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Sexual dimorphism in venom of *Bothrops jararaca*(Serpentes: Viperidae)

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Abstract

Bothrops jararaca is an abundant snake in Brazil, and its venom has been studied exhaustively. The species exhibits adult size dimorphism in which female are larger. We registered the growth in Snout-Vent Length and weight of one litter (with 11 females and 12 males). We compared growth curves and venom profile between male and female of *B. jararaca* in order to establish the relationship of those characters and sex. Their venoms were analyzed when they were 36 months old, concerning SDS PAGE, protein content, proteolytic, hyaluronidasic, phospholipasic, blood-clotting, edematogenic, hemorrhagic, myotoxic activities, and lethality. Differences in the growth curves of the females and the males were significantly different after the 12th month of age, with the females growing faster. Females produced five times more venom than males. The electrophoretic patterns were variable: the venom from males had more protein bands than females. Venom composition varied significantly between males and females. Venom from females is more potent for hyaluronidasic, hemorrhagic, and lethality activities, whereas venom from males is more potent for coagulant, phospholipasic, and myotoxic activities. The variability of proteolytic and edematogenic activities were not significant. The important sexual dimorphism in body size and mass, amount of venom produced, and venom composition in *B. jararaca* may reflect a divergence in niche partitioning.

Keywords: Snake size growth; Sexual dimorphism; Venom; *Bothrops jararaca*

1. Introduction

The pitviper *Bothrops jararaca* is one of the most abundant viperid snake in Brazil, and the most important species concerning the number of accidents, since it causes about 90% of all snakebite recorded in its area of occurrence (Ribeiro and Jorge, 1997). This terrestrial lancehead occupies a diversity of habitats including tropical deciduous (broadleaf) forests, open areas, as well as semitropical upland forests (Campbell and Lamar, 1989; Sazima, 1992).

Clinical manifestations shown by patients bitten by *B. jararaca* include local and systemic envenoming. Local inflammatory process with solid edema is the most frequent sign accompanied by local pain, and local necrosis is another important complication, followed many times by secondary infection (Ribeiro and Jorge, 1990). Concerning systemic

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envenomation, the consumption coagulopathy is the clinical result of the coagulant activity. Its effect is a disseminated intravascular coagulation-like syndrome with partially or completely unclottable blood (Kamiguti and Sano-Martins, 1995). Hemorrhagic phenomena are relatively common consequences of *B. jararaca* bites, but are less important in most cases. The commonest cause of deaths is acute renal failure (Fan and Cardoso, 1995; França and Málaque, 2003).

Due to its epidemiological importance, *B. jararaca* venom has been exhaustively studied and as other snake venoms it contains many different constituents including toxic and nontoxic, enzymatic and non-enzymatic fractions. Such components interfere with blood coagulation as with the case of thrombin-like enzymes (Nishida et al., 1994), the Bothrojaracin, a thrombin inhibitor (Zingali et al., 1993), the activator of factor X (Furukawa and Hayashi, 1977), von Willebrand Factor modulator, botrocetin (Fujimura et al., 1991), or activators and inhibitors of platelet aggregation (Nishida et al., 1994; Serrano et al., 1999). Many proteases, some of them fibrinolytic enzymes (Maruyama et al., 1992), and other components such as Zn metalloproteinase are capable of provoking haemorrhagic action (Mandelbaum and Assakura, 1988; Kini and Evans, 1992). On the other hand, the phospholipase A_2 has been found to be a myotoxic toxin (Moura-da-Silva et al., 1991; Machado et al., 1993), and bradykinin-potentiating peptide (Ferreira, 1965) is another important molecule present in the venom of this pitviper.

Several reports have demonstrated both individual intra-specific and geographical differences in the composition of *B. jararaca* venom. Schenberg (1961) found more than 50 varieties in that venom, and one of the most remarkable difference regards the coagulant activity (Rosenfeld et al., 1959).

Differences in composition and activity also depend on snake age. Venom of the immature *B. jararaca* was reported to contain higher coagulant activity than that of mature specimens (Rosenfeld et al., 1959), due to a higher level of prothrombin and factor X activators (Kamiguti and Hanada, 1985; Furtado et al., 1991). Bites caused by young or immature *B. jararaca* are usually not so severe, even though coagulation disorders have been recorded (Kamiguti et al., 1987; Maruyama et al., 1990). This ontogenetic variation in venom composition may reflect the ontogenetic shift in prey types (Martins et al., 2002).

