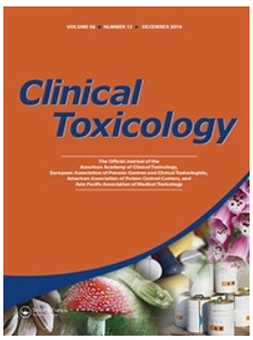

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"*Bad things come in small packages*": predicting venom-induced coagulopathy in *Bothrops atrox* bites using snake ontogenetic parameters

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BASIC RESEARCH

"*Bad things come in small packages*": predicting venom-induced coagulopathy in *Bothrops atrox* bites using snake ontogenetic parameters

Jorge Carlos Contreras Bernal[a,b], Pedro Ferreira Bisneto[c], João Pedro Tavares Pereira[b], Hiochelson Najibe dos Santos Ibiapina[a,b], Lybia Kássia Santos Sarraff[b], Cláudio Monteiro-Júnior[b], Handerson da Silva Pereira[b], Bruno Santos[b], Valeria Mourão de Moura[b,d], Sâmella Silva de Oliveira[a,b], Marcus Lacerda[b,e], Vanderson Sampaio[b,f], Igor Luis Kaefer[c], José María Gutiérrez[g], Paulo Sérgio Bernarde[h,i], Hui Wen Fan[j], Jacqueline Sachett[a,k], Ana Maria Moura da Silva[j] and Wuelton Marcelo Monteiro[a,b]

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ABSTRACT

Introduction: Snake venom composition shows significant inter- and intra-species variation. In the case of the viperid species *Bothrops atrox*, responsible for the majority of snakebites in the Amazon region, geographical and ontogenetic variables affect venom composition, with ecological and medical implications. Previous studies had shown that venom from neonate and juvenile *Bothrops* specimens have a higher *in vitro* coagulant activity. The aim of this investigation was to assess the association of clinical outcomes, such as venom-induced coagulopathy and local complications, with *B. atrox* ontogenetic variables.

Methods: This study explored the relationship between some clinical parameters in patients suffering envenomations by *B. atrox* in the Amazon and several morphometric parameters of the snake specimens causing the bites.

Results: There were 248 specimens confirmed as agents of envenomation, mostly female snakes (70.5%) and classified as juveniles (62.7%). Patients bitten by neonates compared to adult snakes [OR = 2.70 (95%CI 1.15-6.37); $p = .021$] and by snakes with white tail tip [OR = 1.98 (95%CI 1.15–3.41); $p = .013$] were more likely to develop coagulopathy. Time from patient admission to the unclottable blood reversion was not affected by the snake gender ($p = .214$) or age ($p = .254$). Patients bitten by neonate ($p = .024$) or juvenile snakes ($p < .0001$) presented a lower frequency of moderate to severe edema, as compared to those bitten by adult snakes. In agreement with experimental observations, patients bitten by neonates and by snakes with a white tail tip were more likely to develop coagulopathy than those bitten by adult snakes. In contrast, envenomations by adult snakes were associated with a higher incidence of severe local edema.

Conclusion: Despite these variations, no difference was observed in the time needed to recover blood clotting in these patients after *Bothrops* antivenom administration.

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Introduction

Bothrops atrox, the Amazonian lancehead, is the main species responsible for snakebites in the Amazon, causing 80–90% of the envenomations in the region [1]. This species inhabits mostly forests, although it may be occasionally found in agricultural and urban environments [2,3]. *B. atrox* venom includes metalloproteinase as the major toxin family followed by phospholipases A_2, serine proteinases, cysteine-rich secretory proteins, L-amino acid oxidases and C-type lectin-like toxins [4–6]. Hypofibrinogenemia is a major systemic complication from *B. atrox* bites, affecting more than 80% of the patients [7]. This condition results from the action of serine proteinases having thrombin-like activity, which converts fibrinogen to fibrin, and also due to the procoagulant activity of metalloproteinases, which activate factors II and X of the coagulation cascade, resulting in the formation of endogenous thrombin [8]. In addition, PI and P-III metalloproteinases, such as batroxase [9], Atroxlysin-Ia [10] and Batroxrhagin [11], cause microvascular damage by proteolytic degradation of basement membrane [12]. These processes combined are

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Supplemental data for this article can be accessed here.

responsible for systemic bleeding observed in *B. atrox* snakebites [7,13].

The variability in *Bothrops* venom is related to the age [14], sex [15], geographical variation [5,16] and diet [17] of the snakes. *B. atrox* venom variability is mostly related to the expression level of each group of toxins rather than to the presence or absence of major families of venom proteins [5]. Remarkable differences in biological activities of juvenile and adult pit vipers have been observed, with venoms from juveniles having higher coagulant and lower proteolytic activities as compared to those of adults [18]. Some reports described that coagulopathy is more frequent in envenomation caused by juveniles, whereas local effects, including edema and necrosis, are more common in envenomations by adult snakes [19–21]. In general, this type of study is of dubious interpretation, since the number of envenomations with snake characterization is small, and snake morphometrics to confirm the ontogenetic stage are poorly assessed. The aim of this study is to assess the association of clinical outcomes, such as venom-induced coagulopathy and edema with ontogenetic stages of the *B. atrox* specimens perpetrating the bites.

Methods

Study population

We analyzed museum-preserved snake specimens causing human envenomations in the *Fundação de Medicina Tropical Doutor Heitor Vieira Dourado* (FMT-HVD), from 2009 to 2017. By consecutive sampling, every patient who brought the snake to the hospital, with full corresponding medical information obtained in previous prospective studies, was included. This study was approved by the Ethical Committee of the FMT-HVD (approval number 53192516.8.0000.0005).

