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Influence of size, sex and age on venom yield of *Bothrops leucurus* (Serpentes, Viperidae) under captivity conditions

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Snake venom is an expensive metabolic weapon used for digestion and defense. Detailed studies on the production of venoms are important for the manufacture of antivenoms and for the therapeutic management of snakebites. *Bothrops leucurus* is one of the snakes of medical importance responsible for a large number of accidents in Northeast Brazil. To establish the correlation between *Bothrops leucurus* venom (Blv) yield, under captive conditions, and the morphological characteristics (body mass and length), sex and age, 31 specimens were milked during one year, grouped by sex and age (juvenile, adult and long-lived), totaling 106 extractions in that period. We evaluated the electrophoretic profile (SDS-PAGE) under reducing conditions, the 50% lethal dose (LD50) and the minimum coagulant dose (DMC) of the extracted venoms. The body size was positively correlated with venom production in *B. leucurus* snakes. Regardless of sex and age, the venom showed no differences between liquid and solid composition or between right and left fang, however, the production of venom in females was twice the one found in males and more lethal. The clotting ability was lost as the animals aged, indicating that older snakes are not the best choice for venom pools in the production of antivenoms. These results are important for the choice of animals to antivenom production, and to understand the biological effects of snake venoms under captive conditions.

Key words: antivenom; lancehead; sexual dimorphism.

Venom is a powerful weapon used by snakes to digest prey and defend against predators. However, venom is a metabolically expensive product to produce, being a precious resource used by snakes to obtain maximum benefit (Morgenstern & King, 2013). The amount of venom secreted by snake venom glands can be altered by several factors such as morphological characteristics, sex, age, distance between prey, seasonality, temperature, habitat, diet, taxonomy, population origin and milking (Dissanayake *et al.*, 2015; Healy *et al.*, 2019). The severity of envenomation is influenced by the amount of venom injected into prey or victims, and this venom

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yield is also important for the supply of raw material in the manufacture of antivenoms (HEALY *et al.*, 2019).

In Brazil, about 90% of the reported snakebites are caused by species of the *Bothrops* genus, which makes these animals of great interest for public health (RORIZ *et al.*, 2018). In view of this situation, the keeping and breeding of *Bothrops* specimens in captivity is very important for the production of antivenoms and for scientific studies of venom toxicity (GREGO *et al.*, 2006; LIRA-DA-SILVA *et al.*, 2009).

Bothrops leucurus (WAGLER, 1845), popularly known as the white-tailed viper, is distributed in the north of the state of Maranhão, south of Ceará, southeast of Espírito Santo and across the entire state of Bahia (LIRA-DA-SILVA *et al.*, 2009). This species has records for Atlantic Forest and Caatinga domains, inhabiting forests, anthropic environments, as well as open areas in semiarid regions (ARANDA-SOUZA *et al.*, 2014; COSTA & BÉRNILS, 2018). This viper is the major causative agent of venomous snakebites in Northeastern Brazil, can reach around 1.70 meters (CARDOSO *et al*, 2003; GREGO *et al.*, 2006), remaining active during most of the year, mainly from March to October (CAMPBELL & LAMAR, 2004).

Previous studies have shown that *B. leucurus* venom is quite similar to other species from *Bothrops* genus, but there seems to be a high intraspecific variability, mainly in high molecular mass proteins (LIRA-DA-SILVA *et al.*, 2009; GREN *et al.*, 2019). Studies evaluating the yield of *B. leucurus* venom are still scarce, given its restricted distribution in Brazil, and because it does not compose the pool of venoms used in the manufacture of the national reference anti-bothropic serum, despite the large number of accidents it causes, especially in the northeast region (BRAGA *et al.*, 2020). Once injected into the human body, the *B. leucurus* venom can cause damage to local tissues, necrosis, edema, hemorrhage and changes in blood clotting (DE ARAÚJO *et al.*, 2017).