Sexual dimorphism concerning size and scalation has been widely recorded in snakes, including some species of *Bothrops*, as *B. moojeni* (Leloup, 1975), *B. atrox* (Federsoni, 1978/1979), *B. asper* (Solórzano and Cerdas, 1989), and *B. pauloensis* (Valdujo et al., 2002). *B. jararaca*, particularly, presents significant sexual dimorphism in Snout-Vent Length (SVL) and weight, in which females exceed the size of males (Vanzolini, 1946; Sazima, 1992) and number of ventral scales (Hoge et al., 1976/1977).

Sexual dimorphism is usually interpreted in terms of reproductive adaptations, but the degree of sex divergence also may be affected by sex-based niche partitioning. In gape-limited animals like snakes, the degree of sexual dimorphism in body size or relative head size can determine the size spectrum of ingestible preys for each sex (Shine, 1991; Shetty and Shine, 2002). Adult males and females may diverge strongly in dietary composition: males may consume smaller preys, while females usually take larger mammals (Forsman, 1991; Pearson et al., 2002).

It is well established nowadays that snake venom composition is influenced by a multiple factors, including phylogeny, geographic distribution of different populations, age, sex, and prey items ingested (Chippaux et al., 1991). However, information on the relationship between sex and venom variation composition are scarce or completely unknown.

Thus, this work aims to compare the venom profile of both male and female *B. jararaca* regarding yields and quality of activities in an attempt to discuss the causes of venom variation, as well as the influence of sex upon its composition.

2. Materials and methods

2.1. Snakes and venom samples

One pregnant female *B. jararaca* from Ibiuna city (23°34′S, 47°13′W), São Paulo state, was kept in captivity in Laboratório de Herpetologia, Instituto Butantan, until she gave birth to 23 neonates (11 females and 12 males). The young had the SVL, total length (TL) and weight (W) taken to the nearest centimeter. Those biometric data were recorded for all young every 2 months, during all the 48 months of observation. They were kept individually in cages at room temperature (24 ± 2 °C), and were fed "ad libitum" every 15–20 days (with neonate mice, about 5 g each) until the

age of 2 months. After that, they were fed on adult mice or rats (about 25 g each) at the same frequency. Individual venom milking started when snakes were 36 months old. Liquid and dry (freeze-dried) amounts of venom produced were measured individually. Venom samples were stored at −20 °C until analysis when they were reconstituted in sterile saline solution (0.9% NaCl). Animal care and procedures used were in accordance with guidelines of the Animal Ethics Committee of Instituto Butantan.

2.2. Animals

Outbreed Swiss mice (18–22 g), adult male Wistar rats (200–280 g) were obtained from "Biotério de Criação de Animais do Instituto Butantan, SP, Brazil". The animals were maintained and used under strict ethical conditions according to the international welfare recommendations.

2.3. Protein content determination

Protein was measured by the method of Markewell et al. (1978) using albumin as standard, by spectrophotometric absorption at 280 nm.

2.4. SDS–polyacrylamide gel electrophoresis

Venoms (30 μl; 25 mg/ml 0.0l M glicine phosphate buffer, pH 8.7) and molecular weight calibration markers (MERCK) were subjected to 7.5–17.5% SDS-Glicine electrophoresis (25). Proteins were stained with Coomassie Blue R-250 (Laemmli, 1970).

2.5. Proteolytic activity determination

Proteolytic activity was estimated using casein as substrate, as described by Lomonte and Gutiérrez (1983). Venom samples were used in 50, 100, or 200 μg concentration. Blank was made without venom sample. The caseinolytic activity was expressed at $U/mg = (A_{280}/mg\ venom) \times 100$.