Patients' information

Clinico-epidemiological information was collected through a standardized questionnaire (*Epi Info*™, CDC). Clinical severity of envenomation was classified according to the Brazilian Ministry of Health guidelines [22]: (i) mild envenomation, with pain and edema in one segment of the bitten limb; (ii) moderate envenomation, with evident edema involving two segments of the limb and local or systemic bleeding without hemodynamic repercussion; (iii) severe envenomation, with severe pain, severe edema in the limb, severe hemorrhagic conditions with hemodynamic repercussion, compartment syndrome and renal failure. Clotting time was evaluated by the modified Lee-White clotting time test (LWCT) [22]. With a plastic syringe, 1 mL of venous blood was collected and placed into a glass tube (13×75 mm) without anticoagulants, at 25 °C. Using a stopwatch, timing started as soon as the blood was drawn into the tube. The tube was left undisturbed for 5 min and then checked for clots every minute by gently tilting the tube. Unclottable blood was defined when the blood was not clotted until 10 min [7]. Thrombocytopenia was defined as platelet counts <150,000 platelets/μL. Mild thrombocytopenia was defined by a platelet count of 100,000 to 150,000/μL, moderate thrombocytopenia as a platelet count of 50,000 to 100,000/μL and severe thrombocytopenia as a platelet count below 50,000/μL. For edema assessment, the bitten limb was divided into three segments. The upper limb was divided into (i) hand and wrist; (ii) forearm and elbow, and (iii) arm. Lower limb was divided into (i) foot and ankle; (ii) leg and knee and (iii) thigh. The presence of edema only in the segment of the bite was considered as mild, in two segments as moderate and in three segments as severe [23]. Secondary bacterial infection was defined according the guidelines of the Infectious Diseases Society of America [24]. Cellulitis was defined by the presence of local inflammation signs (erythema, edema, bruising and pain) associated with fever, leukocytosis, lymphangitis and/or lymphadenitis. An abscess was characterized by individual injuries, floating, presenting purulent or serous-purulent secretion. For edema assessment and secondary infection diagnosis, two independent examiners blinded to snakes' stage evaluated the patients and came to a final agreement. Acute renal failure was defined according to the Acute Kidney Injury Network (AKIN), as the increase of at least >0.3 mg/dL or up to 199% of baseline creatinine levels [25].

Morphometric characterization of snakes

Sex was verified by examination of the reproductive organs through an incision in the first subcaudal scales [26]. The following morphometric variables were recorded: snout-vent length (SVL), tail length (TaL), total length (ToL), head length (HL), head width (HW), nasal-ocular distance (OND), ocular-loreal distance (OLD), loreal-nasal distance (LND), inter-fang distance (IFD), ventral-symphysal distance (VSD), rostral-labial distance (RLD), loreal width (LW), nasal width (NW), ocular width (OW) and cloacal width (CW) (Figure 1). SVL, TaL and ToL were obtained using a measuring tape, whereas the remaining variables with analogue digital caliper. The individuals were grouped by age as follows: neonate males and females (SVL < 300 mm), juvenile males (SVL between 300-460 mm) and juvenile females (SVL between 300-800 mm), adult males (SVL > 470 mm) and adult females (SVL > 850mm) [27]. Snake weight (g) (W) was obtained by a digital precision scale. An incision was made on the ventral side of the snake to check the presence of the gastric and intestinal contents. Presence of white tail tip (WTT), an indicative of juvenile stage, was recorded. The measurement of the snakes was made by a single trained herpetologist. Three measurements were made for each parameter and the mean obtained was used in this study. Some snakes were brought to the hospital with damaged structures, impairing the measurement of all morphometric variables. However, the possible measures to obtain from these specimens were used in the analysis.

Statistical analyses

The primary endpoint was a prolonged clotting time. Snake variables were compared between patients presenting

