# 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 $A_2$ 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. **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 $A_2$ 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 $A_2$ 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. **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

**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 $A_2$ 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. **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 $A_2$ 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 $A_2$ 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. **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.

41 ***Keywords*****:** snake plasma; plasmatic protein; protease inhibitors; anti-venom; *Bothrops jararaca*;

42 gene expression.

43

## 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 *B. jararaca* envenomation are due to three main venom activities: 1) proteolytic, resulting in inflammatory edema at the bite site; 2) hemorrhagic, related to endothelial damage and systemic bleeding; and 3) coagulant, responsible for the consumption of coagulation factors and consequent homeostasis disruption (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

67 contribute to the development of new approaches for the treatment of ophidic accidents (de
68 Morais-Zani et al. 2013; Lizano et al. 2003).
69 It is believed that studies on the natural resistibility of snakes began with Fontana (1781) who
70 stated that "the venom of the viper is not venomous to its species", more than 230 years ago.
71 Eighty years after this pioneer report, Guyon (1861) discovered that the natural immunity is not
72 species-specific. Since the observations made by Fontana (1781), a number of "plasma factors"
73 have been identified, isolated and characterized, not only from venomous and non-venomous
74 snakes (Thwin et al. 2000) but also from different animals (Fortes-Dias 2002; Omori-Satoh et al.
75 2000; Thwin & Gopalakrishnakone 1998).
76 In this context, Nahas *et al*. (1973) were the first to identify the presence of a natural inhibitor in
77 the plasma of *B. jararaca* in 1973. Later, Nahas *et al*. (1983) have also described the
78 "inactivating effect" of *B. jararaca* plasma upon the coagulant activity of venom from 27
79 different snake species. Several inhibitors have already been identified in *B. jararaca* plasma and
80 serum. The first molecule isolated from the plasma of this species, to our knowledge, was
81 described by Tanizaki et al. (1991) and has the ability to inhibit the hemorrhagic and caseinolytic
82 activity of *B. jararaca* whole venom. Further, this molecule was reported to also inhibit the
83 venom pro-coagulant activity and lethality (de Oliveira & Tanizaki 1992). Besides, an anti-
84 hemorrhagic factor, Bj46a, a potent inhibitor of metalloproteinases and venom hemorrhagic
85 activity, was also purified from *B. jararaca* serum (Valente et al. 2001). In addition, some $PLA_2$s
86 inhibitors (PLIs) are identified in *B. jararaca* plasma through proteomic analysis (2D SDS-
87 PAGE and mass spectrometry) (de Morais-Zani et al. 2013). Interestingly, a comparative study
88 of the plasma composition of juvenile and adult *B. jararaca* snakes showed that the inhibitors
89 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 construct a liver cDNA library from *B. jararaca* adults and compare 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 ºC until use.

113

114 **cDNA library construction and sequencing**

115 The mRNA was isolated from the liver of two *B. jararaca* adults using the RNAeasy Mini Kit
116 (QIAGEN). Thereafter the cDNA library was constructed using the SMART cDNA Library
117 Construction Kit (Clontech) as described by Buarque et al. (2013).

118

119 **Bioinformatics analysis**

120 Bioinformatics analysis was performed as previously described (Karim et al. 2011). The software
121 was written and provided by Dr. José Marcos Ribeiro (NIAID – NIH) in Visual Basic 6.0
122 (Microsoft). The functional annotation of CDS was performed through Blastn and Blastx
123 (Altschul et al. 1990) against several databases (non-redundant protein, refseq-invertebrate,
124 refseq-protozoa, refseq-vertebrate from NCBI and the custom made LEPIDOSAURIA database).
125 The functionally annotated sequences were plotted in a excel spreadsheet (Supplementary data
126 1).