2.6. Hyaluronidase activity

Hyaluronidase activity was determined turbidimetrically by the method of Pukrittayakamee et al. (1988). Venom samples were dissolved on assay mixture (200 mM acetate buffer pH 6.0 containing 150 mM NaCl) to a final concentration of 50 μg, and the substrate (hyaluronic acid 0.5 mg/ml) was added. The mixture was incubated for 15 min at 37 °C, and reaction was stopped by the addition of 1 ml of 2.5% cetyltrimethylammonium bromide in 2% NaOH solution. Absorbance of tubes was recorded at 400 nm against a blank in which no venom was added. Turbidity reducing activity was expressed as a percentage of the remaining hyaluronic acid, taking the absorbance of a tube in which no venom was added as 100%. Specific activity was expressed using turbidity reducing units (TRU)/mg of venom.

2.7. Phospholipase A_2 activity

Phospholipase A_2 activity was measured following the method described by Holzer and Mackessy (1996). Samples of 300 μg of venoms, dissolved in 0.85% NaCl solution, were added at a 10 mM Tris-HCl buffer containing 10 mM $CaCl_2$, 100 mM NaCl. Then, 100 μlof the substrate, 0.3 mM of 4-nitro-3-octanoloixy acid in acetonitrile, were added. The mixture was incubated at 37 °C for 10 min and the reaction stopped by the addition of 2.5% Triton X-100 solution. The absorbance of the samples was recorded at 425 nm against a blank in which no venom was added. Specific activity was expressed as nM of substrate produced/min/mg of venom.

2.8. Coagulant activity

Coagulant activity was determined in human plasma (Theakston and Reid, 1983). Citrated blood samples (1 part of 129 mM trisodium citrate and 9 parts of blood) were centrifuged at 1700 g for 15 min at 4 °C for obtaining plasma. The coagulation time was estimated after the addition of serial dilutions of venom. The minimum coagulant dose (MCD-P) of venom is defined as the minimum amount of venom resulting in clot formation of human plasma within 60 s at 37 °C.

2.9. Edematogenic activity

The time-course of edema was determined in mice injected into the sub plantar surface of left hind paw with 50 μl sterile saline containing different doses of venom. The contra lateral paw received the same volume of sterile saline. Oedema was measured by micrometer at 3 h. Results were calculated as difference between values obtained in both paws, and oedema was expressed as percentage of increase

in paw volume. The edematogenic minimal dose was calculated (dose of venom that produces 30% oedema) (Gutiérrez et al., 1986). For that, the animals were injected with 0.4, 0.6, 0.8, 1, and 1.2 μg of venom.

2.10. Hemorrhagic activity

Hemorrhagic activity was quantitatively determined through intradermal injections of serial dilutions of each venom in 100 μl of saline solution into the shaven backs of Wistar rats (200–230 g, males). They were killed after 24 h, the dorsal skins were removed, and the hemorrhagic lesions were measured (Theakston and Reid, 1983). The minimum hemorrhagic dose (MHD) is defined as the least amount of venom (μg of protein) that results in a hemorrhagic lesion of 10 mm diameter.

2.11. Myotoxic activity

For evaluation of myotoxicity, a solution of 50 μg of venom in 50 μl of physiological saline was injected i.m. (thigh muscle) of male's mice (18–22 g). After 3 h, blood was collected by orbital plexus puncture. Sera were separated and immediately assayed for creatine phosphokinase (CPK) activity at 25 °C, and measured at 340 nm. Six mice were assayed for each venom. For control test, mice were injected with physiological saline. One unit of myotoxic activity per liter (U/l) corresponds to the amount of venom, which hydrolyzes 1 μmol of the substrate per minute in the conditions given in the Sigma CK-520 method.

2.12. Lethal toxicity

Lethal toxicity was assessed in outbreed Swiss mice (18–22 g) by intraperitoneal injection of venom in 0.5 ml of physiological saline. Six mice were used at each venom dose. The LD_{50} was calculated by probit analysis of deaths occurring within 48 h of venom injection (Finney, 1971). Confidential limits (95%) were also calculated.

2.13. Histopathological analysis

For the light microscopic study, all the mice were injected i.m. with 50 μg/50 μl of venom solution in the dorso lateral part of the right thigh (gastrocnemius muscle). The control was injected with saline solution. The animals were killed by CO_2 inhalation after 3 h injection. The gastrocnemius muscle was immediately removed, placed in 10% formaldehyde fixative solution. The tissues were processed, and included in paraffin. Sections were stained with hematoxilin-eosin, and examined under a light microscope.

