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ONTOGENETIC CHANGES IN THE VENOM OF *BOTHROPS INSULARIS* (SERPENTES: VIPERIDAE)AND ITS BIOLOGICAL IMPLICATION

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Abstract. We here report on the venom composition of *Bothrops insularis,* an endemic snake from the island of Queimada Grande (São Paulo, Brazil) that shows ontogenetic change on diet. Electrophoretic profiles showed similar patterns among samples at distinct ages. Densitometric analysis showed an increase of expression of proteic band at 24 kDa of molecular mass range. Venom toxicity was higher for bees (just-emerged *Apis mellifera*) and chicks (one day age Bovans white chicks) than for swiss mice, although a direct relation with ontogeny was not established. Venom selectivity towards local preys was demonstrated by the strong toxicity upon arthropods and chicks in comparison to mammals. Results suggest that the venom of *Bothrops insularis* has peculiarities on its composition that supports a specialized diet on its insular habitat and, although a conspicuous ontogenetic diet change occurs in the species, venom toxicity towards local preys is a characteristic present during the whole life cycle. Our results also provide strong evidence of the presence of taxon-specific toxicity in the venom of *B. insularis,* and a tendency of the adult venom to be more toxic than the one from young individuals.

Keywords. *Bothrops insularis,* Island species, snake venom, ontogeny, diet

Introduction

Snake venoms are important on several biological and ecological processes, such as feeding (throughout digestive enzymes activity), self-defense and prey subjugation (Thomas and Pough, 1979; Kochva *et al.,* 1983). Venom composition can vary according to several factors, such as ontogeny, geographical distribution, sex, and inter and intra-specific variations (Minton and Weinstein, 1986; Gutiérrez *et al.,* 1990; Furtado *et al.,* 1991; Chippaux *et al.,* 1991; Daltry *et al.,* 1996). In this context, environment and natural history traits exert important selective pressure, which reflects, among others features, on venom composition (Daltry *et al.,* 1996; Chippaux *et al.,* 1991; Li *et al.,* 2005a; Li *et al.,* 2005b; Mackessy *et al.,* 2006).

Islands are appropriate places to study several types of evolutionary processes, and speciation is one of the most important ones. Williams *et al.* (1988) showed differences in protein profiles of insular populations of Australian tiger snakes *(Notechis scutatus* and *Notechis ater niger),* suggesting that the main differences are related to time of isolation of the Islands. Daltry *et al.* (1996) found significant variability in venom protein profiles (using isoeletric focalization techniques) among insular populations of *Callose-lasma rhodostoma.* According to these authors, the geographical variability reflects natural selection for feeding on local prey.

Venom selectivity towards specific preys has been reported by Zimmerman *et al.* (1992), Andrade *et al.* (1996) Andrade and Abe (1999), and Mackessy *et al.* (2006). Taxa-specific toxicity is of great importance in the study of snake toxinological and ecological aspects. Additionally, ontogenetic changes on diet are a common feature among members of the genus *Bothrops* (Martins *et al.,* 2002), and are often related with changes on venom composition. However, the physiological mechanisms that lead to variability remain poorly understood (Minton and Weinstein, 1986; Gutiérrez *et al.,* 1990; Gutiérrez *et al.,* 1991; Chippaux *et al.,* 1991; Chaves *et al.,* 1992; Furtado *et al.,* 1991; Mackessy, 1993; Tan *et al.,* 1993; Daltry *et al.,* 1996; Daltry *et al.,* 1997; Andrade and Abe, 1999; López-Lozano *et al.,* 2002; Saravia *et al.,* 2002; Saldarriaga *et al.,* 2003; Mackessy *et al.,* 2003; Furtado, 2003).