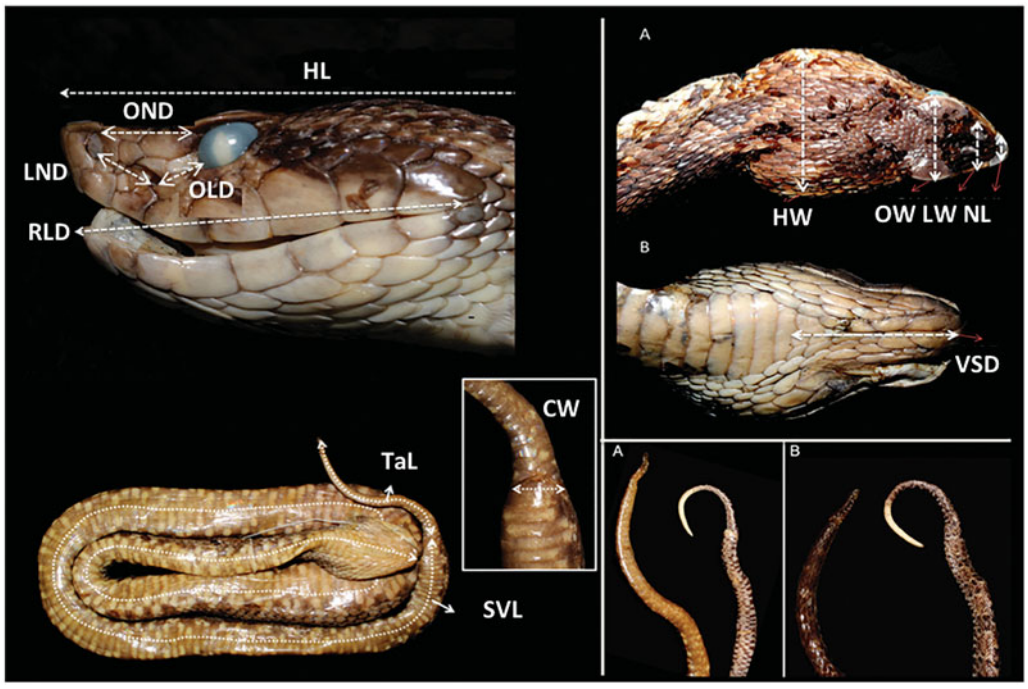

Figure 1. Description of *Bothrops atrox* morphometrics: Left part of picture; head length (HL), nasal-ocular distance (OND), ocular-loreal distance (OLD), loreal-nasal distance (LND), rostral-labial distance (RLD), tail length (TaL), snout-vent length (SVL) and cloacal width (CW). Right upper corner of the picture, (A) head width (HW), ocular width (OW), loreal width (LW) and nasal width (NW). (B) ventral view of head: ventral-symphysal distance (VSD). (A and B) ventral and dorsal view of non-white tail, and white tail (TW), respectively. Total length (ToL), is the sum of TaL and SVL.

unclottable or clottable blood and edema severity using Chi-square test. Odds ratios with the corresponding confidence interval were presented. Snake morphometrics were compared between patients presenting unclottable or clottable blood using Student's *t* test. A classification model for clotting status was performed based on the creation of decision trees using snake morphometrics and weight as independent variables. Receiver operating characteristic (ROC) analysis was conducted to verify potential morphometric markers. Time until unclottable blood reversion was analyzed using Kaplan-Meier estimates, using snake sex and age as independent variables. All the analyses considered a 5% significance level and were conducted using the STATA package version 13 (Stata Corp. 2013).

Results

Patients' parameters

Table 1 presents the characteristics of the 247 patients included in the study.

The most frequent local manifestations were pain (87.9%) and edema (83.9%). Systemic bleeding was observed in 3.6% of the patients. Snakebites were mostly classified as moderate (50.2%). Secondary infection (21%) was the most frequent local complication observed. Acute kidney injury was observed in 12.8% of the patients. Unclottable blood was observed in 61.1% of the patients (Figure 2). Mild thrombocytopenia was reported in 4.7% of the patients. Case fatality rate was 0.4% during hospitalization.

Table 1. Epidemiological characteristics of the 247 patients.

Variables	Cases	%
Sex		
Male	195	79
Female	52	21
Age (years)		
0–10	20	8.1
11–20	42	17.0
21–30	57	23.1
31–40	50	20.2
41–50	31	12.6
50–60	34	13.8
>60	13	5.3
Area of occurrence		
Rural	188	78.7
Urban	33	13.8
Others	27	10.9
Anatomical region of the bite		
Foot	145	61.2
Leg	41	17.3
Toe	26	11
Hand	14	5.9
Others	22	8.9
Time from bite to medical assistance (hrs)		
0–3	154	72
4–6	32	15
7–12	18	8.4
>12	10	4.7
Use of topical medications		
Yes	30	12.4
Use of oral medications		
Yes	46	18.6
Use of tourniquet		
Yes	24	9.7
Incision in the bite site		
Yes	10	4.1

Snake descriptive analysis

Out of 247 *B. atrox* individuals confirmed as agents of envenomation, sex was identified in 193, being 57 males