2.14. Statistical analysis

We used parametric tests, and the results are presented as mean ± standard deviation. We used the Student's test in order to verify differences between the two experimental groups, with $\alpha = 0.05$ (Zar, 1999). We used linear regressions to explore venom yield and SVL (Vanzolini, 1993; Zar, 1999).

3. Results

New born *B. jararaca* females had minimum 23.5 and maximum 26.5 cm SVL whereas males varied from 24.0 to 27.0 cm. Concerning body weight new born females showed a variation from 7.0 to 8.5 g and new born males from 6.0 to 9.0 g (Table 1). The new born did not differ significantly in mean SVL ($t = 1.74$; $P = 0.09$) and mass ($t = 0.43$; $P = 0.67$).

Table 1
Sexual dimorphism in body size during development (36 months) of a *B. jararaca* litter kept in captivity–Average ($\bar{x} \pm$ SD) body size in length (SVL) (cm) and weigth (g), and ranges

Biometric data	Females		Males	
	New born	36 months old	New born	36 months old
SVL (cm)	24.8 ± 8.3	119.8 ± 76.1	25.4 ± 9.0	79.4 ± 38.3
Range	23.5–26.5	102.0–132.0	24.0–27.0	70.0–82.0
Weight (g)	8.0 ± 0.5	576.3 ± 125.5	7.9 ± 0.7	120.8 ± 12.1
Range	7.0–8.5	355–755	6.0–9.0	105–140

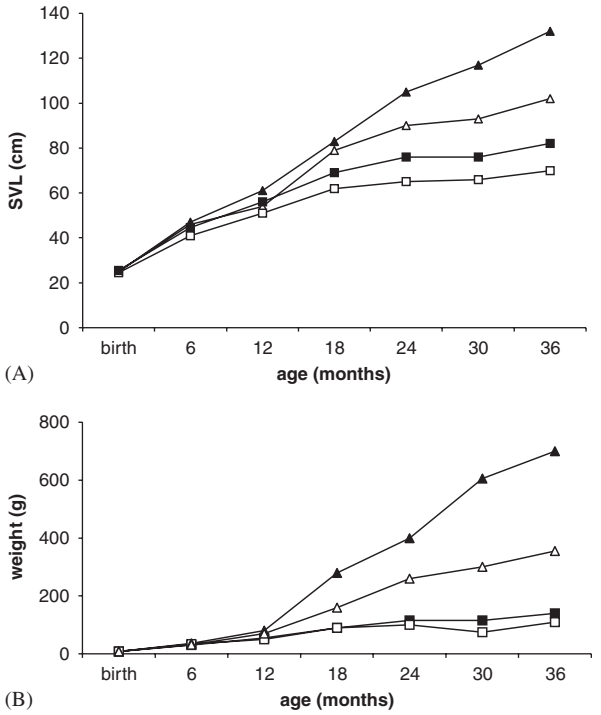

Fig. 1. Growth in SVL (cm) (A) and weight (g) (B) of the smallest and the largest male and female of *B. jararaca* litter kept in captivity during 36 months. ■, largest male. □, smallest male. ▲, largest female. △, smallest female.

Table 2
Range and average ($\bar{x} \pm$ SD) of venom production, both liquid (g) and frozen dried (g), in adult male and female *B. jararaca* litter kept in captivity

Venom	Snake sex	N	Yield (mg)
Liquid	♂	12	75–270 (170±80)
	♀	11	647.3–1108.6 (940±130)
Frozen dried	♂	12	17.8–65.4 (40±20)
	♀	11	179.3–264.1 (220±20)

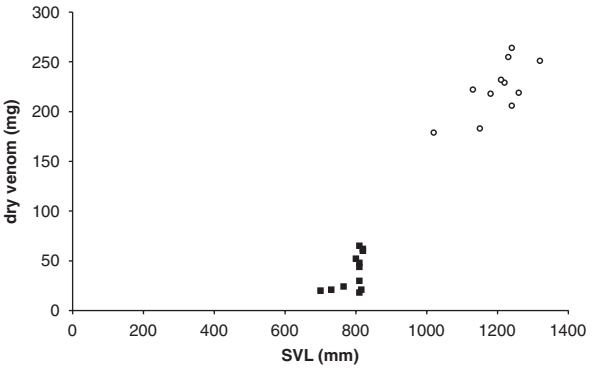

Fig. 2. Yields of freeze dried (g) venom produced × SVL (cm) by male and female of *B. jararaca* litter kept in captivity. ■, males. ○, females.

