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Clinical implications of ontogenetic differences in the coagulotoxic activity of *Bothrops jararacussu* venoms

Caroline Fabri Bittencourt Rodrigues[a,b], Christina N. Zdenek[b], Lachlan A. Bourke[b], Lorenzo Seneci[b,c], Abhinandan Chowdhury[b], Luciana Aparecida Freitas-de-Sousa[d], Frederico de Alcantara Menezes[e], Ana Maria Moura-da-Silva[d,f], Anita Mitico Tanaka-Azevedo[a], Bryan G. Fry[b,*]

[a] *Laboratório de Herpetologia, Instituto Butantan, São Paulo, 05508-040, SP, Brazil*
[b] *Venom Evolution Lab, University of Queensland, St. Lucia, 4072, QLD, Australia*
[c] *Institute of Biology Lieden (IBL), Lieden University, 2333 BE Lieden, the Netherlands*
[d] *Laboratório de Imunopatologia, Instituto Butantan, São Paulo, 05508-040, SP, Brazil*
[e] *Laborato'rio de Coleçoes Zoolo'gicas, Instituto Butantan, Sao Paulo, 05508-040, SP, Brazil*
[f] *Instituto de Pesquisa Clínica Carlos Borborema, Fundação de Medicina Tropical Dr. Heitor Vieira Dourado, 69040-000 Manaus, AM, Brazil*

HIGHLIGHTS

- Procoagulant toxicity in *Bothrops jararacussu* is a function of size.
- Smaller snakes have more potent venom.
- Antivenom is effective against all size groups.

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ABSTRACT

Is snake venom activity influenced by size? This is a long-standing question that can have important consequences for the treatment of snake envenomation. Ontogenetic shifts in venom composition are a well-documented characteristic of numerous snake species. Although snake venoms can cause a range of pathophysiological disturbances, establishing the coagulotoxic profiles related to such shifts is a justified approach because coagulotoxicity can be deadly, and its neutralisation is a challenge for current antivenom therapy. Thus, we aimed to assess the coagulotoxicity patterns on plasma and fibrinogen produced by *B othrops jararacussu* venoms from individuals of different sizes and sex, and the neutralisation potential of SAB (anti bothropic serum produced by Butantan Institute). The use of a metalloproteinase inhibitor (Prinomastat) and a serine proteinase inhibitor (AEBSF) enabled us to determine the toxin class responsible for the observed coagulopathy: activity on plasma was found to be metalloprotease driven, while the activity on fibrinogen is serine protease driven. To further explore differences in venom activity, the activation of Factor X and prothrombin as a function of snake size was also evaluated. All the venoms exhibited a potent procoagulant effect upon plasma and were less potent in their pseudo-procoagulant clotting effect upon fibrinogen. On human plasma, the venoms from smaller snakes produced more rapid clotting than the larger ones. In contrast, the venom activity on fibrinogen had no relation with size or sex. The difference in procoagulant potency was correlated with the bigger snakes being proportionally better neutralized by antivenom due to the lower levels of procoagulant toxins, than the smaller. Thus, while the antivenom ultimately neutralized the venoms, proportionally more would be needed for an equal mass of venom from a small snake than a large one. Similarly, the neutralisation by SAB of the pseudo-procoagulant clotting effects was also correlated with relative potency, with the smaller and bigger snakes being neutralized proportional to potency, but with no correlation to size. Thromboelastography (TEG) tests on human and toad plasma revealed that small snakes' venoms acted quicker than large snakes' venom on both plasmas, with the action upon

* Corresponding author at: School of Biological Sciences, Gehrmann Building #60, The University of Queensland, St. Lucia, QLD 4067, Australia.
E-mail address: bgfry@uq.edu.au (B.G. Fry).

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amphibian plasma consistent with smaller snakes taking a larger proportion of anuran prey than adults. Altogether, the ontogenetic differences regarding coagulotoxic potency and corresponding impact upon relative antivenom neutralisation of snakes with different sizes were shown, underscoring the medical importance of investigating ontogenetic changes in order to provide data crucial for evidence-based design of clinical management strategies.

1. Introduction

Snakebite envenomation is a global crisis, being included by the World Health Organization (WHO) as a neglected tropical disease due to its high morbidity and mortality (Chippaux, 2017; Fry, 2018; Gutiérrez et al., 2010). One of the most critical effects of envenomation is upon the blood clotting system (coagulotoxicity) since disturbances in hemostasis are one of the most effective ways of immobilising and capturing prey (Kini et al., 2011).

The blood clotting system involves the sequential activation of coagulation factors, which culminates in a blood clot (Kini et al., 2011). Snake venom can disrupt this process via procoagulant or anticoagulant mechanisms (Kini, 2006; Kini et al., 2001). Procoagulant toxicity is caused by the direct activation of the coagulation factors, such as prothrombin and Factor X (Kini, 2005; Kini et al., 2001). Anticoagulant toxicity is due to either inhibition of the coagulation factors (Kini, 2006; Youngman et al., 2019a), or depletion of fibrinogen levels through destructive non-clotting cleavage or cleaving fibrinogen in a pseudo-procoagulant manner such that weak, transient fibrin clots are formed, which rapidly degrade thereby contributing to the net anticoagulant state (Bittenbinder et al., 2019, 2018; Bourke et al., 2020; Fry, 2015; Kini, 2006; Youngman et al., 2019b).