Early studies made by Afranio do Amaral have shown that the venom of *Bothrops insularis* was the most powerful venom known among Brazilian snakes of the genus *Bothrops* (Amaral, 1921, 1927). *Bothrops insularis* is an endemic snake of the Island of Queimada Grande, in the South coastline of the state of São Paulo, Brazil (24°30'S, 43°42'W). The Island was isolated from the continent during the late Pleistocene, at approximately 11.000 years ago, after the last glaciation and sea level elevation (Vanzolini, 1973). The species is peculiar due to the high incidence of intersexual individuals within the population

of normal bisexual individuals (Hoge *et al.,* 1959; Beçak *et al.,* 1990). Genetically, these intersexes are females with a rudimentary bilateral or unilateral non-functional hemipenis. Such high incidence of sexual abnormality might be due to inbreeding processes or eventual mutations that led to the appearance of intersexuality (Beçak *et al.,* 1990). There are no mammals on the island and *B. insularis* has a well-recorded ontogenetic shift on its diet, with newborns feeding on ectothermic animals (such as insects, lizards and amphibians) while adults shift their diet to feed on endothermic migratory birds (Amaral, 1921; Duarte *et al.,* 1995; Martins *et al.,* 2002).

Since the pioneering studies made by Amaral, several works have been made with the venom of this species, either related to biological/ecological characteristics or to isolated toxins. A thrombin-like enzime (Selistre and Giglio, 1987), hemorrhagic, and myonecrotic toxins (Selistre *et al.,* 1990) were isolated from the venom of *B. insularis.* Cintra *et al.* (1990) isolated and determined the primary structure of eight Bradikinin Potentiating Peptides (BPPs). Cogo *et al.* (1993) found a presynaptic action in the venom of *B. insularis* that seems to be due to a phospholipase A2 fraction (Rodrigues-Simioni *et al.,* 2004). A new member of the VEGF (Vascular Endothelium Growth Factor) family of proteins, named svVEGF (snake venom Vascular Endothelium Growth Factor), was cloned and expressed from a library built with the venom gland of *B. insularis.* The transcripts diversity of its venom gland was recorded by Junqueira-de-Azevedo and Ho (2002, 2003) through the generation of expressed sequence tags (ESTs). Guimarães-Gomes *et al.* (2003, 2004) identified and characterized a C-type lectin named BiL, with hemagglutinating activity. Modesto *et al.* (2005) characterized a prothrombin activator named Insularinase A from the venom of the species. Finally, we reported the ontogenetic variability in expression of *Bothrops insularis* venom proteases (Zelanis *et al.,* 2007).

The aim of this work is to verify the variability of the venom of *B. insularis,* given the geographic isolation of this snake, and to correlate the results with prey consumption throughout the life cycle (ontogeny) of the species.

Material and Methods

Venoms of Bothrops insularis – A pregnant specimen of *B. insularis* was captured on the Island of Queimada Grande. Three newborn specimens (1 male and 2 intersexes) were kept in captivity for 41 months in the bioterium of the Laboratory of Herpetology of the Instituto Butantan, São Paulo. The snakes were milked individually and venoms were lyophilized. Extracted venoms corresponding to 15 and 41 months after birth were used for assays (juvenile and adult specimens, respectively). This extraction protocol was carried out due to the low venom production of specimens of *B. insularis* in ages below 15 months after birth. The venom of the mother (also an intersex) as well as extracts from 13 adults of *B. insularis,* 150 newborns (one month old) and 30 adults of *Bothrops jararaca* were used as comparison parameters.

Protein content – Protein content of each sample was determined (Markwell *et al.,* 1978) using bovine albumin as a standard.

Sodium Dodecil Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Densitometric analysis – SDS-PAGE was carried out according to Laemmli (1970). Venom samples (120 µg/lane) were resuspended on SDS-PAGE sample buffer under reducing conditions, applied on a 4% polyacrylamide stacking gel and running on gradient gels (7.5 to 17.5%) at 10°C. Gels were stained with Comassie Brilliant Blue R-250 (SIGMA) and destained on methanol/acetic acid solution in water (30/10, v/v). Protein standards were: Phosphorilase b (94.000 Da), Albumin (67.000 Da), Ovalbumin (43.000 Da), Carbonic anhidrase (30.000 Da), trypsin inhibitor (20.100 Da) and α-Lactalbumin (14.400 Da) (Pharmacia, USA). Densitometric analysis was performed using Quantity One 1-D Image analysis® software (Bio-Rad).