Development showed similar pattern among all animals studied until approximately 12 months old, since when the curves of snout-vent and, especially, body weight diverged considerably between sexes (Fig. 1). On average, the females exhibited a more significant growth, increasing from 35 cm (±6 cm) and 58 g (±11 g) in the first year, to 43 cm (±5 cm) and 244 g (±60 g) in the second year and to 29 cm (±6 cm) and 266 g (±266 g) in the third one. The males presented the average of 30 cm (±4 cm) and 43 g (±8 g) in the first year, 23 cm (±3 cm) and 53 g (±10 g) in the second, and 10 cm (±2 cm) and 17 g (±10 g) in the third one. Male grow slowly after the first year, different from females that present a tendency to remain increasing in size (Fig. 1A and B). Thirty-six months after birth, *B. jararaca* female snakes are significantly larger and heavier than males (SVL: $t = 16.9$; $P < 0.0001$; Weight: $t = 13.0$; $P < 0.0001$).

Data showed that females produce significant larger amounts of venom, both liquid and freeze dried, in relation to males at the same age (Table 2). The mean amounts of frozen dried venom for females was 220 mg, and for males it was 40 mg, or five times more in females than in males. Statistical analysis showed significant differences between both liquid ($t = 16.81$; $P < 0.001$) and frozen dried ($t = 31.03$; $P < 0.001$) venom yields between male and female *B. jararaca*. Regression analysis pointed out positive correlations between yields of venom and SVL in male and female *B. jararaca* (Fig. 2). The relationship between average of mg of weights of dried venom per 100 cm of TL also showed that females producing significantly more venom than males ($T_{21} = 17.47$, $P < 0.0001$).

The electrophoretic mobility of eight individual males and six females *B. jararaca* venom samples components were compared after separation under reducing conditions in SDS-PAGE (Fig. 3). The *B. jararaca* venoms showed electrophoretic similarity. There were, however, minor individual variations, particularly in the relative intensity of the protein bands. Three bands of protein in molecular weight of 60, 28, and 14 kDa are present in all *B. jararaca* venoms samples, however, at 28–40 kDa region, in females there are four distinct bands, while in venom from males samples there are four to eight bands superposed. In males the electrophoretic patterns are more variable.

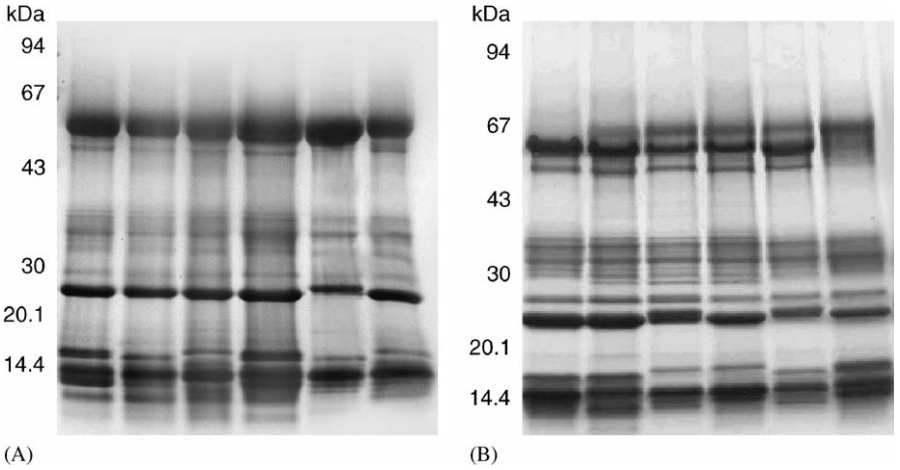

Fig. 3. Electrophoretic pattern (SDS-PAGE) of *Bothrops jararaca* venoms. Females venoms samples (A) and Males venoms samples (B). Numbers on the left correspond to the position of molecular weight markers.