The disturbances on the hemostatic system occur due to particular protein families within the venom, such as snake venom metalloproteinases (SVMPs), snake venom serine proteinases (SVSPs), phospholipases A_2 (PLA_2), and C-type lectins (CTL) (Fry, 2015; Kini and Koh, 2016; Morita, 2005; Prezoto et al., 2018; Serrano, 2013). Although venom composition is restricted to a limited number of protein families, the variation on the protein isoforms, relative abundance in the venom, and pharmacological actions are wide (Gren et al., 2019).

Venom variation is a well-established phenomenon and can occur, among other factors, due to geographical variation (Alape-Girón et al., 2008; Massey et al., 2012), sex (Amorim et al., 2018; Menezes et al., 2006; Zelanis et al., 2016), individual variation (Amazonas et al., 2018; Galizio et al., 2018) and ontogenetic shifts (Antunes et al., 2010; Gutiérrez et al., 2009; Pla et al., 2017; Zdenek et al., 2019). More specifically, on generalist species of the *Bothrops* genus, e.g. *B. jararaca* and *B. moojeni*, an age-dependent variation in coagulotoxicity has been noted, in which the neonates have more coagulant activity than the adults (Antunes et al., 2010; Furtado et al., 1991). Also, concerning clinical manifestations, coagulopathy is more often reported in envenomation by juveniles than in adults (Bernal et al., 2020; Guércio et al., 2006; Milani et al., 1997).

Beyond that, intraspecific variation in snake venom poses a challenge for snakebite treatment since snakes from different regions, sex, or ages may be differentially neutralized by antivenom (Amorim et al., 2018; Antunes et al., 2010; Sousa et al., 2018, 2017). Thus, the knowledge of venom variation is essential to predict potential clinical effects and the relative amount of antivenom needed (Gutiérrez et al., 2009; Harrison et al., 2011).

Belonging to the *Bothrops* genus, which is responsible for the majority of snakebite accidents in Brazil (Chippaux, 2015), *B. jararacussu* is included as a Category 1 by WHO as a species that has medical relevance, due to the number of accidents and morbidity of the snake envenomation (WHO, 1999). *B othrops jararacussu* is characterized by its sexual dimorphism, in which females are larger than males, thus being able to produce a high volume of venom (Melgarejo, 2009; Milani et al., 1997; Silva et al., 2020). This species is distributed in tropical forests in Brazil, southern Bolivia, Paraguay, and Northeastern Argentina (Melgarejo, 2009; Milani et al., 1997). *B othrops jararacussu* is a generalist species, with a predominance of mammals and anurans in its diet, having an ontogenetic shift from anurans to mammals already reported (Fenwick et al., 2009; Martins et al., 2002). The characteristic envenomation by *B. jararacussu* has as a clinical manifestation a high local effect, with a high degree of myotoxicity, in addition to the typical systemic effects of *Bothrops* envenomation, such as coagulopathy (Milani et al., 1997).

Despite the coagulotoxic aspects of the ontogenetic shift on *B. jararacussu* snakes being previously investigated (Furtado et al., 1991), there is a lack of understanding the mechanisms underlaying the coagulotoxic shift and the consequences to the antivenom neutralisation. Therefore, we aimed to fill this knowledge gap by investigating the correlation of coagulotoxicity and size of *B. jararacussu* upon plasma and fibrinogen clotting, antivenom neutralisation potency, clotting factors activation, and taxon-specific effects upon toad and human plasmas.

2. Material and methods

2.1. Venom samples and stocks preparation

All venom work was performed under University of Queensland Approval #IBC134BSBS2015. All venoms were collected between 2017 and 2019 in the States of Rio de Janeiro, Santa Catarina, and São Paulo, Brazil, under ICMBio/SISBIO permits 56576, 57585, and 66597; and INEA permit 025/2018. The procedures involving live snakes were carried out under the approval of Butantan Institute Ethics Committee on Animal Use (Protocol Number: 4479020217). We analysed seventeen venom samples from Brazilian *Bothrops jararacussu* of different sizes, sex, and localities (Table 1). For the relation of biometrics to venom actions, we used the snout-vent length (SVL) since males have longer tails than females (in order to house the hemipenes) and thus SVL is a more accurate way of comparing body size.

The protein concentration of the venoms was measured with a Thermo Fisher Scientific™ NanoDrop 2000 UV–vis Spectrophotometer (Thermofisher, Sydney, NSW, Australia). Based on Nanodrop values, we added deionised water: glycerol (50 %) to make stocks of 1 mg/mL for further analysis. Glycerol was added to preserve enzymatic activity, and the stocks were stored in a −20 °C freezer until use.

