophysical properties of three hemorrhagic factors isolated from the venom of Bothrops jararaca (jararaca). *Toxicon* 24:943-946. 0041-0101(86)90094-2 [pii]

Brahma RK, McCleary RJ, Kini RM, and Doley R. 2015. Venom gland transcriptomics for identifying, cataloging, and characterizing venom proteins in snakes. *Toxicon* 93:1-10. 10.1016/j.toxicon.2014.10.022 S0041-0101(14)00353-5 [pii]

Buarque DS, Braz GR, Martins RM, Tanaka-Azevedo AM, Gomes CM, Oliveira FA, Schenkman S, and Tanaka AS. 2013. Differential expression profiles in the midgut of Triatoma infestans infected with Trypanosoma cruzi. *PLoS One* 8:e61203. 10.1371/journal.pone.0061203

Burden SJ, Hartzell HC, and Yoshikami D. 1975. Acetylcholine receptors at neuromuscular synapses: phylogenetic differences detected by snake alpha-neurotoxins. *Proc Natl Acad Sci U S A* 72:3245-3249.

Campos PC, de Melo LA, Dias GL, and Fortes-Dias CL. 2016. Endogenous phospholipase A2 inhibitors in snakes: a brief overview. *J Venom Anim Toxins Incl Trop Dis* 22:37. 10.1186/s40409-016-0092-5 92 [pii]

Castoe TA, Fox SE, Jason de Koning A, Poole AW, Daza JM, Smith EN, Mockler TC, Secor SM, and Pollock DD. 2011. A multi-organ transcriptome resource for the Burmese Python (*Python molurus bivittatus*). *BMC Res Notes* 4:310. 10.1186/1756-0500-4-310

Cidade DA, Simao TA, Davila AM, Wagner G, Junqueira-de-Azevedo IL, Ho PL, Bon C, Zingali RB, and Albano RM. 2006. Bothrops jararaca venom gland transcriptome: analysis of the gene expression pattern. *Toxicon* 48:437-461. S0041-0101(06)00239-X [pii] 10.1016/j.toxicon.2006.07.008
Clark WC, and Voris HK. 1969. Venom neutralization by rattlesnake serum albumin. *Science* 164:1402-1404.

de Morais-Zani K, Grego KF, Tanaka AS, and Tanaka-Azevedo AM. 2013. Proteomic Analysis of the Ontogenetic Variability in Plasma Composition of Juvenile and Adult *Bothrops jararaca* Snakes. *Int J Proteomics* 2013:135709. 10.1155/2013/135709

de Oliveira EP, and Tanizaki MM. 1992. Effect of a proteinase inhibitor from the plasma of *Bothrops jararaca* on coagulant and myotoxic activities of Bothrops venoms. *Toxicon* 30:123-128.

Didisheim P, Hattori K, and Lewis JH. 1959. Hematologic and coagulation studies in various animal species. *The Journal Laboratory and Clinical Medicine* 53:866-875.

Dunn RD, and Broady KW. 2001. Snake inhibitors of phospholipase A(2) enzymes. *Biochim Biophys Acta* 1533:29-37. S1388-1981(01)00138-X [pii]

Estevao-Costa MI, Rocha BC, de Alvarenga Mudado M, Redondo R, Franco GR, and Fortes-Dias CL. 2008. Prospection, structural analysis and phylogenetic relationships of endogenous gamma-phospholipase A(2) inhibitors in Brazilian *Bothrops* snakes (Viperidae, Crotalinae). *Toxicon* 52:122-129. S0041-0101(08)00350-4 [pii]

10.1016/j.toxicon.2008.04.167

Fontana F. 1781. Traité sur le vénin de la vipere, sur les poisons americains, sur le laurier-cerise et sur quelques autres poissons vegetaux. On y a joint des observations sur la structure primitive du corps animal. Différentes expériences sur la reproduction des nerfs et la description d'un nouveau canal de l'oeil. Florence, 23.

Fortes-Dias CL. 2002. Endogenous inhibitors of snake venom phospholipases A(2) in the blood plasma of snakes. *Toxicon* 40:481-484. S0041010101002744 [pii]
Fortes-Dias CL, Barcellos CJ, and Estevao-Costa MI. 2003. Molecular cloning of a gamma-phospholipase A2 inhibitor from Lachesis muta muta (the bushmaster snake). *Toxicon* 41:909-917. S0041010103000734 [pii]

Fox JW, Ma L, Nelson K, Sherman NE, and Serrano SM. 2006. Comparison of indirect and direct approaches using ion-trap and Fourier transform ion cyclotron resonance mass spectrometry for exploring viperid venom proteomes. *Toxicon* 47:700-714.