For the industrial production of antivenoms, the number of specimens required is directly related to the ratio between the amount of venom for immunization, quality control, and each snake's venom production (CHIPPAUX *et al.*, 1991). Venom yield constitutes an important pattern for serum producers; therefore, milked animals' welfare must be maintained during the production of antivenoms. However, there are several problems related to long-term captivity, such as histopathological damage to the glands, hemorrhage and fibrosis caused by mechanical pressure induced by extraction procedures (RODRÍGEZ-ABARCA *et al.*, 2019). Considering that venom production is largely linked to factors such as feeding, morphology, growth conditions and animal welfare, studies with captive snakes are useful in order to develop more efficient breeding and extraction methods (LEÓN *et al.*, 2018). For this reason, our study investigated how body size, sex and age could influence the production of *B. leucurus* venom when manually milked in captivity conditions.

MATERIALS AND METHODS

Animals and housing

Snakes were obtained through donation, capture and animals born in captivity

in the Herpetology Laboratory of the Butantan Institute - SP, Brazil. Thirty-one laboratory-housed specimens of healthy *Bothrops leucurus* were kept in intensive captivity for at least two years in polyethylene boxes under controlled temperature (27°C), humidity (60 - 75%) and 12 h light/dark cycles. The animals were fed once a month with a diet based on live mice or rats (10-20% of snake body mass) from a colony of heterogeneous male and female Swiss mice (*Mus domesticus domesticus*) or rats (*Rattus norvegicus*) of conventional sanitary standard (ILAR, 1996), and had access to water *ad libitum*.

Experimental groups

Experimental procedures were approved by the Ethical Committee of Butantan Institute, permit number 1364/15. Animal care was based on ethical principles of animal experimentation, the Resolution 879 of the Federal Council of Veterinary Medicine, the Law 11764/08 and following the rules of the International Council for Laboratory Animal Science, of the Brazilian Society of Laboratory Animal Science. The 31 used specimens of *B. leucurus* snakes, were kept in the Herpetology Laboratory of the Butantan Institute, and divided into groups according to sex (male and female) and age: juvenile (2-4 years old; n = 12; 7 females and 5 males), adult (5-7 years old; n = 8; 4 females and 4 males) and old (10-22 years old; n = 11; 9 females and 2 males). For all assays, venom pools were produced with male and female specimens from each experimental group.

Body size

For milking, weighing and body measuring, dry ice cooling was used for partial anaesthetization of snakes (SANCHEZ *et al.*, 1992). The larger snakes (over 250 g) were weighed on a mechanical scale (0.01 g), whereas the smaller ones (below 250 g) weighed on an electronic scale (0.001 g). For body length (Snout-Vent-Length or SVL), a metric ruler (0.1 cm) or a portable ruler (0.1 cm) was used (DE ROODT *et al.*, 1998; GREGO *et al.*, 2006).

Venom milking

B. leucurus crude venoms (Blv) were selectively milked with the same extractor, through manual gland compression, separated by each fang (right and left), packed in tubes and weighed separately (AG 200 analytical balance- Gehaka). Due to the size and reduced yield in juvenile snakes, there was no separation by fang in this group. In total, four quarterly milking was performed per specimen for one year. The specimens were used as pseudoreplications, totaling 106 extractions. All venoms were lyophilized (Liotop L101 bench lyophilizer- Liobras) and refrigerated (-20°C) until the moment of use. For the analysis of venom yield, we included only samples that did not display venom loss during milking. After each extraction, the snakes were left to rest for a week, and subsequently fed.

Venom yield

To analyze the Blv yield, animals' weight, length, sex and stage of development were considered. The amount of venoms in liquid and dry phase between the right and left fang was also assessed. Ven-

oms in liquid and dry phase were weighed on an analytical balance (0.001g). To evaluate the predictive value regarding venom production, we applied the linear relationship y = mx + b, where y = dependent variable (venom production), x = independent variable (length or body mass), m = slope, and b = intercept y (ZAR, 2009).