2.2. Antivenom

We used the polyvalent *Bothrops* antivenom (SAB) (batch: 1305077, Expiry date - ED: 05/16) produced by Instituto Butantan, Brazil. Even though the antivenom used is expired, the antivenoms have been shown to be stable over time, with powdered antivenoms shown to be particularly resilient, but even liquid antivenoms have been shown to be active for at least sixty years

Table 1
Identification, sex, location, and biometric data of *B. jararacussu* samples used in this work. ID: code used to differentiate the samples (the code contains: Sex, SVL in cm, Location code). Sex: Male/ Female Location: City and State (SC – Santa Catarina; SP – São Paulo; RJ – Rio de Janeiro). All the samples were from Brazil. Snout-vent length (SVL - cm), Tail length (TL - cm) Total Length (TotalL - cm): Length of the individuals.

ID	Sex	Location	SVL (cm)	TL (cm)	TotalL (cm)
M25.7FLR	Male	Florianópolis - SC	25.7	4.5	30.2
M36.4ILB	Male	Ilhabela – SP	36.4	6.3	42.7
M41.6IGP	Male	Iguape - SP	41.6	8.0	49.6
M51.0AGM	Male	Águas Mornas - SC	51.0	9.3	60.3
M60.3ILB	Male	Ilhabela – SP	60.3	10.0	70.3
M69.8ILB	Male	Ilhabela – SP	69.8	13.5	83.3
M77.0SJB	Male	São João da Barra - RJ	77.0	N.D.	N.D.
F35.0ILB	Female	Ilhabela – SP	35.0	5.0	40
F44.5SSB	Female	São Sebastião – SP	44.5	5.6	50.1
F48.0ILB	Female	Ilhabela – SP	48.0	6.3	54.3
F53.9ILB	Female	Ilhabela – SP	53.9	9.5	62.5
F73.2SJB	Female	São João da Barra – RJ	73.2	10.0	83.2
F94.2JQB	Female	Juquitiba – SP	94.2	13.3	107.5
F108.6STB	Female	Sete Barras – SP	108.6	14.8	123.6
F109.9JQB	Female	Juquitiba – SP	109.9	14.8	124.7
F112.0IGP	Female	Iguape-SP	112.0	N.D.	N.D.
F123.0JQB	Female	Juquitiba – SP	123.0	15.0	138

SVL-Snout-Vent Length; TL- Tail Length; TotalL- Total Length; N.D.- not determined.

(Al-Abdulla et al., 2013; Lister et al., 2017; O'Leary et al., 2009). The serum was obtained from horses immunised with a pool containing venoms of five *Bothrops* species with different percentages: *B. jararaca*, (50 %), *B. neuwiedi* complex (12.5 %), *B. jararacussu* (12.5 %), *B. alternatus* (12.5 %), and *B. moojeni* (12.5 %). The antivenom is composed of soluble IgG F(ab')2 fragments, and, according to the manufacturer, 1 mL neutralises 5 mg of the *Bothrops jararaca* venom reference. For use in the neutralisation assays, SAB was centrifuged (12,000 RCF, 10 min at 4 °C), the supernatant removed, filtered (0.45 nm filter), aliquoted (1 mL tubes), and stored at 4 °C until use.

2.3. Plasma and Fibrinogen aliquots

All human plasma work was performed under University of Queensland Biosafety Approval #IBC134BSBS2015 and Human Ethics Approval #2016000256. Citrated (3.2 %) human plasma collected from healthy donors and obtained from the Australian Red Cross (Research agreement #18-03QLD-09) was used. The plasma (one pooled plasma bag: label #A540020103540, A+) was defrosted at 37 °C, aliquoted into 1 mL tubes, flash-frozen in liquid nitrogen, and then stored at −80 °C until needed, at which time an aliquot was defrosted at 37 °C for 5 min. The plasma was replaced every 1 h during the analysis to avoid degradation.

For fibrinogen analysis, human fibrinogen was obtained from Sigma Aldrich (St. Louis, Missouri, US, catalog #F3879), diluted with 150 mM NaCl, 50 mM Tris, pH 7.4 buffer and aliquoted as a 4 mg/mL solution into 1 mL quantities, flash-frozen in liquid nitrogen and kept at −80 °C until use. The aliquots were defrosted at 37 °C for 5 min prior to use.