Fries E, and Blom AM. 2000. Bikunin--not just a plasma proteinase inhibitor. *Int J Biochem Cell Biol* 32:125-137.

Fries E, and Kaczmarczyk A. 2003. Inter-alpha-inhibitor, hyaluronan and inflammation. *Acta Biochim Pol* 50:735-742. 035003735

Ghannam A, Sellier P, Fain O, Martin L, Ponard D, and Drouet C. 2016. C1 Inhibitor as a glycoprotein: The influence of polysaccharides on its function and autoantibody target. *Mol Immunol* 71:161-165. 10.1016/j.molimm.2016.02.007 S0161-5890(16)30021-9 [pii]

Gibbs HL, and Mackessy SP. 2009. Functional basis of a molecular adaptation: prey-specific toxic effects of venom from Sistrurus rattlesnakes. *Toxicon* 53:672-679. 10.1016/j.toxicon.2009.01.034

Guyon J. 1861. Le venin des Serpents exerce-t-ils sur eux-mêmes l'action qu'il exerce sur d'autres animaux. *C R Ac des Sc LIII*:12.

Inoue S, Shimada A, Ohkura N, Ikeda K, Samejima Y, Omori-Satoh T, and Hayashi K. 1997. Specificity of two types of phospholipase A2 inhibitors from the plasma of venomous snakes. *Biochem Mol Biol Int* 41:529-537.

Karim S, Singh P, and Ribeiro JM. 2011. A deep insight into the sialotranscriptome of the gulf coast tick, Amblyomma maculatum. *PLoS One* 6:e28525. 10.1371/journal.pone.0028525
Kini RM. 1998. Proline brackets and identification of potential functional sites in proteins: toxins to therapeutics. *Toxicon* 36:1659-1670. S0041-0101(98)00159-7 [pii]

Kinkawa K, Shirai R, Watanabe S, Toriba M, Hayashi K, Ikeda K, and Inoue S. 2010. Up-regulation of the expressions of phospholipase A2 inhibitors in the liver of a venomous snake by its own venom phospholipase A2. *Biochem Biophys Res Commun* 395:377-381. 10.1016/j.bbrc.2010.04.024

Kobayashi H. 2006. Endogenous anti-inflammatory substances, inter-alpha-inhibitor and bikunin. *Biol Chem* 387:1545-1549. 10.1515/BC.2006.192

Lim YP. 2013. ProThera Biologics, Inc.: a novel immunomodulator and biomarker for life-threatening diseases. *R I Med J (2013)* 96:16-18.

Livak KJ, and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-408. 10.1006/meth.2001.1262 S1046-2023(01)91262-9 [pii]

Lizano S, Domont G, and Perales J. 2003. Natural phospholipase A(2) myotoxin inhibitor proteins from snakes, mammals and plants. *Toxicon* 42:963-977. 0.1016/j.toxicon.2003.11.007 S0041010103003337 [pii]

Maruyama M, Tanigawa M, Sugiki M, Yoshida E, and Mihara H. 1993. Purification and characterization of low molecular weight fibrinolytic/hemorrhagic enzymes from snake (*Bothrops jararaca*) venom. *Enzyme Protein* 47:124-135.

Menezes MC, Furtado MF, Travaglia-Cardoso SR, Camargo AC, and Serrano SM. 2006. Sex-based individual variation of snake venom proteome among eighteen *Bothrops jararaca* siblings. *Toxicon* 47:304-312. S0041-0101(05)00399-5 [pii]
Ministério da Saúde. 2016. Casos de acidentes por serpentes. Brasil, Grandes Regiões e Unidades Federais. 2000 a 2015. Brasil.

Mora-Obando D, Fernandez J, Montecucco C, Gutierrez JM, and Lomonte B. 2014. Synergism between basic Asp49 and Lys49 phospholipase A2 myotoxins of viperid snake venom in vitro and in vivo. *PLoS One* 9:e109846. 10.1371/journal.pone.0109846 PONE-D-14-25968 [pii]

Morais KB, Vieira CO, Hirata IY, and Tanaka-Azevedo AM. 2009. *Bothrops jararaca* antithrombin: isolation, characterization and comparison with other animal antithrombins. *Comp Biochem Physiol B Biochem Mol Biol* 152:171-176.

Nahas L, Betti F, Kamiguti AS, and Sato H. 1973. Blood coagulation inhibitor in a snake plasma (*Bothrops jararaca*). *Thrombosis et Diathesis Haemorrhagica* 30:106-113.

Nahas L, Kamiguti AS, Sousa e Silva MC, Ribeiro de Barros MA, and Morena P. 1983. The inactivating effect of *Bothrops jararaca* and *Waglerophis merremii* snake plasma on the coagulant activity of various snake venoms. *Toxicon* 21:239-246.