127

128 **Quantitative PCR (qPCR)**

129 Quantitative PCR was performed using three biological samples for each group (juveniles and
130 adults). Total RNA was extracted from the liver of adults and juveniles *B. jararaca* snakes using
131 Trizol (Invitrogen) and quantified using NanoVue equipment (GE Healthcare). Total RNA was
132 treated with 1 Unit of DNase (Fermentas) for 1 h at 37°C. Reactions were stopped by adding
133 EDTA and heating for 10 min at 65°C. cDNA synthesis was performed using the ImProm-II™
134 Reverse Transcription System (Promega) following the manufacturer's guidelines and qPCR was
135 performed according Livak and Schimittgen (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), PLI-α (PLI-α forward and PLI-α 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 total volume. Primers sequences are listed in supplementary data (*Table 1*). 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 the most abundant in the cDNA library of *B. jararaca* liver (data not shown), including the anti-hemorrhagic factor BJ46a, which presents inhibitory activity against venom metalloproteases. Quantitative analyses obtained by qPCR showed no significant differences between juvenile and adult *B. jararaca* snakes (Figure 1A). The partial BJ46a sequence deduced from our cDNA library (amino acids residues 137 to 345) was aligned against similar proteins described in other snake species (Figure 2). The deduced amino acid sequence confirms the identity of the transcript and reinforces the similarity among BJ46a and related-inhibitors described in different Viperidae snakes.

159

160 **Gamma phospholipase $A_2$ inhibitor**

161 γ-phospholipase $A_2$ inhibitor (γ-PLI) expression profile analysis by qPCR reveals a up-regulation
162 around 30 fold in adults in relation to juvenile specimens (figure 1B). From our cDNA library, it
163 was possible to deduce the whole inhibitor amino acid sequence (Figure 3). When aligned to the
164 sequence of a previously reported *B. jararaca* γ-PLI, the two sequences differ only by four
165 amino acids residues in the positions 48 (G→A), 200 (F→I), 201 (K→R) and 203 (T→A). Note
166 that the amino acid position numbers correspond to the alignment of several γ-PLI displayed in
167 Figure 3, which showed a high degree of similarity. It is interesting to observe the high incidence
168 of amino acid substitutions found in the C-terminal region, not only between the two γ-PLI
169 described in *B. jararaca*, but among the nine inhibitors aligned, described in three different
170 genera of snakes from Viperidae (*Bothrops* and *Protobothrops*) and Colubridae families
171 (*Elaphe*).

172

173 **Inter-alpha inhibitor**

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

182

183 **Plasma protease C1 inhibitor**

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

193

194 **DISCUSSION**

195 Although a number of snake venom gland transcriptomes have been characterized and are
196 accessible in databases (for review, see (Brahma et al. 2015)) studies concerning gene expression
197 in other tissues are scarce and only recently became available (Castoe et al. 2011; Schwartz et al.
198 2010). However, none of these studies focused on the quantitative analysis of inhibitors that
199 might be involved on venom neutralization, with comparison of adult and juvenile profile. This
200 comparative analysis may contribute to the elucidation of the physiology and anti-venom
201 mechanisms described in *B. jararaca* plasma.

202 Snake venom metalloproteinases (SVMPs) are the most abundant components in adult and
203 juvenile *B. jararaca* venom proteome and venom gland transcriptome (Zelanis et al. 2012;
204 Zelanis et al. 2016) which displays hemorrhagic activity, as described for jararhagin (Paine et al.
205 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 transcript levels between juvenile and adult *B. jararaca* snakes. These contradictory findings could be explained by the lack of correlation between transcriptome and proteome composition observed in other situations. In this context, Durban et al. (2013) have demonstrated that the ontogenetic changes in venom composition observed in *Crotalus simus simus* are controlled by post-transcriptional mechanisms, since the transcriptome profile of the venom glands of neonate and adult specimens exhibit similar toxin family composition. Nevertheless, the mechanisms underlying the regulation of BJ46a expression in juvenile and adult *B. jararaca* snakes need to be elucidated.

Three structural classes of PLIs have been described in snake plasma: (1) α-PLIs, which inhibit specifically acidic $PLA_2$s from group II (found in the venom of Viperidae snakes), (2) β-PLIs, which inhibit specifically basic $PLA_2$s from group II, and (3) γ-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 the present work, we analyzed the transcriptional profile of a γ-PLI in the liver of juvenile and adult *B. jararaca* snakes. qPCR results showed that the levels of transcripts encoding to γ-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 γ-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 α-, β- and γ-PLIs was induced by the intramuscular injection of venom in the venomous snake *Gloydius brevicaudus*. Therefore, the higher expression levels of γ-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.