2.4. Coagulation assays (Plasma or fibrinogen)

The coagulation assays were performed on a Stago STA-R Max haemostasis analyser (Stago, Asnières Sur Seine, France), which measures clotting times with an automated viscosity-based (mechanical) system. To investigate the effect of the venom on plasma and fibrinogen coagulation, we diluted 30 μL of the venom samples in 270 μL of Owren Koller (OK) buffer (Stago catalog #00360) (1:10 dilution). The clotting time (seconds) was measured in triplicate at eight different final venom concentrations (20 μg/mL, 10 μg/mL, 4 μg/mL, 1.66 μg/mL, 0.66 μg/mL, 0.25 μg/mL, 0.125 μg/ ml, and 0.05 μg/mL). For the 20 μg/mL point, we used 50 μl of venom, 50 μl of phospholipid solubilised in OK buffer (Stago catalog #00597), 50 μl of $CaCl_2$ 25 mM (Stago catalog #00367), and 25 μl OK buffer. The solution was incubated in a cuvette for 120 s before the addition of 75 μl of plasma or fibrinogen, depending on the analysis. The negative control replaced the venom for deionised water: glycerol 50 %. The positive control on plasma was evaluated with 50 μl of Kaolin (Satago catalog #00597) as an activator of the clotting cascade, that was added to the plasma, and, after 120 s, the reaction was initiated with 50 μl of CaCl 25 mM (Stago catalog #00367). For the venom samples, the concentration was automatically diluted with OK buffer by the machine for subsequent dilutions. Clotting time was measured up until the max of 999 s.

2.5. Antivenom assays

The AV assays were run on a Stago STA-R Max haemostasis analyser (Stago, Asnières Sur Seine, France), as described for the coagulation assays. The AV was diluted with OK buffer into a 5% concentration, based on previous studies (Sousa et al., 2018). The resulting final dilution of AV in the assay was 1:200 (1.25 μl antivenom in 250 μl total cuvette volume). The coagulation tests were modified in the AV assays by replacing the 25 μl of OK buffer for 25 μl of the antivenom/OK buffer solution, thereby maintaining the final 250 μl test volume. Also, the negative control for the antivenom replaced the venom sample for deionised water: glycerol 50 %.

2.6. SVMP and SVSP inhibition assays

Prinomastat is a metalloproteinase (SVMP) inhibitor (Howes et al., 2007; Preciado and Pereañez, 2018), so, to test the involvement of those proteins in the coagulation on plasma and fibrinogen, we selected one of the venoms (F53.9ILB) to further test and modified the coagulation assay by substituting the 25 μl of OK buffer for 2 mM of Prinomastat (catalog #PZ0198, Sigma Aldrich, St. Louis, Missouri, US) diluted into deionised water (final concentration of the inhibitor into the 250 μl cuvette was 0.2 mM). The negative control replaced the venom for deionised water: glycerol 50 % Only one venom was tested due to the limited availability of venom. To test the involvement of SVSPs on the fibrinogen-clotting activity, we ran a similar assay using AEBSF (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, catalog #A8456, Sigma Aldrich, St. Louis, Missouri, US), an SVSP inhibitor (Debono et al., 2019a), but the concentration of the inhibitor used

was 20 mM (2 mM final concentration) and the incubation time was 20 min, according to previous studies (Debono et al., 2019a). The AEBSF assay was only performed on fibrinogen because, as an SVSP inhibitor, the AEBSF interferes with plasma coagulation by itself, inhibiting the existing serine protease factors, e.g., thrombin, and thus would not be used as a therapeutic to neutralise snake venom as AEBSF itself would cause coagulopathy.

2.7. Prothrombin activation

Once differences on the clotting-times was addressed, to assess the extent of prothrombin activation by the venom, we selected three representative samples regarding differences on clotting speed and neutralization, regardless of sex (Fastest/least neutralised; and slowest/best neutralised: M41.6IGP; M36.4ILB F48.0ILB; and F112.0IGP; F109.9JQB; F108.6STB, respectively). The venom stocks (1 mg/mL) were diluted to a 0.1 μg/mL concentration and incubated with fluoroskan buffer without calcium (150 mM NaCl, 50 mM Tris-HCl, pH 7.4). Subsequently, 10 μl of the samples were plated out in triplicate on a 384-well plate (black, Thermo Fisher, Rochester, NY, US), with or without 10 μl of the zymogen (1 μg/mL) per well. 10 μl of thrombin (1 μg/mL) was used as a positive control. On the plate, 10 μl of fluoroskan buffer and 10 μl of PPL (cephalin prepared from rabbit cerebral tissue adapted from STA C. K Prest standard kit, Stago Catalog # 00597) were also included on the well. Activation from venoms was measured by adding 70 μl substrate (ES011 (Boc-Val-Pro-Arg-AMC. Boc: t-Butyloxycarbonyl; 7-Amino-4- methyl coumarin, diluted into fluoroskan buffer, but with 10 mM $CaCl_2$, with the final ratio 1:500) per well. Fluorescence was monitored by a Fluoroskan Ascent™ Microplate Fluorometer (Cat#1506450, Thermo Scientific, Vantaa, Finland) at excitation 320 nm and emission 405 nm over 300 min, at 37 °C. The machine was programmed to shake the plate for three seconds before each round of reading to maintain homogeneity in the wells. Data were collected using Ascent® Software v2.6 (Thermo Scientific, Vantaa, Finland).

2.8. Factor X activation

To evaluate the potential of the Factor X activation by the selected venom samples, we used an assay similar to the prothrombin activation (Section 2.7). We substituted the zymogen from prothrombin to Factor X and the positive control from thrombin to Factor Xa. Due to different affinities to the substrate between the factors, we also changed the concentration of the zymogen (10 μg/mL) and venoms (1 μg/mL).