Ohana B, Fraenkel Y, Navon G, and Gershoni JM. 1991. Molecular dissection of cholinergic binding sites: how do snakes escape the effect of their own toxins? *Biochem Biophys Res Commun* 179:648-654. 0006-291X(91)91421-8 [pii]

Okroj M, Holmquist E, Sjolander J, Corrales L, Saxne T, Wisniewski HG, and Blom AM. 2012. Heavy chains of inter alpha inhibitor (IalphaI) inhibit the human complement system at early stages of the cascade. *J Biol Chem* 287:20100-20110. 10.1074/jbc.M111.324913 M111.324913 [pii]
Omori-Satoh T. 1977. Antihemorrhagic factor as a proteinase inhibitor isolated from the serum of *Trimeresurus flavoviridis*. *Biochim Biophys Acta* 495:93-98.

Omori-Satoh T, Sadahiro S, Ohsaka A, and Murata R. 1972. Purification and characterization of an antihemorrhagic factor in the serum of *Trimeresurus flavoviridis*, a crotalid. *Biochim Biophys Acta* 285:414-426.

Omori-Satoh T, Yamakawa Y, and Mebs D. 2000. The antihemorrhagic factor, erinacin, from the European hedgehog (*Erinaceus europaeus*), a metalloprotease inhibitor of large molecular size possessing ficolin/opsonin P35 lectin domains. *Toxicon* 38:1561-1580.

Paine MJ, Desmond HP, Theakston RD, and Crampton JM. 1992. Purification, cloning, and molecular characterization of a high molecular weight hemorrhagic metalloprotease, jararhagin, from *Bothrops jararaca* venom. Insights into the disintegrin gene family. *J Biol Chem* 267:22869-22876.

Pidde-Queiroz G, Furtado Mde F, Filgueiras CF, Pessoa LA, Spadafora-Ferreira M, van den Berg CW, and Tambourgi DV. 2010. Human complement activation and anaphylatoxins generation induced by snake venom toxins from *Bothrops* genus. *Mol Immunol* 47:2537-2544. 10.1016/j.molimm.2010.07.003 S0161-5890(10)00504-3 [pii]

Queiroz LS, Santo Neto H, Assakura MT, Reichl AP, and Mandelbaum FR. 1985. Pathological changes in muscle caused by haemorrhagic and proteolytic factors from *Bothrops jararaca* snake venom. *Toxicon* 23:341-345.

Rosenfeld G. 1971. *Symptomatology, pathology and treatment of snake bites in South America*. New York.
Santoro ML, Sano-Martins IS, Fan HW, Cardoso JL, Theakston RD, and Warrell DA. 2008. Haematological evaluation of patients bitten by the jararaca, Bothrops jararaca, in Brazil. *Toxicon* 51:1440-1448. S0041-0101(08)00110-4 [pii] 10.1016/j.toxicon.2008.03.018

Schwartz TS, Tae H, Yang Y, Mockaitis K, Van Hemert JL, Proulx SR, Choi JH, and Bronikowski AM. 2010. A garter snake transcriptome: pyrosequencing, de novo assembly, and sex-specific differences. *BMC Genomics* 11:694. 10.1186/1471-2164-11-694

Serrano SM. 2013. The long road of research on snake venom serine proteinases. *Toxicon* 62:19-26. 10.1016/j.toxicon.2012.09.003 S0041-0101(12)00746-5 [pii]

Straight R, Glenn JL, and Snyder CC. 1976. Antivenom activity of rattlesnake blood plasma. *Nature* 261:259-260.

Tanaka-Azevedo AM, Tanaka AS, and Sano-Martins IS. 2003. A new blood coagulation inhibitor from the snake Bothrops jararaca plasma: isolation and characterization. *Biochemical and Biophysical Research Communications* 308:706-712.

Tanaka-Azevedo AM, Torquato RJ, Tanaka AS, and Sano-Martins IS. 2004. Characterization of Bothrops jararaca coagulation inhibitor (BjI) and presence of similar protein in plasma of other animals. *Toxicon* 44:289-294.

Tanizaki MM, Kawasaki H, Suzuki K, and Mandelbaum FR. 1991. Purification of a proteinase inhibitor from the plasma of Bothrops jararaca (jararaca). *Toxicon* 29:673-681.