Protein quantification

One milligram (weight of dry venom) of each venom pool was diluted in 1 mL of saline (0.85%) and the protein content was measured by the method of MARKWELL *et al.* (1978) using albumin as standard. The final absorbance was read at 660 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Venom pool samples (20 μg) were subjected to SDS-PAGE according to LAEMMLI (1970), under reducing conditions, using 12% SDS-polyacrylamide gels. Gels were stained with Coomassie blue R-250. The molecular mass standard used was Dual Color Precision Plus (Bio Rad), ranging from 10 to 250 kDa. Densitometric analysis was performed using Gel Analyzer program.

Minimum coagulant dose on the citrated equine plasma (MCD- P)

The samples were tested using concentrations ranging from 0.015 to 2.0 mg of Blv / mL in 200 μL of equine plasma. Coagulation time (seconds) was evaluated in a Coagulometer (Stago Start) at 37ºC. The MCD-P was recorded as the lowest concentration of venom pool (μg of venom / mL of plasma) capable of coagulating a solution of equine plasma in 60s at 37°C. All tests were performed three times and the average time of the obtained values was considered. To verify coagulation inhibition in each venom groups, 100 μL of Blv (1.0 mg/ mL) was incubated with the Brazilian bothropic antivenom (B-BAV - Batch 0811177 / A - IB) (1:1) for 30 minutes. Subsequently, 200 μL of equine plasma were added and the clotting times at 37ºC were evaluated.

Median Lethal Dose 50% (LD50)

The 50% lethal dose (LD50) was calculated using the method described by SILES-VILLARROEL *et al.* (1979) . Two experiments were carried out using groups of five male Swiss mice (*Mus domesticus domesticus*) (18-22 g), from the Central Serpentarium of Butantan Institute, for each age group (juveniles, adults and old). One mg of lyophilized venom pool was diluted in 1.0 mL saline solution (0.9%) and injected (0.5 mL ip) into each group of mice (n = 5), using five sampled venom dilutions (33.0; 48.0; 70.0; 101.5; 147.0 μg/mL). The number of dead mice was recorded 48 hours after injection. The recorded data represented the mean values of the two experiments, and the calculations were done with "Full Probit Analysis" (FINNEY, 1971).

Statistical analysis

The data were analyzed descriptively (95% confidence intervals) and expressed as mean ± SD, followed by the number of observations. Prior to any statistical analyses, all recorded data recorded was tested for normality ($p > 0.05$) running a Shapiro-Wilk test, with the aim to confirm the data normality ($p > 0.05$). To study the relationships between different variables (body

mass and SVL), a Multiple Regression test was used, as well as the Kruskal-Wallis test, with the aim to verify intra and inter-group variation in venom yield. Differences with $p < 0.05$ were considered statistically significant (ZAR, 2009). All analysis were carried out using the software Prism 4.0 (Graph Pad, CA, USA).

RESULTS AND DISCUSSION

The group of snakes at the present study was composed of 12 juvenile animals (7 females, 53.8%; 5 males, 46.2%), 8 adults (4 females, 50%; 4 males, 50%) and 11 old (9 females, 81.8%; 2 males, 18.2%). The arithmetic means of dry venom in 19 adults and oldest *B. leucurus* snakes ranged from 0.5 to 1.2 g (0.8 ± 0.2g) (Kruskal-Wallis = 5.54; df = 1; p = 0.01); and in the 12 juvenile snakes ranged from ~0.07 to 0.1 g (0.08 ± 0.02g) (Kruskal-Wallis = 0.27; df = 1; p = 0.5) (Table 1).

There is variability in the amount of venom injected by a snake during an incident and it seems obvious that a juvenile specimen would inject a smaller amount of venom, when compared to an adult snake, even considering that not all of the gland's contents are injected during the bite. However, previous studies have already shown that large snakes have more venom, and produce more serious envenomations than smaller snakes, overturning the popular belief that smaller snakes are more dangerous than larger ones (DE ROODT *et al.*, 2016; DE ROODT *et al.*, 1998; JANES JR. *et al.*, 2010). In our study, for all extractions we recorded a positive correlation between body size (body mass and length) and the venom volume of each specimen.