2.9. Thromboelastography (TEG) on human and toad plasma

Clot strength using human or toad (*Rhinella marina*) plasma was assessed using a TEG® 5000 Thrombelastograph® Hemostasis Analyzer System. The TEG method uses a pin suspended from a torsion wire, placed into a cup connected to a mechanical-electrical transducer, at 37 °C. The strength of the clot alters the resistance in the pin, generating the results. Volume ratios here matched coagulation assays. Seventy-two microliters of 25 mM $CaCl_2$ (Stago catalog #00367), 72 μl PPL (Stago Catalog # 00597), 20 μl of OK buffer (Stago Catalog # 00597), and 7 μl of 1 mg/mL venom glycerol stock were initially added together. Then, 189 μl of human or toad plasma was added, the whole solution pipette-mixed, and the test immediately started. Positive controls were performed with the addition of kaolin (Satago catalog #00597) instead of venom samples. The negative control replaced the venom for deionised water: glycerol 50 %. We did not run the negative control on toad plasma because it did not form spontaneously clot after the recalcification (Sousa et al., 2018). Tests were run for 30 min (n = 3).

2.10. Statistical analysis

All graphs and analyses were made in GraphPad Prism, 8.0. We tested the values for normality with Shapiro-Wilk or D'Agostino-Pearson omnibus (K2). Values of $p < 0.05$ were considered statistically significant. All raw data are included in Supplementary Dataset 1 and all statistical analysis results in Supplementary Dataset 2.

2.11. Venom potency and neutralisation

We calculated the area under the curve (AUCs) to evaluate the potency of the different venom samples. We calculated the x-fold shifts between the AUCs of venom vs AV, which indicates the neutralisation potential of the AV relative to the venom, by the formula: (AV AUC/Venom AUC)-1, where the value 0 indicates no neutralisation. Then, the AUCs and X-fold shifts were compared with simple linear regression, to ascertain the differences in coagulotoxicity and neutralisation ability across venoms and the size effects between them. As the *B. jararacussu* species have sexual dimorphism, the linear regressions were performed separated by sex. Values of r^2 greater than 0.7 were considered positive strongly related. To ratify if the slope was different from zero, values from $p < 0.05$ were considered significant. Due to the weak potency of some venoms on fibrinogen-clotting, the X-fold shifts were not calculated because they are unreliable, e.g. a venom that is well neutralised may still have a low x-fold shift value due to the measurement limitation (999 s).

2.12. Clotting factors activation activity

We calculated the AUCs to evaluate the factor's activation activity by the selected venom samples (fastest and least neutralised; slowest and well neutralised). To compare different factors, we normalised the AUC values by dividing the AUCs of the venoms samples by the AUCs of the positive control, multiplying them by 100, which results in the relative percentage. Thus, the Student's *t*-test was performed to assess the activation of Factor X and prothrombin by the venom samples grouped by clotting velocity, which also corresponds to small vs large individuals, regardless of sex).

2.13. TEG

To compare the differences between fast-clotting and slow-clotting snakes within and between the different plasmas, we first divided the parameters by the positive control (Kaolin), multiplying by 100, getting the relative percentage of each parameter (SP, R, MA, MRTGG, TGG). To compare the differences between fast vs. slow clotting, which conversely corresponds to small vs. large individuals, regardless of sex, within plasma and between the plasma, we grouped the representative individuals and analysed each parameter indenpendently (SP, R, MA, MRTGG, TGG). We used Multiple Student's *t*-tests, with Holm-Sidak method correction for multiple comparisons.

3. Results

3.1. Plasma coagulation assays and AV neutralisation

All venoms exhibit strong procoagulant effects in a dose-dependent manner (Fig. 1). To promote a better visualization of the