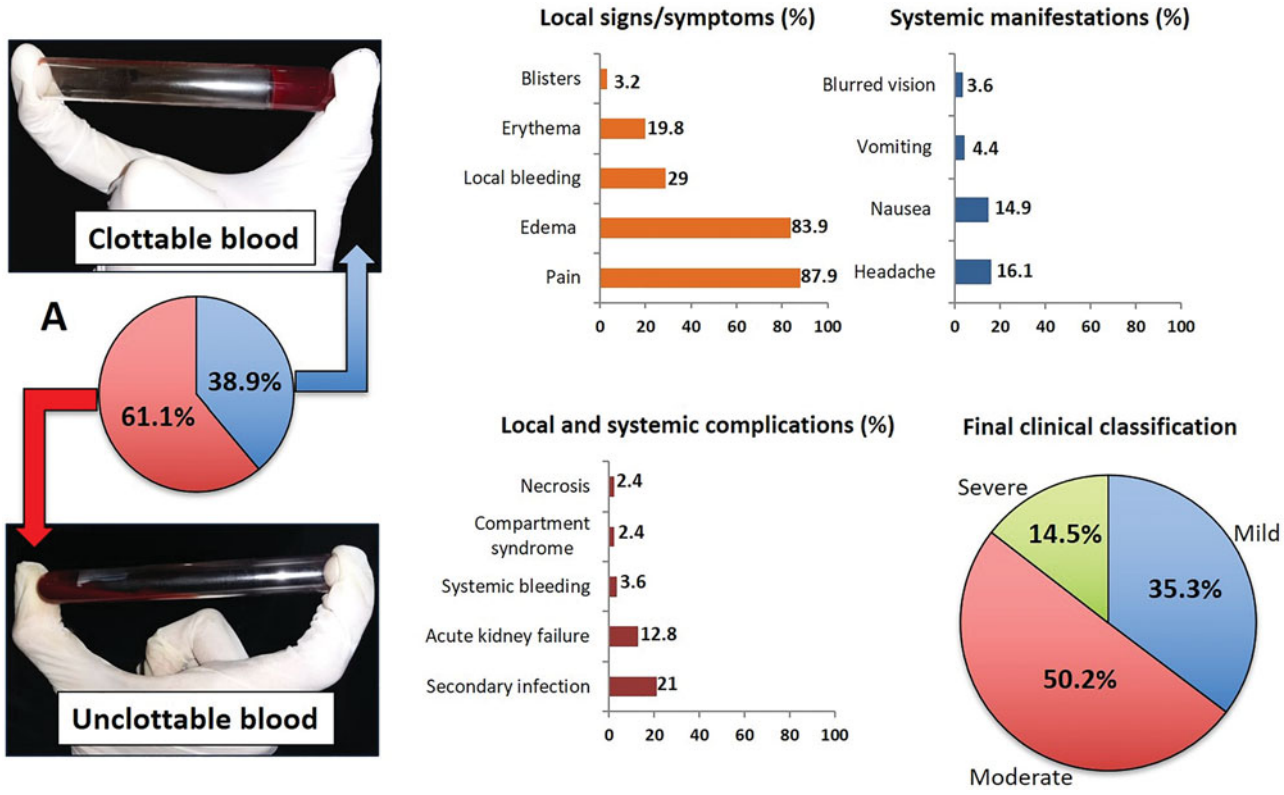

Figure 2. Clinical characteristics of patients. Blood was considered unclottable when sample lasted more than 9 minutes to clot in the modified Lee–White clotting time (LWCT) method [7]. Clinical classification was made according to the guidelines of the Brazilian Ministry of Health [6]. Acute kidney injury was defined according to the Acute Kidney Injury Network (AKIN) Guideline [25].

(29.5%) and 136 females (70.5%). Detailed morphometric information is presented in Supplementary file 1. Most of the snakes were classified as juveniles (62.7%). Considering males, 68.5% of the snakes were adults, while females were mostly juveniles (57.9%). White tail tip was present in 56.3% of the snakes. A proportion of 24.2% of the snakes had gastric or intestinal contents (Figure 3).

Variables associated with coagulopathy

Patients bitten by neonates compared to adult snakes [OR = 2.70 (95%CI 1.15-6.37); $p = .021$] and by snakes with white tail tip [OR = 1.98 (95%CI 1.15-3.41); $p = .013$] presented a higher probability of developing coagulopathy (Table 2). No snake variable was found to be associated with thrombocytopenia.

Predicting coagulopathy from snake morphometrics

For female snakes, mean weight and most morphometric variables distinguished between the two types of patients except NOD, OLD, LDN and NW (Figure 4). For patients bitten by male snake specimens, the only variable significantly different between the two groups of patients was LND, presenting good diagnostic ability to classify blood clotting status (AUC = 0.802 (95%CI 0.676-0.928)) (Figure 5).

Decision trees generated from total and female snakes presented a greater number of branches in relation to those from male snakes. Supplementary file 2 presents rules with $\geq$90% of discriminatory power and at least five patients in the branch. Snake weight was the most discriminatory attribute in patients bitten by female snakes. Snake LND was the most discriminatory attribute in patients bitten by male snakes.

Time until restoration of blood coagulability

Time from patient admission to the unclottable blood reversion was not affected by the snake sex ($p = .214$) nor age ($p = .254$) (Figure 6).

Snake age and local complications

Patients bitten by neonate ($p = .024$) or juvenile snakes ($p < .0001$) presented a lower frequency of moderate/severe edema, as compared to those bitten by adult snakes (Figure 7). Although in low frequency for robust analysis, other local complications such as blistering, compartment syndrome and necrosis were not seen among patients bitten by neonate snakes.

In this study, one patient died. A 91 year-old male was bitten in the right hand and the right foot. Immediately after the bite the patient reported an intense acute pain in the bitten sites. Eleven hours after the snakebite, patient was hospitalized presenting intense pain in the right foot and edema extending to the whole limb. Eight vials (80 mL) of *Bothrops* antivenom were administered. He presented with acute renal injury. Patient's health status deteriorated and