Median Lethal Dose (LD_{50}) with different experimental animals – In order to verify the lethal toxicity of *B. insularis* venom samples upon its prey items, we decided to use three kinds of experimental models: Swiss mice *(Mus musculus),* Bovans white chicks *(Gallus domesticus),* and honeybees *(Apis mellifera),* representing two endothermic and one ectothermic preys, respectively.

Lethal toxicity was assessed by intraperitoneal injection (0.5 mL in 0.15M NaCl) into male Swiss strain mice (18-22 g), using groups of six mice for each venom dose (five doses). The dose that killed 50% of animals was calculated by probit analysis (Finney, 1971), with a computer program, taking into consideration deaths occurring within 48 h after venom injection. Groups of just-emerged honeybees (weighing ~82 mg each one; n = 5, for each dose),

collected in the apiary of UNESP at Rio Claro, SP, were subjected to an intra-celomatic injection (2 µl in 0.15M NaCl), in the pronotum, with several concentrations of venom samples (five doses) according to the method of Manzoli-Palma *et al.* (2003). The LD_{50} values were calculated as mentionated above, taking into consideration deaths occurring 24 hr after venom injection. Groups of one-day old male chicks, (weighing 40-45 g each one, n = 4, for each dose) provided by chicken farm Granja Kunitomo (Mogi das Cruzes Municipality, State of São Paulo) were subjected to an intra-muscular injection (100 µl in 0.15M NaCl) on left pectoralis muscle with several concentrations of venom samples (five doses). The LD_{50} values were calculated as mentioned above, taking into consideration deaths occurring 24 hr after venom injection. In the comparative analysis of LD_{50} values, results were expressed as micrograms of venom per grams of tested animal. Food and water for the offered preys were available *ad libitum.*

Results

Ontogenetic phases of *Bothrops insularis* have shown marked differences, either on protein profile, evidenced by electrophoretic profiles, or in LD_{50} assays with different experimental models. The development of the animals in captivity showed expressive sexual dimorphism among male and intersexes, mainly in relation to length, body mass and venom yield (data not shown), a feature that was also described for other species of the genus *Bothrops,* like *B. jararaca* (Travaglia-Cardoso, 2001; Furtado *et al.,* 2006).

Regardless of age, all venoms showed a similar protein profile (Fig. 1) with major protein bands located at 50, 45, 24 and 14 kDa ranges. These four major protein bands correspond to the four major peaks verified on densitometric analysis (Fig. 1). Peaks from densitometric analysis (Fig. 1) clearly showed an increasing of intensity on the adult phase (41 months old). Moreover, an increase of expression of a protein band at the 24 kDa range was observed in all venoms analyzed, a characteristic that was also verified through densitometric analysis.

In relation to toxicity, LD_{50} values suggest distinct sensitivity among the experimental animals. We observed an age-dependent toxicity of venom in mice, for which the venom from adult animals (41 months old) was more toxic than the one from young individuals (15 months old). In contrast, the venom of *B. jararaca* did not show ontogenetic change on LD_{50} values for mice (Table 1). On the other hand, chicks have shown the lowest LD_{50} values. Toxicity results suggest a taxon-specific toxicity, with the highest LD_{50} values for mice and the lowest ones for bees and chicks.

Discussion

Results obtained in this work provide important subsidy for a better understanding of the venom/prey relationship in *Bothrops insularis* (see also, Zimmerman *et al.,* 1992; Andrade *et al.,* 1996; Andrade and Abe, 1999; Mackessy *et al.,* 2006).