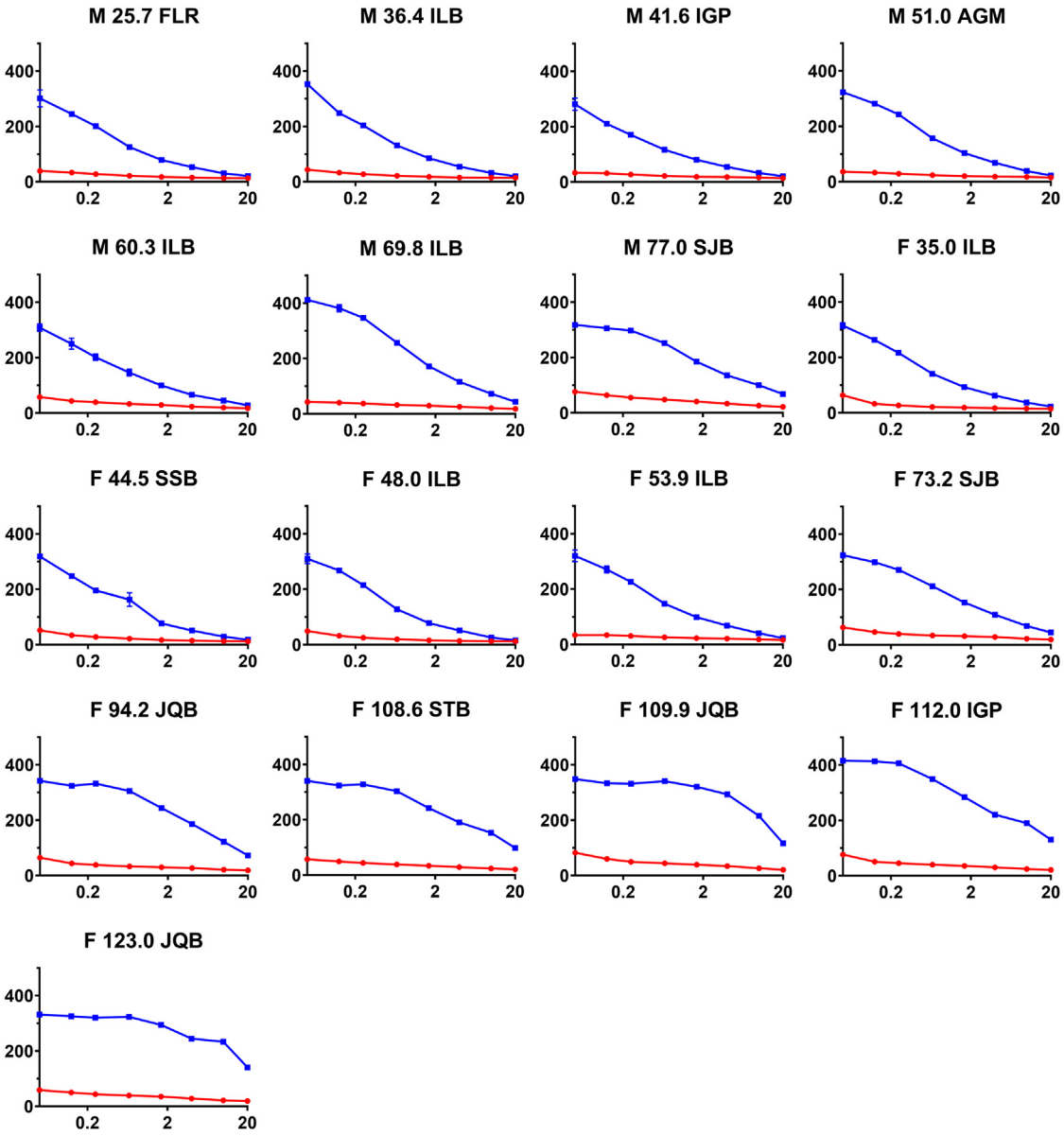

Fig. 1. Log view of human plasma clotting times of *B. jararacussu* venoms from multiple sexes and ages (red line) and *B. jararacussu* venoms with antivenom (blue line) over eight different venom concentrations (20 μg/mL, 10 μg/mL, 4 μg/mL, 1.6 μg/mL, 0.66 μg/mL, 0.25 μg/mL, 0.125 μg/mL, and 0.05 μg/mL). Y axis represents clotting time (seconds), and X axis represents the venom concentration (μg/mL). All venoms were tested in triplicate (N = 3). Positive control = 45.75 ± 0.45 s, Negative control venom = 349.6 ± 13.52 s, Negative control antivenom = 429.13 ± 69.4 s. Sample codes: M25.7FLR – Male, 25.7 cm, Florianópolis-SC; M36.4ILB – Male, 36.4 cm, Ilhabela-SP; M41.6IGP – Male, 41.6 cm, Iguape-SP; M51.0AGM – Male, 50.1 cm, Águas Mornas-SC; M60.3ILB – Male, 60.3 cm, Ilhabela -SP; M69.8ILB – Male, 69.8 cm, Ilhabela – SP, M77.0SJB – Male, 77.0 cm, São João da Barra-RJ; F35.0ILB – Female, 35.0 cm, Ilhabela-SP; F44.5SSB – Female, 44.5 cm, São Sebastião-SP; F48.0ILB – Female, 48.0 cm, Ilhabela-SP; F53.9ILB – Female, 53.9 cm, Ilhabela-SP; F73.2SJB – Female, 73.2 cm, São João da Barra-RJ; F94.2JQB – Female, 94.2 cm, Juquitiba-SP; F108.6STB – Female, 108.6 cm, Sete Barras-SP; F109.9JQB – Female, 109.9 cm, Juquitiba-SP; F112.0IGP – Female, 112.0 cm, Iguape-SP; F123.0JQB – Female, 123.0 cm, Juquitiba-SP.

venom dilutions on the graph area, we plotted the x-axis in a log-scale view.