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-

252 inflammatory mediators and block complement activation during systemic inflammation (Fries
253 & Kaczmarczyk 2003; Okroj et al. 2012). These inhibitors can be found in plasma as inter alpha
254 inhibitor, which is composed by two heavy chains (H1 and H2) and one light chain (LC), or as
255 pre-alpha inhibitor, which consists of one heavy (H3) and one light chain (LC) (Fries & Blom
256 2000). In this work, we found that transcripts related to the heavy chain of pre-alpha inhibitor
257 (H3-like) presented a 5-fold up-regulation in the liver of adult *B. jararaca* snakes. It seems
258 plausible that this inhibitor can play a role on the neutralization of the major and minor activities
259 of SVSP, such as disturbance of hemostasis and induction of inflammatory reactions,
260 respectively.
261 Another important plasma serine proteinase inhibitor is C1 inhibitor, a multi-functional molecule
262 that acts inactivating a number of serine proteases in different enzymatic cascades, as
263 complement, coagulation, and fibrinolytic systems (Ghannam et al. 2016). It was hypothesized
264 that this inhibitor could be involved in the neutralization of venom components in case of
265 accidental envenomation. Considering that, in addition to the impact on blood coagulation, *B.*
266 *jararaca* SVSP can activate the complement system. Consequently, generates anaphylatoxins
267 that might play a key role in the inflammatory process and also contribute to the spreading of
268 other venom toxins (Pidde-Queiroz et al. 2010). Therefore, we decided to evaluate the levels of
269 transcripts related to C1 inhibitor in the liver of *B. jararaca* snakes. Results described herein
270 showed that transcripts encoding to this plasma inhibitor showed a 3-fold increase in the liver of
271 adult specimens in comparison to juvenile individuals.

272 The analysis of ontogenetic variation in venom activities of *B. jararaca* showed that the activity
273 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.

However, it is noteworthy that 3 out of 4 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. Nevertheless, it is important to emphasize that complementary studies are necessary to support this hypothesis.

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.

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## ADDITIONAL INFORMATION AND DECLARATIONS

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**Competing Interests**

There are no competing interests to declare regarding this manuscript.

**Author Contributions**

- Cícera Maria Gomes performed the experiments, analyzed the data, wrote the manuscript.
- Karen de Morais-Zani wrote the manuscript.
- Stephen Lu analyzed the data, wrote the manuscript.
- Diego de Souza Buarque performed the experiments.
- Glória Regina Cardoso Braz analyzed the data.
- Kathleen Fernandes Grego planned the experiments, extracted the liver.
- Aparecida Sadae Tanaka planned the experiments, analyzed the data, contributed reagents and other essential materials, wrote the manuscript.
- Anita Mitico Tanaka-Azevedo planned the experiments, contributed reagents and other essential materials, wrote the manuscript.