Present results show a remarkable feature on electrophoretic profiles, in which an increase of a 24 kDa proteic band becomes evident. This pattern was observed in all three individual venoms analyzed, suggesting an ontogenetic expression of proteins within this molecular mass range. Such molecular mass range comprises mainly proteases (generally metalloproteases) which have broad actions upon several biological substrates, like blood coagulation factors, extracellular matrix components, and endothelial cells, that lead to several envenomation processes such as, dermonecrosis, myonecrosis, edema, and hemorrhage (Bjarnason and Fox, 1995; Gutiérrez and Rucavado, 2000; Serrano and Fox, 2005).

Table 1. LD_{50} values for *Bothrops insularis* offspring and *Bothrops jararaca* venoms towards different experimental models. Numbers in parenthesis means 95% confidence intervals; n.e. = not evaluated. Values in µg/g animal.

Experimental Models	Intersex 1		Intersex 2		Male		Offspring Mother	Pool *B. insularis*	*B. jararaca* (newborns)	*B. jararaca* (adults)
	15	41	15	41	15	41				
Mammals (Mice)	4.6 (4.5-5.8)	1.9 (1.4-2.8)	3.7 (3,4-4,6)	2.6 (2.0-3.9)	7.1 (6.0-9.3)	3.6 (2.9-4.6)	2.8 (2.3-3.1)	3.3 (2.7-5.2)	1.9 (1.6-2.2)	1.8 (1.5-2.4)
Arthropods (Honeybees)	1.6 (1.4-1.9)	1.7 (1.4-1.9)	2.3 (1.8-3.1)	1.7 (1.3-1.9)	2.6 (1.9-3.4)	1.4 (1.0-1.9)	1.9 (2.4-2.2)	2.4 (0.5-4.0)	n.e.	n.e.
Aves (Chicks)	1.0 (0.9-1.2)	0.8 (0.5-1.2)	1.2 (0.7-2.3)	2.0 (1.5-2.3)	2.6 (1.9-3.4)	0.9 (0.5-2.5)	0.9 (0.5-1.6)	2.4 (1.9-2.7)	n.e.	1.3 (0.5-2.5)

Lopéz-Lozano *et al.* (2002) reported ontogenetic variability on coagulant activity of *Bothrops atrox* snake venom from the Amazon Rain Forest. These authors verified an ontogenetic increase of a 23 kDa proteic band on venoms and, according to sequence analysis, this protein showed to be a metalloprotease.