The venom yield in adult female snakes (1.1 ± 0.24 g) was twice that found in males (0.5 ± 0.13g) (Kruskal-Wallis = 5.54; df = 1; p = 0.01), whereas among juvenile animals, this difference was not statistically significant (Kruskal-Wallis = 0.27; df = 1; p = 0.5). Regarding the venom production, adults and oldest snakes, showed from four to eight times higher production of venom than the juvenile ones (Table 1) (Kruskal-Wallis = 66.89; df = 1; $p < 0.001$). Our results corroborate previous studies in other species as *Naja naja* (DISSANAYAKE *et al.*, 2015), *Notechis scutatus* and *Pseudonaja textilis* (MIRTSCHIN *et al.*, 2002), as well as other species from the *Bothrops* genus (DE ROODT *et al.*, 1998), which found that the venom yield is more correlated to the body size and total length than the sex or other morphological characters. However, a venom yield study of *Naja atra* revealed that the amount of venom produced is different between the sexes, with male snakes expelling more venom than females of this species (GAO *et al.*, 2019).

We did not find significant variance between the venom production by the right and left fang of the snakes in this study. According to solid and liquid percentage in the venom, it was possible to observe that, regardless of the life stage (juvenile, adult and old), the venom display variations from 70% to 75% of liquid, whereas solid components vary from 25% to 30%. Despite those variations in venom composition, there was no significant variation between groups (p = 0.09). For adult and old snakes, those percentages in venom composition were recorded for both left (LF) and right fang (RF) (n = 18; t = -1.66, p = 0.11) (Table 2).

Considering healthy animals, both

Table 1: Biometric data and liquid venom yield of juvenile, adults and oldest *B. leucurus* snakes kept in captivity. Mean and standard deviation (x ± SD) in the four extractions. The body size (SVL = Snout-Vent-Length) did not vary during the study in the adults and oldest snakes.

Age class	ID (n=19)	Sex	Weight (g) ± SD	SVL (cm) ± SD	Venom (g) ± SD
Juvenile (2-4 years old)	Bl 1201-02	♀	75±15.8	68.0±4.0	0.086±0.038
	Bl 1201-03	♀	77±17.4	69.0±4.8	0.075±0.030
	Bl 1201-05	♂	66±11.7	63.0±3.5	0.078±0.023
	Bl 1201-06	♂	64±11.7	64.0±3.3	0.085±0.019
	Bl 1201-08	♀	80±18.5	71.5±5.0	0.099±0.023
	Bl 1201-09	♀	76±16.5	69.0±4.1	0.083±0.030
	Bl 1201-12	♂	65±11.9	65.0±3.9	0.070±0.027
	Bl 1201-14	♀	72±18.7	71.5±6.0	0.082±0.030
	Bl 1201-16	♂	65±10.7	64.5±4.1	0.088±0.017
	Bl 1201-17	♀	70±18.9	68.0±5.8	0.096±0.020
	Bl 1201-18	♂	69±13.1	66.0±3.5	0.068±0.019
	Bl 1202-06	♀	58±15.1	61.0±6.1	0.087±0.036
Adult (5-7 years old)	Bl 0901-06	♀	550±35.0	111.0	0.743±0.06
	Bl 0901-07	♂	495±0.1	107.0	0.601±0.01
	Bl 0901-08	♂	350±22.5	92.5	0.340±0.10
	Bl 0901-11	♂	381±26.9	96.0	0.363±0.14
	Bl 0901-13	♀	553±43.8	111.0	0.729±0.23
	Bl 0901-12	♀	530±35.0	107.0	0.580±0.28
	Bl 0901-15	♂	269±58.8	95.0	0.316±0.19
	Bl 1201	♀	470±22.5	98.5	0.596±0.14
Old (10-22 years old)	Bl 9417	♀	2201±109.4	152.0	1.708±0.21
	Bl 0202-17	♂	385±0.1	103.5	0.335±0.01
	Bl 0113	♀	1593±32.5	143.0	1.110±0.51
	Bl 0318	♀	1695±53.3	156.0	1.575±0.21
	Bl 0202-16	♀	1630±13.3	149.0	1.528±0.59
	Bl 0801	♀	1465±66.7	146.0	1.239±0.29
	Bl 9905	♀	1545±26.7	142.0	0.975±0.55
	Bl 0104	♀	1865±0.1	149.0	1.070±010
	Bl 0305	♂	313±2.5	106.0	0.457±0.13
	Bl 0204	♀	598±38.9	119.5	0.684±0.12
	Bl 0107	♀	1490±0.1	142.0	1.282±0.01