We determined the potency of the venom samples by the AUCs, with faster clotting venoms having lower values. F48.0ILB had the stronger venom potency (274.3 ± 3.27) while F109.9JQB had the weak venom potency (578.43 ± 25.65) (Table 2). The potency of the venom clotting was positively related with size for both sexes (Linear Regression Male, Y = 5.105*X + 133.9, r^2 = 0.885; p = 0.002; Female Y = 2.977*X + 199.3, r^2 = 0.800; p = 0.001) (Fig. 4 A). Furthermore, the AV increases the clotting times, showing a neutralisation potential. Antivenom-driven neutralisation was evaluated by x-fold shifts in the curves (Table 2).

Corresponding to the relative potency, the same amount of antivenom neutralized less of the effect of small relative to large snake venoms. It is important to underscore that the shift in the curve, as the venom:antivenom ratio changed, clearly demonstrated that the antivenom is effective regardless of size group, it would simply require more antivenom for the same mass of venom for small vs large snakes due to the higher concentration of the procoagulant toxins.

Regarding the neutralization, the females were strongly related with size than the males (Linear regression from males: Y = 0.02389*X + 0.9479, r^2 = 0.4962; p = 0.77; Linear regression from females: Y = 0.06745*X - 1.230, r^2 = 0.912, p < 0.0001) (Fig. 2) which is also consistent with the sexual dimorphism of *B. jararacussu* snakes. It is important to emphazise that, due the sexual dimorphism, we performed the linear regressions separated by sex. For plasma, we observed a size-related profile, which the

Table 2
Venom potency (AUCs) and neutralisation potential upon plasma and fibrinogen. AUCs and x-fold shifts presented as mean ± SD. X-fold shifts not shown for fibrinogen due to the limitation of the measurement by the Stago machine (999 s). Sample codes: M25.7FLR – Male, 25.7 cm, Florianópolis-SC; M36.4ILB – Male, 36.4 cm, Ilhabela-SP; M41.6IGP – Male, 41.6 cm, Iguape-SP; M51.0AGM – Male, 50.1 cm, Águas Mornas-SC; M60.3ILB – Male, 60.3 cm, Ilhabela -SP; M69.8ILB – Male, 69.8 cm, Ilhabela – SP, M77.0SJB – Male, 77.0 cm, São João da Barra-RJ; F35.0ILB – Female, 35.0 cm, Ilhabela-SP; F44.5SSB – Female, 44.5 cm, São Sebastião-SP; F48.0ILB – Female, 48.0 cm, Ilhabela-SP; F53.9ILB – Female, 53.9 cm, Ilhabela-SP; F73.2SJB – Female, 73.2 cm, São João da Barra-RJ; F94.2JQB – Female, 94.2 cm, Juquitiba-SP; F108.6STB – Female, 108.6 cm, Sete Barras-SP; F109.9JQB – Female, 109.9 cm, Juquitiba-SP; F112.0IGP – Female, 112.0 cm, Iguape-SP; F123.0JQB – Female, 123.0 cm, Juquitiba-SP.

	Plasma			Fibrinogen	
ID	AUC venom	AUC Antivenom	X-fold shift	AUC Venom	AUC Antivenom
M25.7FLR	297.77 ± 7.92	878.83 ± 51.90	1.96 ± 0.25	5376 ± 127.57	15,333.67 ± 1012
M36.4ILB	318.67 ± 3.60	912.63 ± 8.02	1.86 ± 0.04	8169 ± 722.00	19,930 ± 0
M41.6IGP	336.07 ± 7.31	891.63 ± 25.23	1.65 ± 0.12	2636.67 ± 109.41	7219.33 ± 912.85
M51.0AGM	361.03 ± 8.66	1097.33 ± 21.94	2.04 ± 0.13	2568.67 ± 474.97	5441.33 ± 317.03
M60.3ILB	433.00 ± 10.88	1135.33 ± 115.68	1.62 ± 0.22	7791 ± 468.71	17,903.67 ± 535.72
M69.8ILB	453.73 ± 18.59	1891 ± 59.81	3.17 ± 0.16	5074.33 ± 149.39	11,536.30 ± 1051.73
M77.0SJB	583.97 ± 5.02	2316 ± 34.22	2.97 ± 0.04	14,708.33 ± 813.20	19,930 ± 0
F35.0ILB	331.57 ± 6.71	1020.33 ± 18.18	2.08 ± 0.07	6388.33 ± 549.15	16,690 ± 408.18
F44.5SSB	284.67 ± 1.10	871.33 ± 38.87	2.06 ± 0.15	3395 ± 79.30	8986.33 ± 326.12
F48.0ILB	274.30 ± 3.27	817.13 ± 14.15	1.98 ± 0.02	4011 ± 299.07	9869.33 ± 181.73
F53.9ILB	390.20 ± 5.33	1,097.67 ± 39.27	1.81 ± 0.08	6738 ± 206.64	16,307.30 ± 121.54
F73.2SJB	493.30 ± 11.82	1751.33 ± 44.66	2.55 ± 0.12	10,248 ± 410.19	19,930 ± 0
F94.2JQB	469.93 ± 15.07	2871.67 ± 98.27	5.12 ± 0.29	3792 ± 240.00	9726.67 ± 1379
F108.6STB	519.57 ± 15.22	3257 ± 168.89	5.27 ± 0.21	6753.33 ± 270.53	17,006.33 ± 298.99
F109.9JQB	578.43 ± 25.65	4440 ± 81.16	6.69 ± 0.49	5542 ± 547.86	11,151.67 ± 274.61
F112.0IGP	541.87 ± 12.40	3980.67 ± 67.32	6.35 ± 0.26	6015 ± 331.18	12,000.33 ± 653.73
F123.0JQB	498.33 ± 21.29	4441.67 ± 69.28	7.93 ± 0.47	6920.67 ± 576.90	14,450 ± 1,343.37