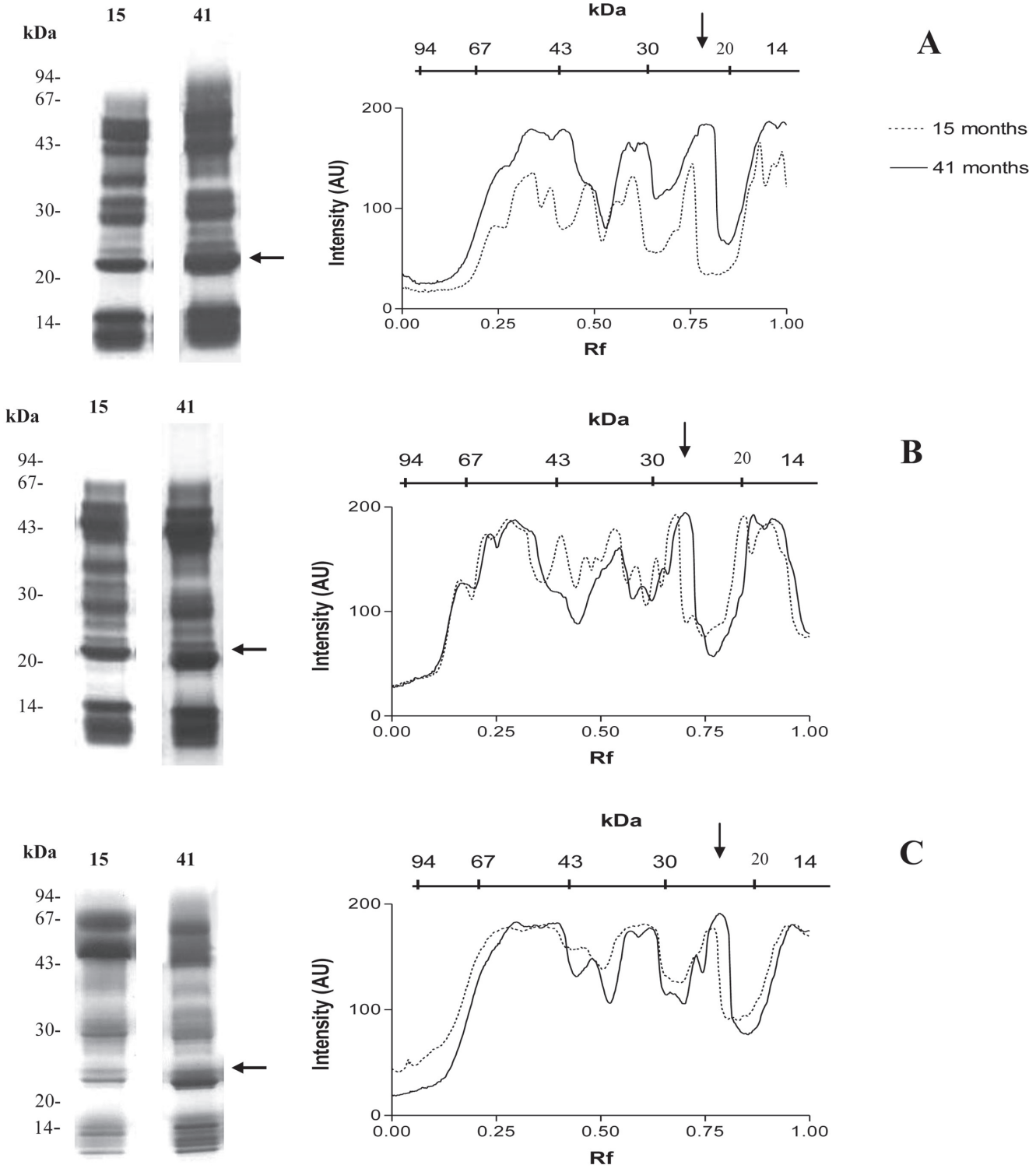

FIGURE 1. Densitometric analysis (Arbitrary Units) of electrophoretic profiles of *Bothrops insularis* venoms (120 µg/lane) under reducing conditions. A: Intersex 1 venom; B: Intersex 2 venom; C: Male venom. Numbers on top of figures correspond to the snake's age (in months). 24 kDa proteic band is indicated by an arrow at right (SDS-PAGE profiles) and on top of the figures (densitometric graphics). Molecular weight markers are shown at left on SDS-PAGE gels and on the top of densitometric profiles. The samples were running together on the same gel with the same running conditions (see Material and Methods section).

Recently, Modesto *et al.* (2005) purified a 22.6 kDa metalloprotease from the venom of *B. insularis,* with prothrombin activation and fibrin(ongen)olytic activity. According to Junqueira-de-Azevedo and Ho (2002, 2003) metalloproteases correspond to almost 42% of toxins transcripts from the venom gland of *B. insularis*. Zymography analysis, with acrylamide-gelatine gels, showed that the band at 24 kDa range had its proteolytic activity inhibited when previously incubated with metalloprotease inhibithors, such as EDTA and 1,10-phenantroline (Zelanis *et al.,* 2007). Proteases play an important role in venom composition, since they are related to several physiological processes that have, as main purpose, to accelerate prey digestion, which may prevent putrefaction and regurgitation of larger prey items (Thomas and Pough, 1979; Kochva *et al.,* 1983; Mackessy, 1993; Daltry *et al.,* 1997; Mebs, 1999). Thus, an increase of protease expression must be extremely important for a species such as *Bothrops insularis,* that has an ontogenetic shift on its diet, feeding on larger preys (birds) during the adult phase. In addition, Junqueira-de-Azevedo *et al.,* (2001) cloned and expressed a Vascular Endothelium Growth Factor, named sv-VEGF, which facilitate venom spreading due to its action of increasing in vascular permeability and, therefore, contributing to a systemic action of the venom as a whole.