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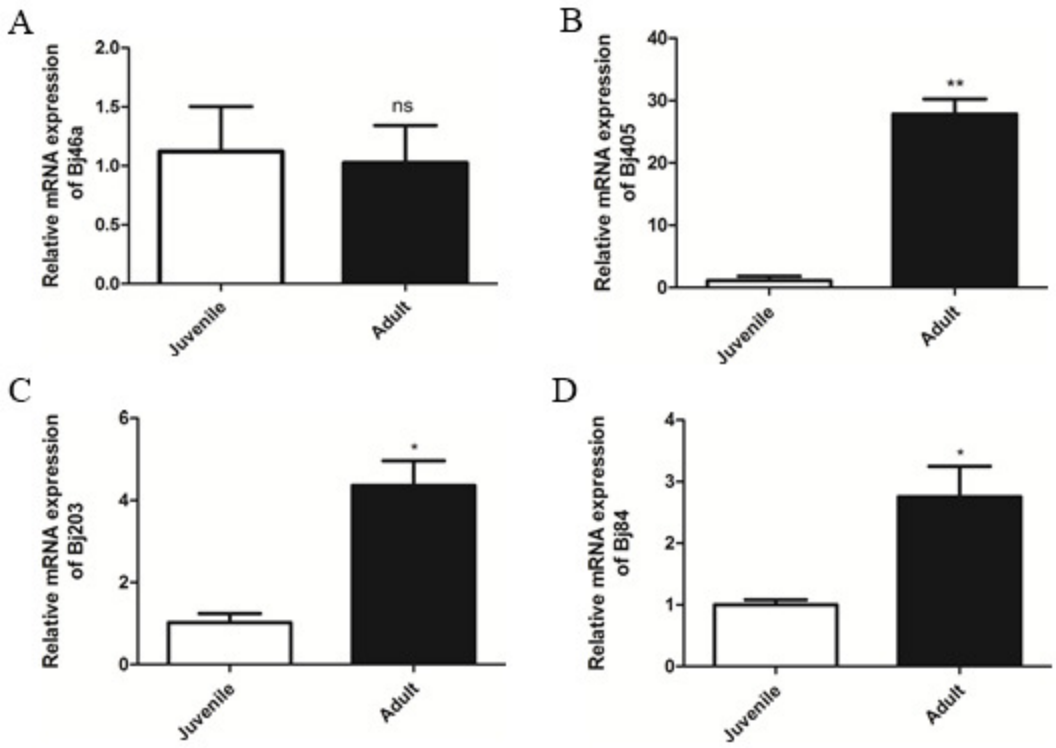
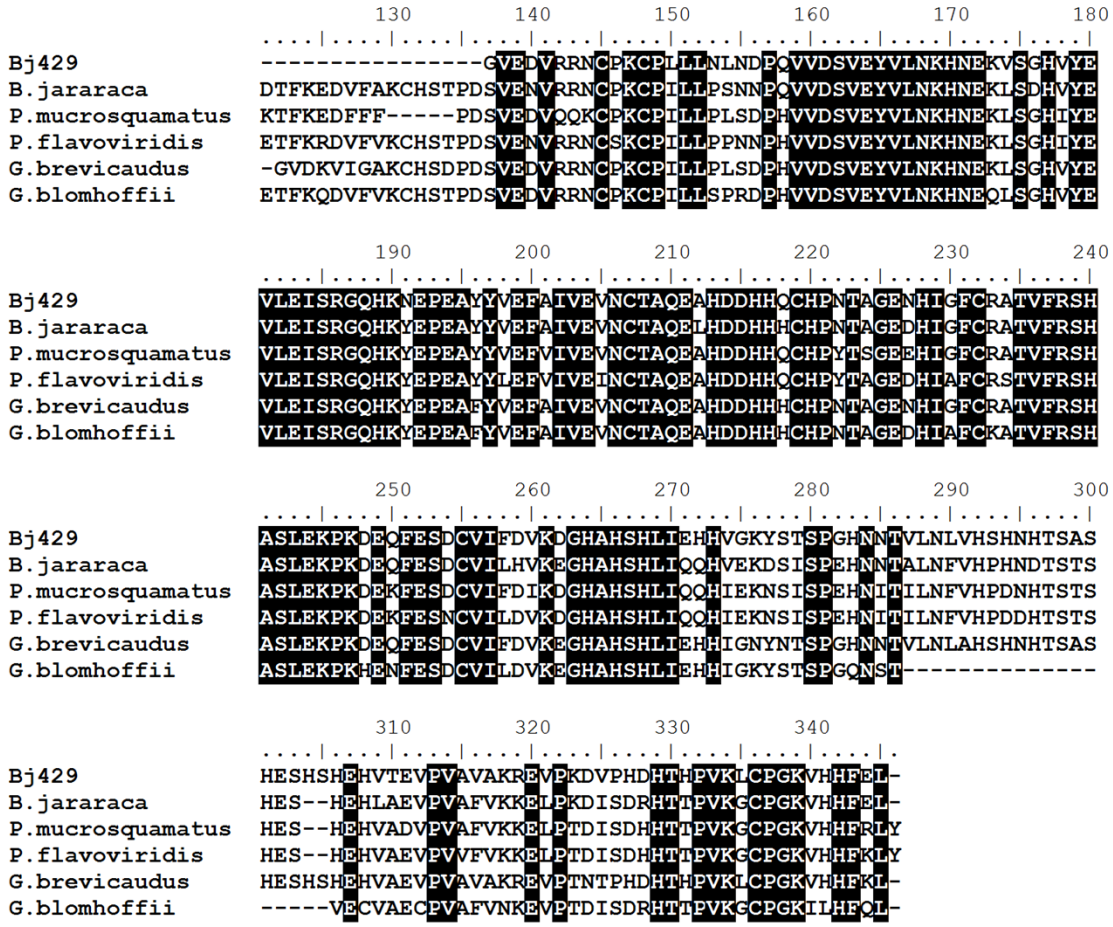