Table 2: Median and standard deviation (x ± SD) of lyophilized (solid) and crude *B. leucurus* venoms of each group, as well as yield of right fang (RF) and left fang (LF). Due to the low yield of juvenile snake venom, extraction was not separated by fang in that group.

Group		Venom			
		Solid (g) ± SD	**Solid (%)**	**Crude (g) ± SD**	**Crude (%)**
Adult	RF	0.068 ± 0.051	30	0.262 ± 0.102	70
	LF	0.071 ± 0.032	26	0.300 ± 0.100	74
Old	RF	0.099 ± 0.073	26	0.503 ± 0.306	74
	LF	0.165 ± 0.076	25	0.646 ± 0.278	75
Juvenile*		0.019 ± 0.009	25	0.084 ± 0.031	75

fangs are functional and it is possible that in nature, where they are more sensitive to environmental changes and diseases, snakes usually display differences for venom expelled by each fang (DE ROODT *et al.*, 2016). Since the captivity maintains stable environment conditions and prevents the appearance of diseases, the variability between fangs tends to reduce considerably (DE ROODT *et al.*, 1998). In a study carried out with adult rattlesnakes (*Crotalus atrox*) kept under diet and controlled environmental conditions revealed only minor changes in the composition of the venom (REX & MACKESSY, 2019). However, LEÓN *et al.* (2014) reported that the productivity of *Bothrops asper* venom changes throughout life in captivity, reducing the yield of 500 mg in newly collected snakes to 200 mg in those kept in captivity for years. COSTA *et al.* (2005) also found variability in dry venom yield of *Bothrops*, *Crotalus* and *Lachesis* snakes kept in captivity for a long time.

In juvenile snakes there was a progressive increase in the venom production correlated with the increase in weight and body length (Fig. 1). Male and female neonates of *Bothrops* snakes are similar in size. However, after 12 months of age, females tend to become larger and more robust than males (MIRTSCHIN *et al.*, 2002; GREGO *et al.*, 2006; DA SILVA *et al.*, 2017). Sexual dimorphism in relation to morphological (color, size, shape) and ecological characteristics (habitat and diet) is a widespread phenomenon among snakes (SHINE, 1993; SHINE, 1994). In some cases, females also have relatively larger heads, differing not

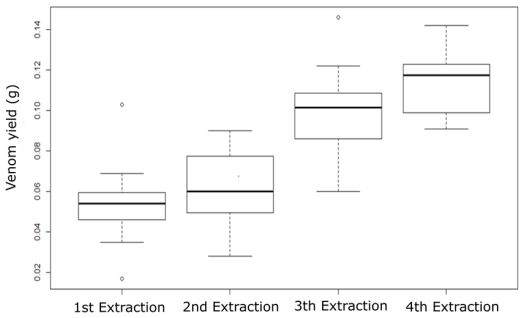

Figure 1: Boxplot representation of median, minimal and maximal yield venom of the four extractions (in an interval of three months each) in juvenile *B. leucurus* specimens. Dots indicate outliers. *$P < 0.05$ was considered statistically significant.