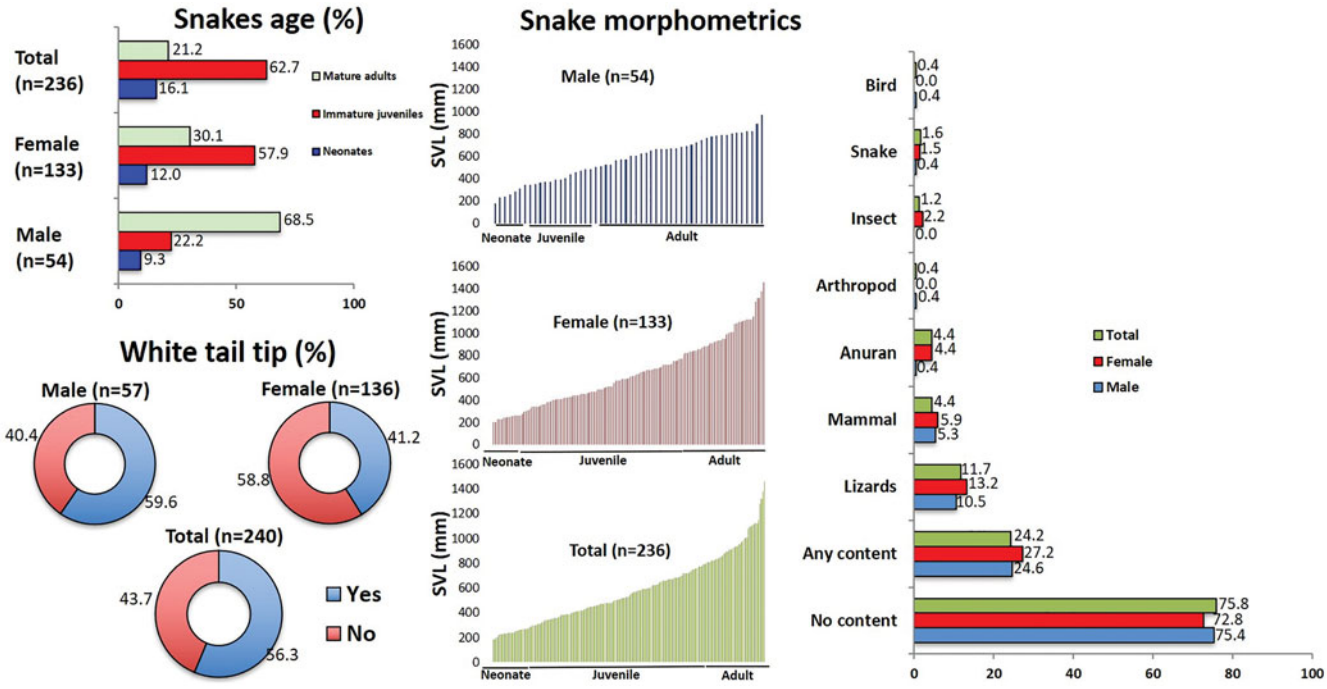

Figure 3. Morphometrics, age group, presence of white tail tip and intestinal contents of *Bothrops atrox* snakes which caused the envenomations. Neonate males and females (SVL < 300 mm); juvenile males (SVL 300-460 mm) and juvenile females (SVL 300-800 mm); adult males (SVL > 470 mm) and adult females (SVL > 850mm) [27].

Table 2. Variables associated with coagulopathy.

Variable	Unclottable blood	Clottable blood	OR (CI95%)	*p*
Patients age (years)				
0–10	16 (11.9%)	4 (4.2%)	Ref.	
11–20	20 (14.8%)	15 (15.8%)	3.00 (0.83-10.83)	0.096
21–40	55 (40.7%)	45 (47.4%)	3.27 (1.02-10.48)	**0.038**
41–60	38 (28.1%)	24 (25.3%)	2.52 (0.75-8.46)	0.132
>60	6 (4.4%)	7 (7.4%)	4.66 (0.99-21.89)	0.060
Patients gender				
Male	105 (77.8%)	76 (80.0%)	Ref.	
Female	30 (22.2%)	19 (20.0%)	0.87 (0.46-1.66)	0.693
Area of occurence				
Rural	108 (86.4%)	75 (85.2%)	Ref.	
Urban	17 (12.6%)	13 (14.8%)	1.10 (0.50-2.40)	0.806
Time to medical assistance (hours)				
0–3	94 (73.3%)	68 (72.3%)	Ref.	
4–6	22 (16.9%)	15 (16.0%)	0.94 (0.45-1.94)	0.880
7–12	12 (9.2%)	6 (6.4%)	0.69 (0.24-1.93)	0.498
>12	2 (1.5%)	5 (5.3%)	3.45 (0.65-18.34)	0.151
Thrombocytopenia				
Yes	9 (6.8%)	8 (9.2%)	Ref.	
No	123 (93.2%)	79 (90.8%)	0.72 (0.26-1.95)	0.529
Hemoglobin				
Above normal values	19 (14.5%)	11 (12.8%)	Ref.	
Normal values	89 (67.9%)	66 (76.7%)	1.28 (0.57-2.87)	0.548
Below normal values	23 (17.6%)	9 (10.5%)	0.68 (0.23-1.97)	0.472
Snake gender				
Male	33 (30.6%)	23 (29.1%)	Ref.	
Female	75 (69.4%)	56 (70.9%)	1.07 (1.57-2.02)	0.832
Snake age				
Neonates	25 (19.4%)	10 (11.2%)	Ref.	
Juveniles	67 (51.9%)	40 (44.4%)	1.49 (0.65-3.43)	0.343
Adults	37 (28.7%)	40 (44.4%)	2.70 (1.15-6.37)	**0.021**
White tail tip				
Yes	76 (57.6%)	37 (40.7%)	Ref.	
No	56 (42.4%)	54 (59.3%)	1.98 (1.15-3.41)	**0.013**
Feeding status				
Fed	29 (21.7%)	29 (31.2%)	Ref.	
Unfed	103 (78.0%)	64 (68.8%)	0.62 (0.34-1.13)	0.125

Bold values represent statistical differences.

48 hours from admission he was diagnosed with compartment syndrome and was submitted to an extensive fasciotomy in the right leg. Secondary bacterial infection was diagnosed and clindamycin was started. After two days, the patient died of septic shock. Abnormalities in clotting time or platelet counts and bleeding were not observed during hospitalization. The snake brought by the patient was an adult female.

Discussion

Several studies have analyzed the epidemiological and clinical aspects of snakebite patients, but did not include the characteristics of snakes responsible for the bites. With the current knowledge about the variability in the composition of snake venoms, the characteristics of the offending snake are relevant for clinical studies.