Thwin MM, and Gopalakrishnakone P. 1998. Snake envenomation and protective natural endogenous proteins: a mini review of the recent developments (1991-1997). *Toxicon* 36:1471-1482. S0041-0101(98)00137-8 [pii]
Thwin MM, Gopalakrishnakone P, Kini RM, Armugam A, and Jeyaseelan K. 2000. Recombinant antitoxic and antiinflammatory factor from the nonvenomous snake *Python reticulatus*: phospholipase A2 inhibition and venom neutralizing potential. *Biochemistry* 39:9604-9611. bi000395z [pii]

Valente RH, Dragulev B, Perales J, Fox JW, and Domont GB. 2001. BJ46a, a snake venom metalloproteinase inhibitor. Isolation, characterization, cloning and insights into its mechanism of action. *Eur J Biochem* 268:3042-3052.

Williams V, and White J. 1992. Variation in the composition of the venom from a single specimen of *Pseudonaja textilis* (common brown snake) over one year. *Toxicon* 30:202-206.

Yamakawa Y, and Omori-Satoh T. 1992. Primary structure of the antihemorrhagic factor in serum of the Japanese Habu: a snake venom metalloproteinase inhibitor with a double-headed cystatin domain. *J Biochem* 112:583-589.

Zelanis A, Andrade-Silva D, Rocha MM, Furtado MF, Serrano SM, Junqueira-de-Azevedo IL, and Ho PL. 2012. A transcriptomic view of the proteome variability of newborn and adult *Bothrops jararaca* snake venoms. *PLoS Negl Trop Dis* 6:e1554. 10.1371/journal.pntd.0001554 PNTD-D-11-01075 [pii]

Zelanis A, Menezes MC, Kitano ES, Liberato T, Tashima AK, Pinto AF, Sherman NE, Ho PL, Fox JW, and Serrano SM. 2016. Proteomic identification of gender molecular markers in *Bothrops jararaca* venom. *J Proteomics* 139:26-37. 10.1016/j.jprot.2016.02.030 S1874-3919(16)30051-3 [pii]

Zelanis A, Tashima AK, Rocha MM, Furtado MF, Camargo AC, Ho PL, and Serrano SM. 2010. Analysis of the ontogenetic variation in the venom proteome/peptidome of *Bothrops*
jararaca reveals different strategies to deal with prey. *J Proteome Res* 9:2278-2291.

10.1021/pr901027r
Figure 1. Expression analysis of plasmatic inhibitors from juvenile and adult *B. jararaca* snakes. The abundance expression of (A) BJ46a-like (Bj429), (B) γ- phospholipase A$_2$ inhibitor (Bj405), (C) inter-alpha-trypsin inhibitor (Bj203) and (D) plasma protease C1-inhibitor (Bj84). Error bars represent the standard deviation of the mean from three independent experiments (n = 3). Statistical analysis was carried with unpaired *t* test. Asterisks represent significant difference: *p < 0.05 and **p < 0.01. NS = non-statistical significant.
Figure 2. Multiple alignments of amino acid sequences of Bj46a-like (Bj429) with similar sequences described in different species of snakes. The sequences used are from Bothrops jararaca (sp|Q9DGI0.1), Protobothrops mucrosquamatus (XP_015681073.1), Protobothrops flavoviridis (sp|P29695.2), Gloydius brevicaudus (sp|Q5KQS2.1) and Gloydius blomhoffii (sp|Q5KQS1.1). Identical residues are black boxed.
Figure 3. Multiple alignments of amino acid sequences of $\gamma$-phospholipase A$_2$ inhibitor (Bj405) with similar sequences described in different species of snakes. The sequences used are from Bothrops jararaca (gb|ABV91331.1), Protobothrops elegans (dbj|BAJ14719.1), Bothrops jararacussu (gb|ABV91333.1), Bothrops alternatus (gb|ABV91326.1), Bothrops moojeni (gb|ABV91334.1), Elaphe climacophora (dbj|BAH47550.1), Bothrops neuwiedi (gb|ABV91336.1) and Bothrops erythromelas (gb|ABV91328.1). Identical residues are black boxed.
Figure 4. Multiple alignments of amino acid sequences of inter-alpha-trypsin inhibitor (Bj203) with similar sequences described in different species of reptiles. The sequences used are from *Gekko japonicus* (XP_015262960.1), *Anolis carolinensis* (XP_003217700.2), *Python bivittatus* (XP_007442992.1), *Protobothrops mucrosquamatus* (XP_015671353.1), *Pelodiscus sinensis* (XP_006127649.1) and from *Chrysemys picta bellii* (XP_008177427.1). Identical residues are black boxed.
Figure 5. Multiple alignments of amino acid sequences of plasma protease C1 inhibitor (Bj84) with similar sequences described in different species of reptiles. The sequences used are from *Anolis carolinensis* (XP_008109235.1), *Python bivittatus* (XP_007423129.1), *Thamnophis sirtalis* (XP_013930568.1), *Protobothrops mucrosquamatus* (XP_015676034.1) and *Alligator mississippiensis* (gb|KYO40723.1). Identical residues are black boxed.