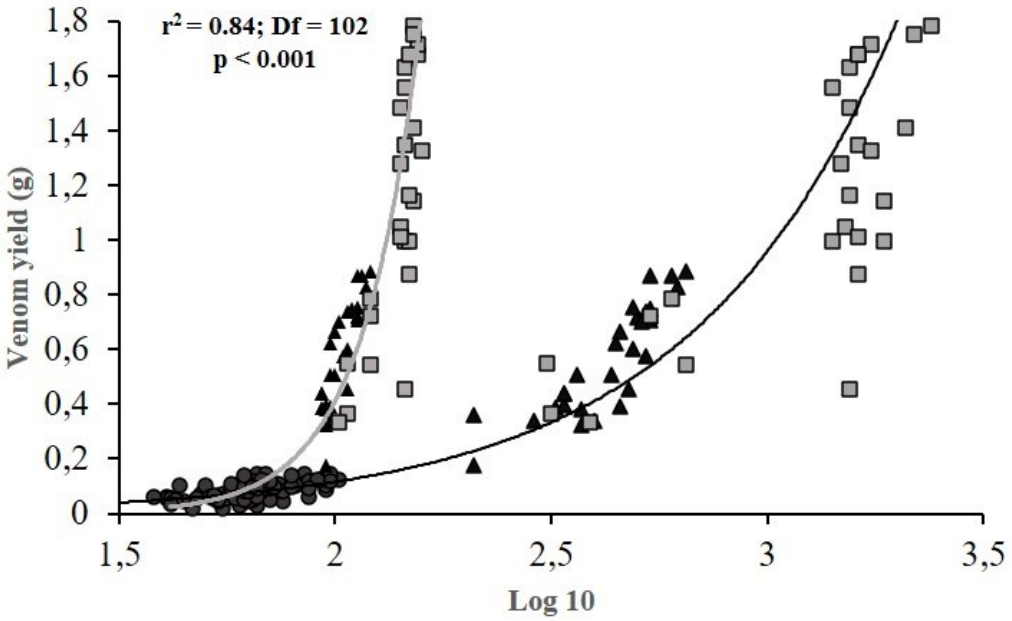

Figure 2: Positive correlation between venom production, log transformed body mass (black trend line) and body length (Snout-Vent-Length or SVL) (grey trend line) of all *B. leucurus* groups: juveniles (circles), adults (triangles) and old people (squares).

only in size but also in shape (DA SILVA *et al.*, 2017). A previous study with *Bothrops jararaca* revealed that venom yield from adult females is five times higher, and the venom is eight times more lethal than male snakes (FURTADO *et al.*, 2006), corroborating with our findings in *B. leucurus.*

Two trend line models were built addressing the variables venom yield, body mass and body length (Snout-Vent-Length or SVL) (Fig. 2). As all snake groups (juveniles, adults and old) were included in the analysis, and the graph demonstrates that there is a strong and positive correlation ($p < 0.001$), regardless of the group studied.

It was recorded a significant variation in the protein content between pooled samples of Blv, with concentrations ranging from 518.7 μg to 1,304.7 μg/ mL (Table 3). Regarding sex, rather more protein concentrations was recorded in females than males. The minimum coagulant dose (MCD) was directly proportional to the age of the snakes, disappearing the coagulant potential in older animals, regardless of sex. In the present study, the coagulation test showed similar results to those found by FURTADO *et al.*, (2006) in *B. jararaca*, showing that females have more coagulant venom than males, but that this biological property tends to be lost with aging. SILVA (2017) demonstrated that clotting times were shorter in the presence of female *B. leucurus* venom, however, the protein profile and the hemolytic and antioxidant effects were similar between male and female snake venoms. Several compo-

nents present in *B. leucurus* venom can act on the coagulation cascade in different pathways, such as the serine protease Leucurolin, which has high fibrinogenolytic activity (MAGALHÃES *et al.*, 2007). The B-BAV was able to neutralize the coagulation activity in all sampled groups (Table 3). These results are similar to studies developed with several species of snakes of the *Bothrops* genus (GAMA, 2000; LORENCETTI *et al.*, 2005; SAAD *et al.*, 2012; ZELANIS *et al.*, 2012; MORA-OBANDO *et al.*, 2021). QUEIROZ *et al.* (2008) demonstrated that the Brazilian bothropic antivenom (B-BAV) could recognize several but not all components present in the different venoms of *Bothrops spp*. Although the efficacy of an antivenom is directly bonded to the species of snake used in the immunization process, the antivenom production of Latin America demonstrates a high degree of cross-neutralization, despite the diversity of the *Bothrops* genus (SEGURA *et al.*, 2010).