One hypothesis was that male snakes cause more bites than females, since males move around more often than females, especially during the reproductive season [28,29]. Otherwise, in this work it was shown that most of the bites were caused by female snakes (2.4 females for each male). However, since only snakes captured and brought by the patients to the hospital were included in this study, a selection bias may have occurred since males, smaller and faster than females, might escape from the patient more often than females, particularly the pregnant ones [29]. Another possible explanation for this finding is that there are more females than males in nature, which is suggested in some studies [30,31]. Moreover, females of *B. atrox* tend to be in general larger than males [27], thus having a higher capacity to reach a person during a bite. On the other hand,

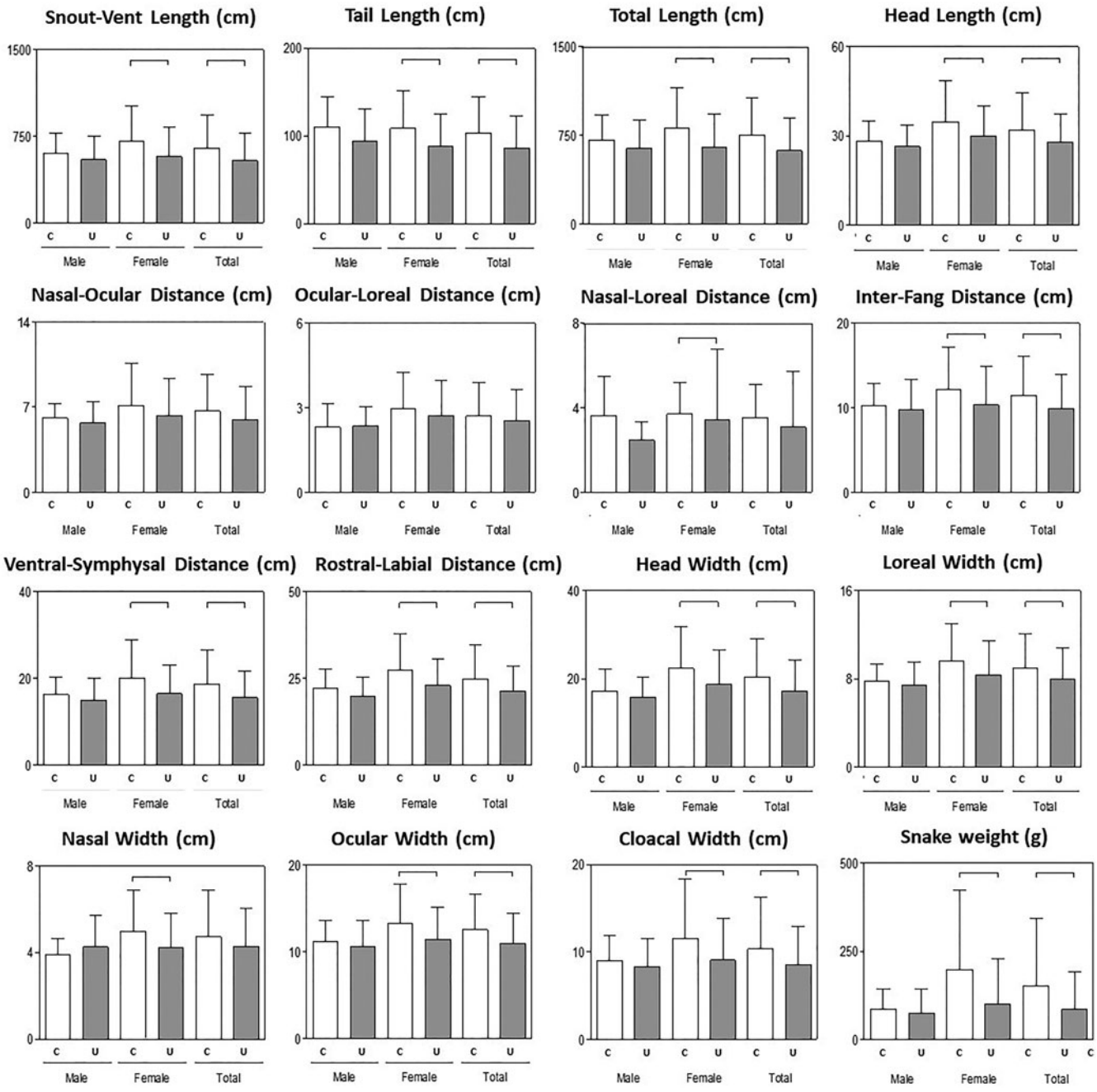

Figure 4. Comparison of morphometrical variables from *Bothrops atrox* snakes according to blood clottability. Calculated by T-test comparing two independent samples, considering $p < .05$ for significance. C: clottable blood, U: unclottable blood.

considering female snakes in reproductive age, as previously observed for *B. jararaca* [29], they present more thermoregulatory activity, needed for the development of the embryos, in open sunny places more often frequented by humans.

Systemic bleeding is a hallmark of the *Bothrops* envenomation, with unclottable blood on admission as its major risk factor [32]. Interestingly, coagulotoxic components are the most variable in the venom of *B. atrox*. A drastic ontogenetic change was observed in the proteome of *B. atrox* in the region of Orinoquia, Venezuela where juveniles present PIII-class metalloproteinases as major toxins in their venoms while PI-class metalloproteinases and phospholipase A2 predominate in the venom of adult specimens [5]. Functional variability on the coagulant activity has been demonstrated in different ontogenetic stage of the snake [33]. For instance, adult snakes from Central Amazonia present a venom with lower lethality but higher dermonecrotic activity while venoms from snakes from Maranhão are more coagulotoxic, with higher lethal activity [34]. It has been accepted that procoagulant activity is a fundamental feature of pit-viper venoms for the purpose of prey capture by small snakes [35].