Table 3
Protein content determination, proteolytic, hyaluronidasic, phospholipasic, blood clotting, edematogenic, hemorrhagic, myotoxic activities, and lethality of females and males venoms of *Bothrops jararaca* litter

Venom Activities	Females	Males	Statistical analysis
Total protein (μg protein/mg venom)	1047.6±134.4	931.8±148.1	$P<0.05$
Proteolytic (U/mg)	113.6±1.8	103.5±1.8	*n.s.*–$P>0.05$
Hyaluronidasic (TRU/mg venom)	32.2±1.4	28.1±1.2	$P<0.02$
Phospholipasic (nM/min/mg venom)	1.40±0.1	4.13±0.09	$P<0.0001$
Clotting MCD (μg/ml)	49.4±0.9	27.2±0.4	$P<0.001$
M E D (μg)	0.57±0.07	0.61±0.17	*n.s.*–$P=0.61$
Hemorrhagic dose (μg/rat)	14.2±2.1	20.2±2.5	$P<0.05$
Myotoxic (U/L)	58.0±13.3	102.0±36.3	$P<0.01$
Lethal dose (μg/mouse)	44.8 (34.7–68.6)	69.8 (56.7–83.6)	$P<0.01$

For venom activities see Materials and Methods

Table 3 depicts conspicuous variations in total protein content, proteolytic, hyaluronidasic, phospholipasic, blood clotting, edematogenic, hemorrhagic, myotoxic activities, and lethality of the *B. jararaca* venom from females and males.

Statistical analysis showed significant differences between protein contents, higher in female venom ($P<0.05$). We utilized the protein contents, in order to standardize the venoms samples for activities determination.

The proteolytic action in casein is the same for males and females venoms, what was confirmed by zymogram (data not shown). The female venoms are significantly more potent in hyaluronidasic ($P<0.02$), hemorrhagic ($P<0.05$) and lethal ($P<0.01$) activities, whereas male venoms are significantly more active in blood clotting ($P<0.001$), myotoxic ($P<0.01$) and phospholipasic ($P<0.0001$) activities. The edematogenic activities are the same for males and females *B. jararaca* venom.

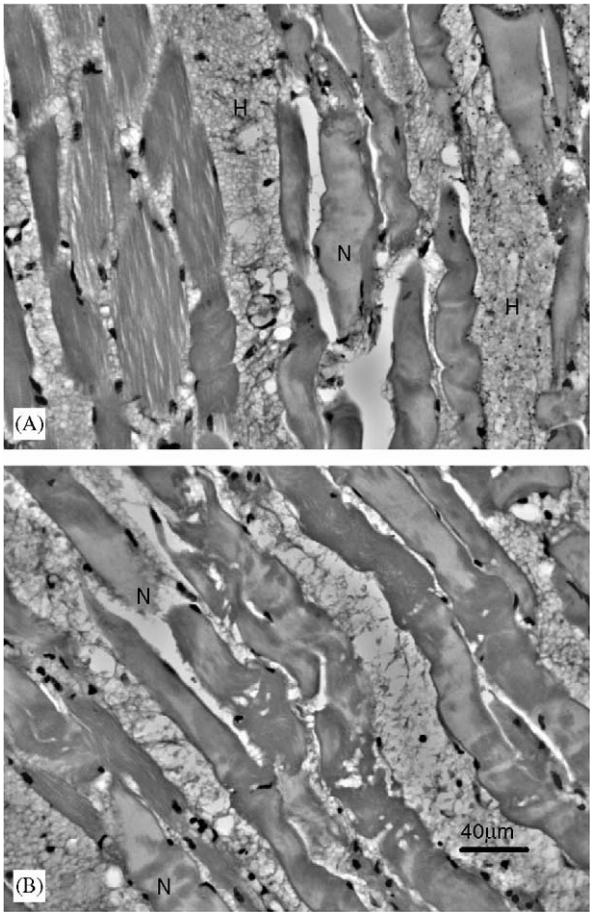

Fig. 4. Light micrographs of mouse skeletal muscle taken 3 h after injection of venom samples. A, Females venom; B, Males venom; H. hemorrhage; N. necrotic muscular fibers.

The histopathological analysis of muscles tissue injected before 3 h with venoms demonstrated that the local tissue damage were different, with females venom causing massive hemorrhage with moderate myonecrosis, and males inducing severe myonecroses with scanty hemorrhagic areas (Fig. 4).