Table 3: Protein content (µg protein/mL Blv) and Minimum Coagulant Dose (MCD) of *Bothrops leucurus* venom (Blv) on equine plasma (200µL) in the different groups tested in the time of 60sec. The Blv (25 µL) and B-BAV (Brazilian Bothropic antivenom) (1:1) were incubated, and 200 µL of equine plasma to verify inhibition of coagulation. n.c.: no coagulation. JF: Juvenile Female. AF: Adult Female. OF: Old Female. JM: Juvenile Male. AM: Adult Male. OM: Old Male.

Group	µg protein/ mL Blv	MCD (µg/mL)	Blv + B-BAV
JF	1123.3	2.0	n.c.
JM	1066.2	3.0	n.c.
AF	1304.7	76.0	n.c.
AM	1040.0	109.0	n.c.
OF	790.7	n.c.	n.c.
OM	518.7	n.c.	n.c.

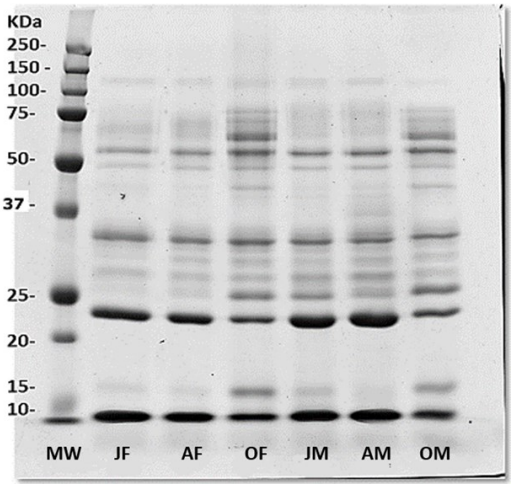

Figure 3: SDS-PAGE profiles of *B. leucurus* venoms pools (20 µg/lane) under reducing conditions. Electrophoresis was carried out in 12% polyacrylamide gels as described by LAEMMLI (1970) followed by staining with Coomassie Brilliant Blue R-250. *Juvenile Female (JF); Adult Female (AF); Old Female (OF); Juvenile Male (JM); Adult Male (AM); Old Male (OM). Molecular mass markers (MW) are shown in the first lane of gel.

The electrophoretic profiles in SDS-PAGE of venoms under reduction conditions showed similar composition and distribution of venom proteins, displaying small variations in number, arrangement and intensity of the bands. The hemorrhagic and cytotoxic character of most envenomations caused by viperids is explained by the action of four families of major toxins, four secondary ones, six minor ones, in addition to several rare ones and peptides (DAMM *et al.*, 2021). Previous studies

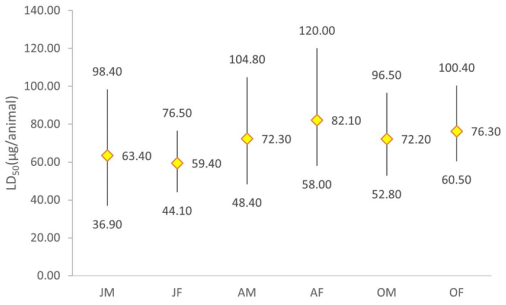

Figure 4: Lethal dose 50% (LD50) of *B. leucurus* pool venom (Blv). The median, minimal and maximal values of LD_{50} were expressed as µg/animal (mice of 18-22g). The highest toxicity of the Blv was obtained in the juvenile snakes ($p < 0.05$). *Juvenile Male (YM); Juvenile Female (YF); Adult Male (AM); Adult Female (AF); Old Male (OM); Old Female (OF).