**Figure 1. Expression analysis of plasmatic inhibitors from juvenile and adult *B. jararaca* snakes.** The abundance expression of (A) anti-hemorrhagic factor BJ46a (Bj429), (B) γ-phospholipase $A_2$ inhibitor (Bj405), (C) inter-alpha-trypsin inhibitor (Bj203) and (D) plasma protease C1-inhibitor (Bj84). 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.




490

491 **Figure 2. Multiple alignments of amino acid sequences of antihemorrhagic factor Bj46a**

492 **(Bj429) with similar sequences described in different species of snakes.** The sequences used

493 are from *Bothrops jararaca* (sp|Q9DGI0.1), *Protobothrops mucrosquamatus* (XP_015681073.1),

494 *Protobothrops flavoviridis* (sp|P29695.2), *Gloydius brevicaudus* (sp|Q5KQS2.1) and *Gloydius*

495 *blomhoffii* (sp|Q5KQS1.1). Identical residues are black boxed.

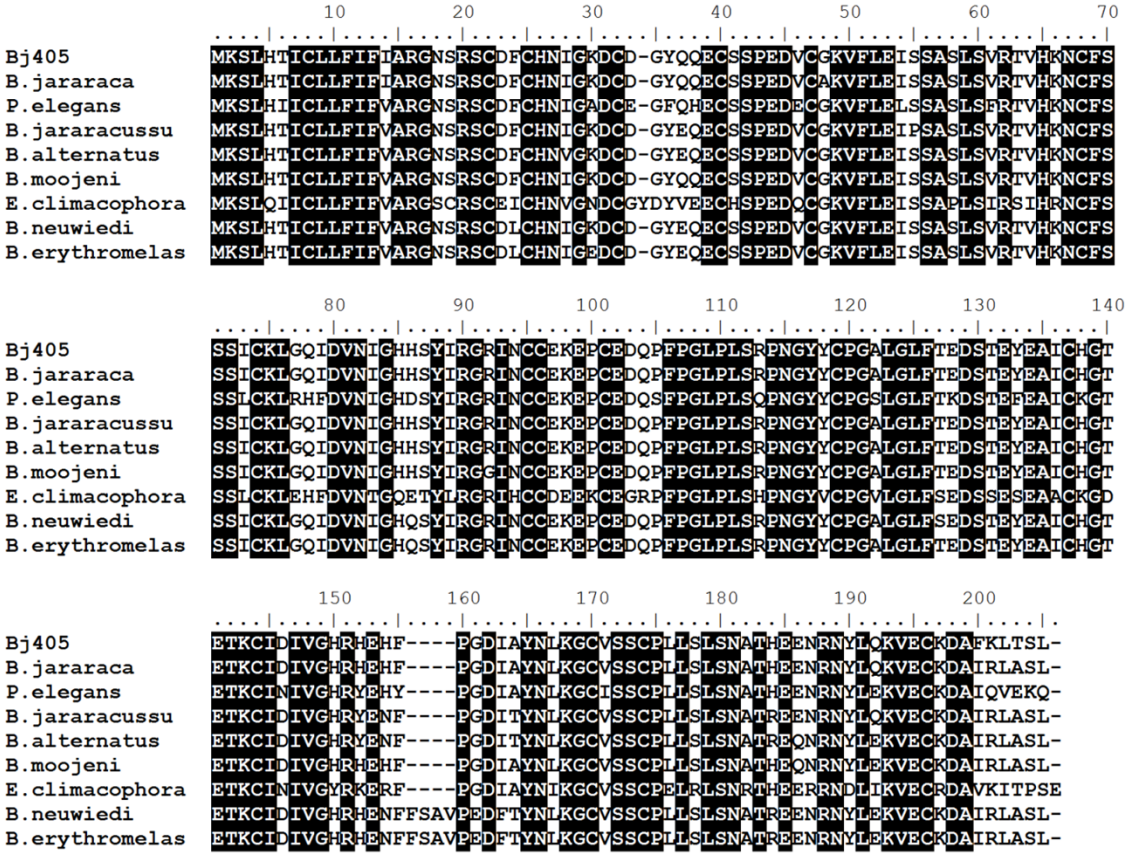



**Figure 3. Multiple alignments of amino acid sequences of γ- phospholipase $A_2$ inhibitor (Bj405) with similar sequences described in different species of snakes.** The sequences used are from *Bothrops jararaca* (gb|ABV91331.1), *Protobothrops elegans* (dbj|BAJ14719.1), *Bothrops jararacussu* (gb|ABV91333.1), *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.