4. Discussion

Sexual dimorphism in size, shape, color and behavior is a widespread phenomenon among animals, and in most snake taxa the females exceed males in body size (Shine, 1993, 1994). The primary causes for sexual dimorphism in body size may be due to selection for fecundity or ecological requirements (Shine, 1986, 1989).

Sexual differences in habitat use and diet are widespread among snakes, and are accompanied by significant divergences in relative head size and head shape in many taxa (Camilleri and Shine, 1990; Shine, 1991). Natural selection acting to reduce resource competition between sexes is a probable evolutionary scenario.

Although many studies have documented differences in body size between male and female snakes, and concomitant differences in prey size (Shine, 1990), studies that explicitly demonstrate the sexual dimorphism in venom are almost inexistent. This issue aims to examine the relationships among growth, amount of venom production, and venom activities in adult male and female of one litter of *B. jararaca*.

The lancehead *B. jararaca* hunts mainly from ambush (Sazima, 1989, 1992), and consume a wide variety of prey. Juvenile *B. jararaca* typically consume centipedes, frogs and lizards whereas adults feed on rodents (Sazima, 1992; Martins et al., 2002).

Male and female neonates are similar in size. Juvenile growth rates also appear to be uniform between sexes. However, after 12 months from birth males and females begin to diverge in size and mass, when female growth raises. This difference apparently resulted most proximally from reduced growth rates of males after the onset of reproductive activity and the continuation of growth in females throughout the mature stage (Brown and Weatherhead, 1999). In *Bothrops* the sexual maturation period is around 24 months age (Leloup, 1975; Almeida-Santos and Salomão, 2002).

An increase in venom yield with the snake body size is not surprising, and similar patterns are probably universal among venomous snakes (Marsh and Whaler, 1984; Mirtschin et al., 2002). But little information is available on sex differences in venom yields (de Roodt et al., 1998).

Adult' females of *B. jararaca* present a production of venom with a mean yield of 220 mg whereas males present 40 mg. The venom from females has higher lethal activity than that of males, and when the amount of venom is correlated with the value of lethal dose, females present about 4850 lethal doses whereas males present 570. Thus, females are potentially eight times more dangerous. The hyaluronidasic and hemorrhagic activities were also more active, confirmed by the histopathological analysis that shows massive hemorrhagic affects in murine muscle tissue.

At the 28–40 kDa region, in electrophoretic profile, venom from males' samples present four to eight superposed bands. In this molecular weight

region we found serine proteases as Bothrojaracin (Zingali et al., 1993) and Bothrombin (Nishida et al., 1994), both related with actions in hemostatic system. This could be involved with the higher coagulant activity in males' venom. They also present intense phospholipasic and myotoxic activities that are responsible for intense myonecrotic action in murine tissue damage.

It was related for females of *Bitis nasicornis* (Marsh and Glatston, 1974), *Crotalus adamanteus* (Mebs and Kornalik, 1984), and *Calloselasma rhodostoma* (Daltry et al., 1996a, b) the production of an extra venom component that is absent from conspecific males analyzed by electrophoretic profiles. It was not described variations in venom activities for those species, and only for *Calloselasma rhodostoma* the enzymatic activities were analyzed, but no consistent differences among the samples were evident (Daltry et al., 1996a, b).

It was observed in many viperid snakes intersexual differences in diet (Shine et al., 1998), including other Brazilian lancehead, *B. moojeni*, in which adult males consume mostly ectotherms, and adult females prey mostly on endotherms (Nogueira et al., 2003).

The sexual size dimorphism with females bigger than males could reflect the tendency for females to eat larger preys, and for males to maintain similar diet as juveniles. This may have provided advantages to each sex in developing adaptations to foraging in different places for different kinds of preys, what is probably occurring with the species *B. jararaca*. Natural selection could thus amplify existing sex differences in some aspects such as body size, and relative head size with implications in amount and composition of venom.

The present study evidenced a conspicuous biochemical and pharmacological variability in the *B. jararaca* venom that seems to be associated with sexual dimorphism in body size and mass. It is relatively easy to deduce the implications concerning snakebite risk, and the caution with the snake extraction for compose the pool to be used in venom research in species where sexual dimorphism occurs as *B. jararaca*.

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