have identified the composition of the *B. leucurus*' major toxins families: venom metalloproteinases (svMP) (25 - 65 kDa), serine protease (svSP) (35 kDa), phospholipases A_2 (PLA_2) (14 and 15 kDa) (Wilson, 2013), C-type lectin (~15 kDa) (Da Silva *et al.*, 2017); and secondary toxin families: L-amino acid oxidase (LAAO) (66 kDa). Disintegrin (DI) triggers the thromboinflammation scenario (Dos Santos Cavalcante *et al.*, 2022). According to sex, both females and males at different ages exhibited variations in the main components (number and intensity of the bands) between ~ 20 and 75 kDa. It was possible to verify bands of ~ 15 kDa in all venoms, which was more evident in older snakes, regardless of sex (Fig. 3).

A previous study comparing *B. jararaca* venom from long-term captive and recently wild-caught, showed no significant differences regarding protein composition, biological function and enzymatic activities, except for edematogenic activity, more prominent in the recently wild-caught venom pool (Da Costa Galízio *et al.* 2018). However, a proteomic study performed by McCleary *et al.* (2016) with recently wild-caught and in long-term captivity *Pseudonaja textilis* revealed that there are limitations to the effect of captivity on the composition of venom toxins. Intraspecific variation in snake venom may occur due to geographic location, eating habits, sex and stages of snake's development (Calvete, 2017). In general, there was no significant differences in the protein composition of the female and male *B. leucurus* venom, as the study by Braga *et al.* (2020) pointed out. However, studies of peptidomic evaluation, such as the one carried out by Pimenta *et al.* (2007) described significant differences in the venoms of males and females of *B. jararaca*. The modulatory expression of *B. jararaca* toxins may be associated with hormonal factors related to sex, justifying the differences in the coagulant potential of the bothropic venoms (Augusto-de-Oliveira *et al.*, 2016).

The potency of snake venom is usually prey-specific (Healy *et al.*, 2019). In this study, although Blv from juvenile female snakes showed higher lethality between groups (LD50 = 59.4µg / animal), the results of the lethal dose 50% (LD50) did not show significant variation among life stages, as well as between sexes (Fig. 4). The study by Healy *et al.*, (2019) also found no relationship between LD50 and snake venom production, and showed that venom is shaped by predator-prey coevolution and by macro ecological forces (body size and habitat structure). However, Rodríguez-Abarca *et al.* (2019) showed that snakes in

captivity have their LD50 and biochemical composition altered, resulting in changes in toxicity. This could happen because prey-specific snake venoms show decreasing potencies (higher LD50), since dietary diversity would tend to modulate the evolution of the generalist venom (LYONS *et al.*, 2020). Venom production is lower in species that inhabit three-dimensional environments, but increases with increasing body size and, consequently, metabolic rate (HEALY *et al.*, 2019). Thus, venom production can be influenced by the structural complexity of a habitat, due to its relationship with the encounter rate and prey escape routes (CARBONE *et al.*, 2014).

According DE ROODT *et al* (2016), there are practical implications for the predictability of the amount of venom that can be extracted from a snake, especially in breeding sites with a large number of animals. Therefore, correlating the size of a snake to the estimated venom production is an important tool for research centers and serpentariums. Despite the variations related to the method of extraction, the experience of the technician who does the milking and structure of the animal's own gland, in intensively bred snakes, such differences tend to be minimized.

To elucidate the evolutionary implications of variation in snake venom production under captive conditions, other causal factors of variation, such as diet and frequency of venom milking, should be further investigated, which was a limitation of the present study.

CONCLUSION

Size, sex and age can influence snake venom production. Such factors must be considered to optimize venom extraction for the *Bothrops* genus. In addition, snakes in captivity for a long time can have the biological properties of the venom affected due to advanced age, which can compromise the production of the venom obtained by antivenom manufacturers. Snake venom yield studies can help to understand how different factors interfere in animal production under captive conditions, elucidate predatory strategies in nature, guide accident treatment protocols, and bring valuable information to seroproducing centers.

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