In prey animals, the rapid formation of endogenous thrombin by *Bothrops* venom could result in prey incapacitation through stroke induction [35]. Dissimilar patterns were observed in the main clot parameters in animal plasmas as models of amphibian, mammalian and avian potential preys [35]. Specific venom effects are consistent with the

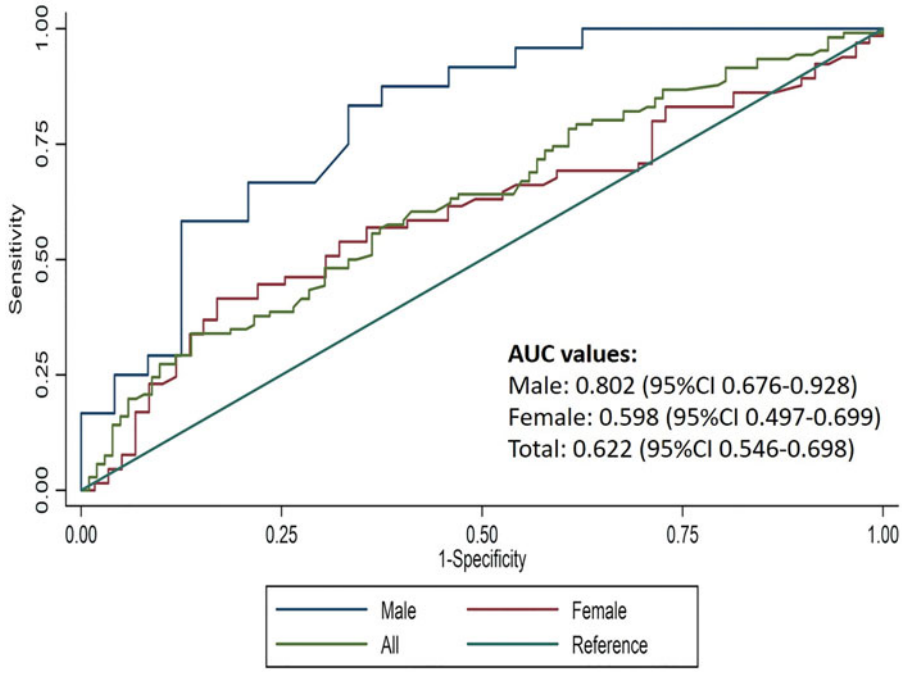

Figure 5. ROC curves of the discriminatory power of the morphometrics and weight for clotting blood status classification. The only morphometric variable presenting good diagnostic ability to classify clotting blood status was the LND for snakebites caused by male specimens (AUC = 0.802 (95%CI 0.676–0.928)). The other variables presented poor diagnostic performance.

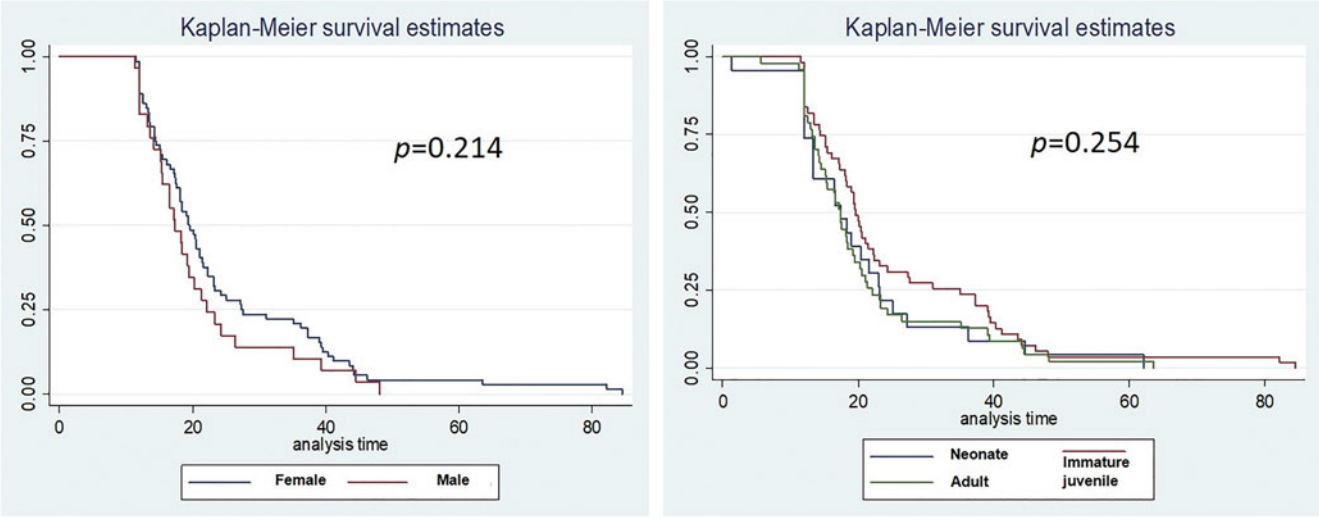

Figure 6. Time until reversion of blood clottability after hospital admission and antivenom administration, according to snake gender and age. Survival analysis demonstrated that the time from patient admission to the unclottable blood reversion was not affected by the morphological features of the snake.

evolutionary history of *B. atrox* populations regarding selection pressure of prey availability in various ecological niches and ontogenetic stages [6]. Young *Bothrops* snakes preferentially eat amphibians, lizards and birds, shifting to mammals when they become adults [17]. Therefore, change in *B. atrox* venom proteome is most likely related to the survival of the snake by adaptation to particular preys. Experimentally, venoms from juveniles displayed higher human plasma clotting activity compared to venoms from adult specimens [33]. Venoms of juvenile specimens of *B. atrox* from the Colombian Amazon possess higher hemorrhagic and coagulant activities than venoms from adults [36].