Toxicity of *B. insularis* snake venom was remarkable toward chicks and bees in comparison to mice, whose LD50 values were higher. This is a predictable characteristic for a species that feeds mainly on arthropods and birds. The status of being the most powerful venom known among Brazilian snakes of the genus *Bothrops* was incorrectly attributed to *B. insularis* by Afranio do Amaral on his early studies with *B. insularis* venom (Amaral 1921, Amaral 1927). The experimental models used in his studies were only birds and the route of venom injection was intravenous, leading to animal shock and a quick intra-vascular disseminated coagulation.

Diet plays an important role on venom characteristics, acting as a selective factor. Li *et al.* (2005a and b) showed the implications of egg-based diet of *Aipysurus eydouxii* (Elapidae, Hydrophiinae) on venom toxins profile. According to these authors, a reduction of selective pressure upon genes encoding potent toxins for fish, and accumulation of mutations on these genes, could be responsible for the low activity of *A. eydouxii* venom upon fish, differing from another species of the same genus *(A. laevis),* which feeds on fish and has an extremely neurotoxic venom.

Furtado *et al.* (1991) showed an increase of toxicity in adult venoms of nine species from the genus *Bothrops.* An expressive relationship with diet and snake venom ontogeny was shown by Andrade and Abe (1999) working with two species of the same genus: *B. jararaca* (generalist, feeding mostly on ectothermic preys when juvenile, and endothermic ones when adult) and *B. alternatus* (specialist, feeding on mammals during the whole life). As expected for mammals (mice), the authors found higher toxicity in the venom of *B. alternatus* (regardless of the age of the animal) in comparison to the venom of *B. jararaca.* The opposite was verified for amphibians (frogs) in which *B. jararaca* venom showed higher toxicity, with juveniles more toxic for amphibians and adults more toxic for mammals. On the other hand, our results did not show ontogenetic variability in lethality of *B. jararaca* venom upon mice.

Although an expressive ontogenetic shift on venom toxicity is apparent in *B. insularis,* it is possible to suggest that toxicity for local preys is probably a characteristic present during the whole life of a specimen, given the values obtained for chicks and bees in comparison to mammals. *Bothrops insularis* venom specificity may be the result of selective pressure related to the particular feeding habit of this species. If the bird is released after being bitten, it may be able to fly some distance and, therefore, does not leave a chemical trail by which it could be relocated by the snake (Hayes *et al.,* 2002).

In conclusion, the results presented here demonstrate the complex intra-specific variability on snake venoms and suggest an ontogenetic variability and taxon-specific activity in the venom of *Bothrops insularis,* two main factors that support the snake's diet on its insular habitat.

Resumo

Nós verificamos mudanças ontogenéticas na composição do veneno de *Bothrops insularis,* uma serpente endêmica da Ilha da Queimada Grande (São Paulo, Brasil) que apresenta mudanças ontogenéticas na dieta. Os perfis eletroforéticos mostraram padrões similares entre as amostras e em diferentes idades. A análise densitométrica mostrou um aumento na expressão de uma banda protéica de massa molecular de 24 kDa. A toxicidade do veneno foi maior para abelhas (*Apis mellifera,* recém emergidas) e aves (pintainhos machos de um dia de vida, linhagem Bovans White), embora uma relação direta com a ontogênese

não tenha sido estabelecida. A seletividade do veneno para presas locais foi demonstrada pela intensa toxicidade sobre artrópodes e aves em comparação com mamíferos. Os resultados sugerem que o veneno de *B. insularis* possui peculiaridades na sua composição que favorecem uma dieta especializada no seu habitat insular e, embora ocorra uma expressiva mudança ontogenética na dieta, a toxicidade do veneno para presas locais é uma característica presente durante toda a vida desta espécie. Nossos resultados fornecem uma forte evidência da presença de uma toxicidade taxon-específica no veneno de *B. insularis* e também de uma tendência de os venenos dos adultos serem mais tóxicos que os dos indivíduos jovens.

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