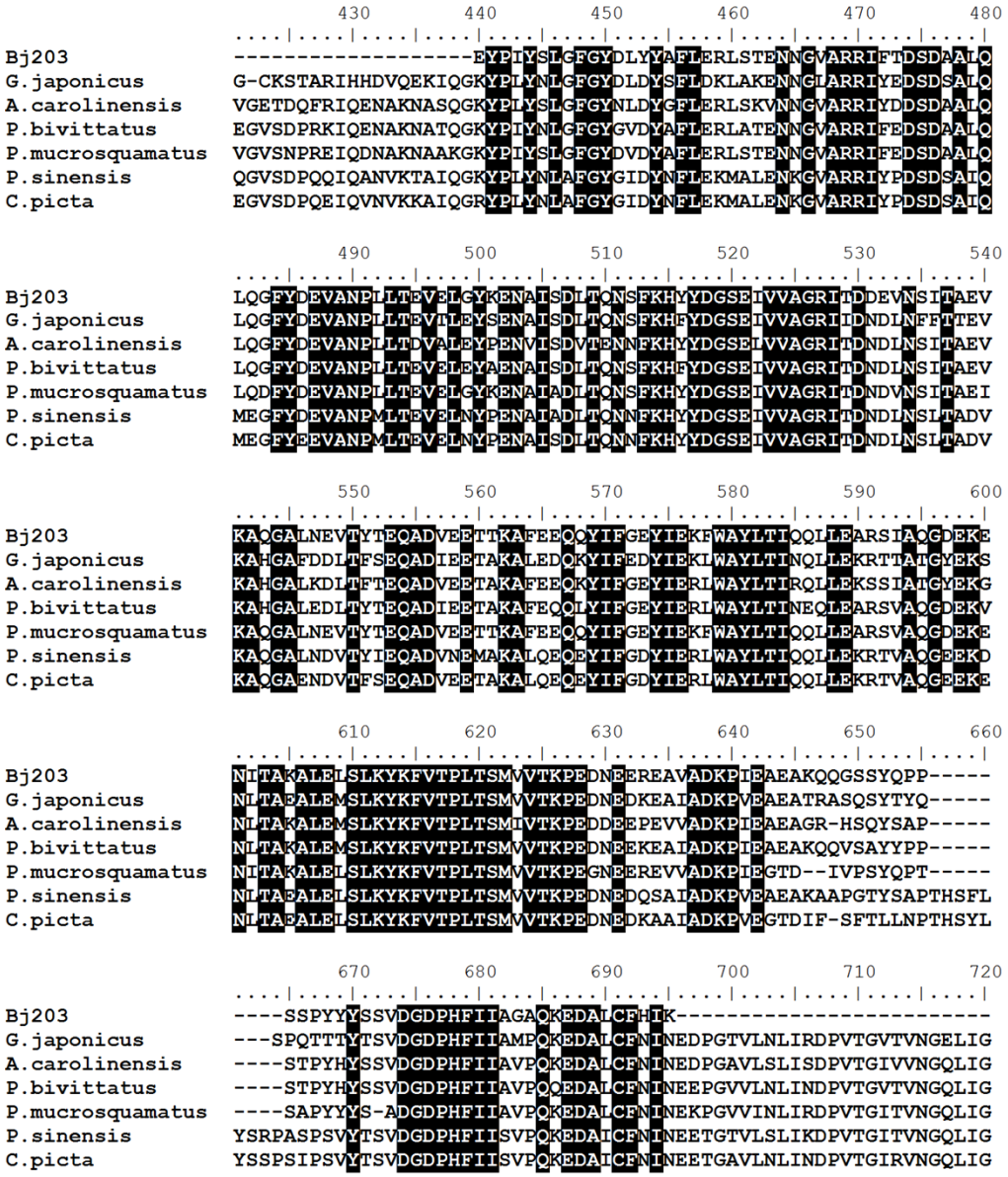



**Figure 4. Multiple alignments of amino acid sequences of inter-alpha-trypsin inhibitor (Bj203) with similar sequences described in different species of reptiles.** The sequences used are from *Gekko japonicus* (XP_015262960.1), *Anolis carolinensis* (XP_003217700.2), *Python bivittatus* (XP_007442992.1), *Protobothrops mucrosquamatus* (XP_015671353.1), *Pelodiscus sinensis* (XP_006127649.1) and from *Chrysemys picta bellii* (XP_008177427.1). Identical residues are black boxed.

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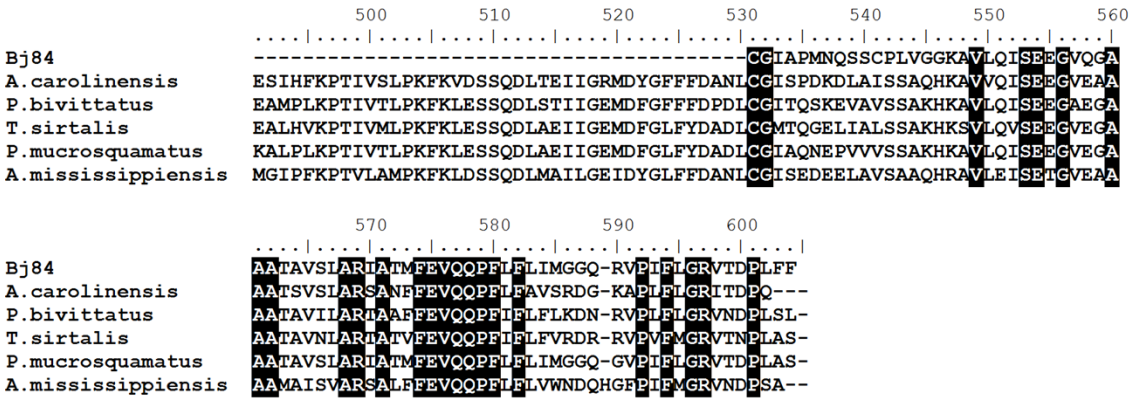


512

513 **Figure 5. Multiple alignments of amino acid sequences of plasma protease C1 inhibitor**

514 **(Bj84) with similar sequences described in different species of reptiles.** The sequences used