In contrast to coagulopathy, local tissue damage was more prevalent in patients bitten by adult snakes. Local signs of *Bothrops* envenomations range from a painless injury to intense pain and swelling at the bite site, evolving in some cases to blistering, tissue necrosis and compartment syndrome [13,37]. Tissue damage is caused by metalloproteinases, which cause the hydrolysis of extracellular matrix components and disruption of capillary vessels. Moreover, metalloproteinases and phospholipase A2 induce up-regulation of pro-inflammatory mediators expression enhancing tissue damage [38,39]. Phospholipases A2, namely BaPLA2I and BaPLA2II, that cause edema and myonecrosis, and a dermonecrotic metalloproteinase, namely Atroxlysin-Ia, have been isolated from *B. atrox* venom [40].

In this study, patients bitten by adult *B. atrox* presented a higher frequency of moderate to severe edema, confirming

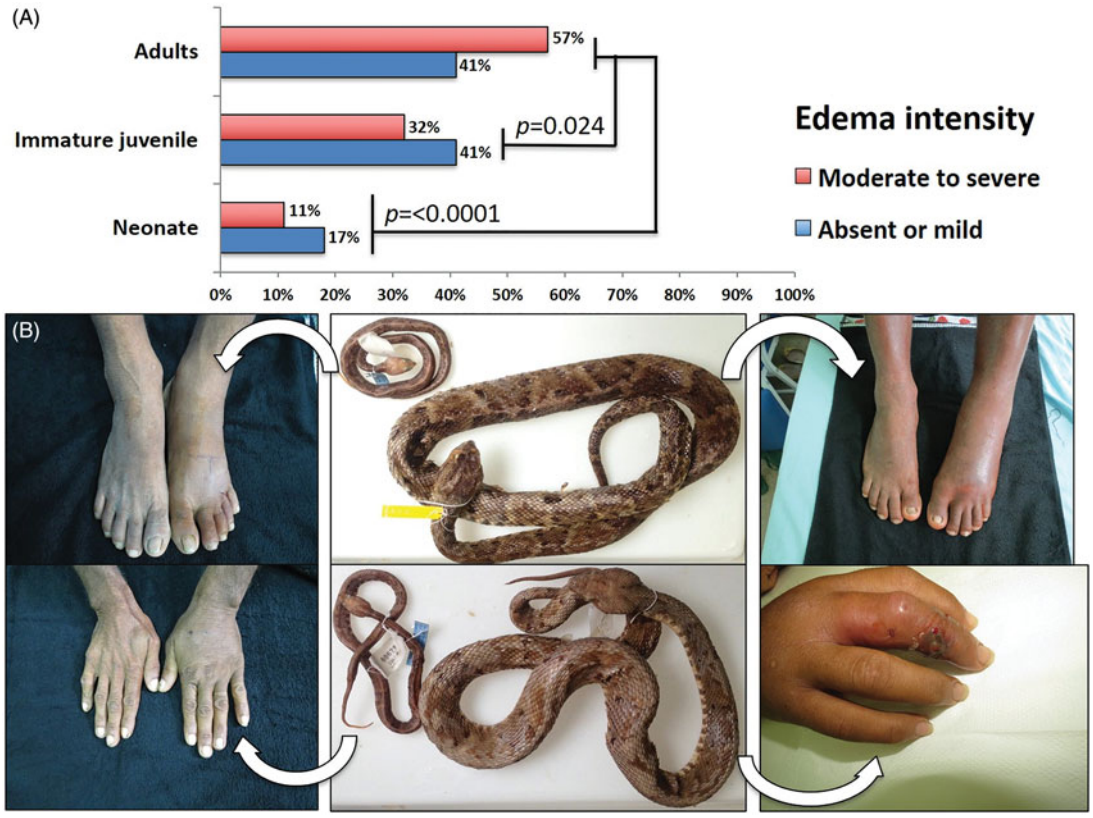

Figure 7. Edema frequency and intensity according to the snake's morphometric features. (A) Patients bitten by neonate ($p = .024$) or juvenile snakes ($p < .0001$) compared to adult snakes presented a lower frequency of moderate to severe edema. (B) Some examples of adult and juvenile snakes causing severe and mild edema, respectively.

previous observations from *B. jararaca* envenomations [19–21]. Moreover, the only death observed in this study was from a patient that presented severe local tissue damage and no manifestation of coagulopathy, which was bitten by an adult snake. This finding is interpreted as a result of the higher expression of metalloproteinases and phospholipases A2 in venoms from adult pit vipers [36,41].

Time from antivenom administration to the clotting activity restoration was not affected by the snake stage and the antivenom was efficient for the treatment of *B. atrox* envenomations although venom from juveniles is not used in the immunization pool for antivenom production. In fact, previous *in vitro* studies showed a high efficacy of commercial *Bothrops* antivenom to neutralize *B. atrox* venoms of different populations, including from Manaus region [35], and the antivenom efficacy was also observed in patients bitten by *B. atrox* in Pará State [13]. Once the antivenom is administered, it effectively neutralizes the toxins of adult or juvenile snakes, even considering the quantitative differences in the procoagulant toxins from the venom, leading to a similar restoration of coagulation parameters [6,34].

Conclusions

Venom-induced coagulopathy is more frequent in envenomations caused by juvenile *B. atrox*, whereas envenomations inflicted by adult snakes cause more severe local tissue damage. These findings are attributed to venom ontogenetic variability in *B. atrox*, since venoms of younger specimens have higher procoagulant activity, whereas those of adults exert stronger local tissue damage. Despite the observed variation in the incidence of coagulopathy, clotting activity restoration after antivenom administration was similar in the two groups of patients.

Disclosure statement

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