515 are from *Anolis carolinensis* (XP_008109235.1), *Python bivittatus* (XP_007423129.1),

516 *Thamnophis sirtalis* (XP_013930568.1), *Protobothrops mucrosquamatus* (XP_015676034.1) and

517 *Alligator mississippiensis* (gb|KYO40723.1). Identical residues are black boxed.

518 **Table 1. Primers used for qPCR.**

| Gene | Primer |
|---|---|
| β- actin *foward* | 5'-GGCCAACAGAGAGAAGATGACCC-3 |
| β- actin *reverse* | 5'-TCGGTCAAGTCACGGCCA-3' |
| Bj46a *foward* | 5'-TCAAGAGGGCAGCACAAGAAT-3' |
| Bj46a *reverse* | 5'-AGTCCGACTCAAACTGTTCATC -3' |
| PLI-γ *foward* | 5'-CCAGAAGATGTATGTGGCAAGG -3 |
| PLI-γ *reverse* | 5'-TTTGGTCGGGAGAGGGGC -3' |
| C1- foward | 5'-TCGCTCCAATGAACCAGTCG-3' |
| C1-reverse | 5'-TGACCCGTCCCAGAAAGATTG-3' |
| Inter-alpha inhibitor foward | 5'- CTTACCTCACCATTCAACAACTTCT-3' |
| Inter-alpha inhibitor reverse | 5'- TGGACCCTTGCTGCTTTGC-3' |

519