smaller being more potent than the larger ones, regardless of the sex (Male $r^2 = 0.885$, $p = 0.002$; Female $r^2 = 0.800$, $p = 0.001$) (Fig. 4 A). This indicates that there is a strong evolutionary selection pressure operating upon this trait in both sexes.

3.2. Fibrinogen coagulation assays and Antivenom neutralisation

B othrops jararacussu venoms showed much lower potency for a pseudo-procoagulant direct action upon fibrinogen, as demonstrated by their AUCs, consistent with this action being a relicital action lingering in the background that was inherited from the pseudo-procoagulant ancestral pit viper (Table 2). Consistent with the pseudo-procoagulant action no longer being under a selection pressure in this lineage, in contrast with the clotting on plasma, the pseudo-procoagulant action upon fibrinogen did not follow any pattern regarding size or sex of the individuals (Fig. 3) and was thus not related with size or sex (Linear regression male: $Y = 116.1*X + 618.5$, $r^2 = 0.262$, $p = 0.240$; Linear regression female: $Y = 10.90*X + 5105$; $r^2 = 0.032$, $p = 0.618$) (Fig. 4 B). Because of the lower potency of the venoms on the fibrinogen clotting, the linear view of the x-axis promotes better visualisation of the curves. Thus, in contrast to the plasma results, the venoms were generally not as potent on fibrinogen in comparison and did not show a size-related pattern (Fig. 4 B).

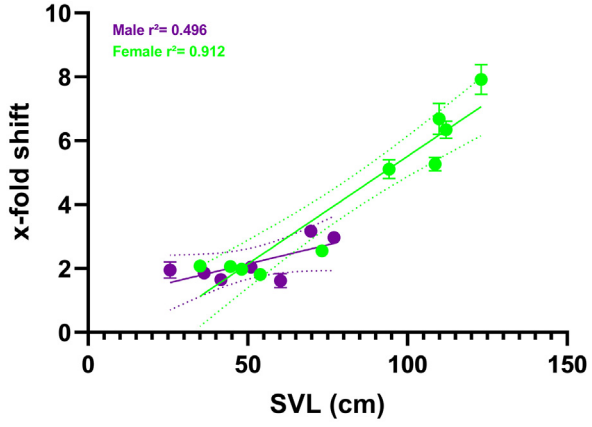

Fig. 2. Linear regression of the Snout-vent length (cm) with the X-fold shifts in plasma clotting time curves, showing antivenom neutralisation across *B. jararacussu* venom of different sex and sizes. The X-fold shift was calculated by dividing the AUC for each venom incubated with antivenom plasma curves by the AUC for venom plasma curves and subtracting the total by 1. A value of 0 is no shift (antivenom does not neutralise venom), while a value above 0 indicates neutralisation. Values of r^2 greater than 0.7 with $p < 0.05$ were considered as a strong positive relation between neutralisation and size. The analyses were separated by sex.

3.3. Inhibitors tests

SVMPs and SVSPs are proteins with coagulotoxic action (Kini et al., 2011, 2001; Kini and Koh, 2016), possessing different mechanisms to affect blood coagulation. As expected, Prinomastat (SVMP inhibitor) promoted a shift on plasma curves (X-fold shift = 2.29 ± 0,09) but did not affect the fibrinogen curves. Conversely, AEBSF (SVSP inhibitor) on fibrinogen increased the clotting times (X-fold shift = 0.5677 ± 0.07) (Fig. 5). This demonstrates that the plasma clotting is SVMP-driven, but the fibrinogen clotting is SVSP-driven. Note: AEBSF was not tested on plasma, as the venom kallikrein enzymes upon fibrinogen is of the same broad serine protease enzyme class as the endogenous clotting enzymes FXa and thrombin. Thus, neutralising the venom enzymes would simultaneously neutralise the endogenous clotting enzymes, so, the AEBSF would interfere with the normal blood clotting by itself, and thus would be uninformative in regard to inhibition of venom enzymes.

3.4. Clotting factors activation

In order to ascertain any differences in factor (prothrombin and Factor X) activation between fastest and slowest-clotting snakes, we selected the three fastest and least neutralised (which also corresponds to smaller snakes) and three slowest and best neutralised (which also corresponds to larger snakes) samples, regardless of sex. For all venoms, Factor X was more activated than the prothrombin, reaching ~60 % of activation relative to the positive control on F48.0ILB (Fig. 6). The results showed higher activity of the neonates in terms of zymogen activation, which was

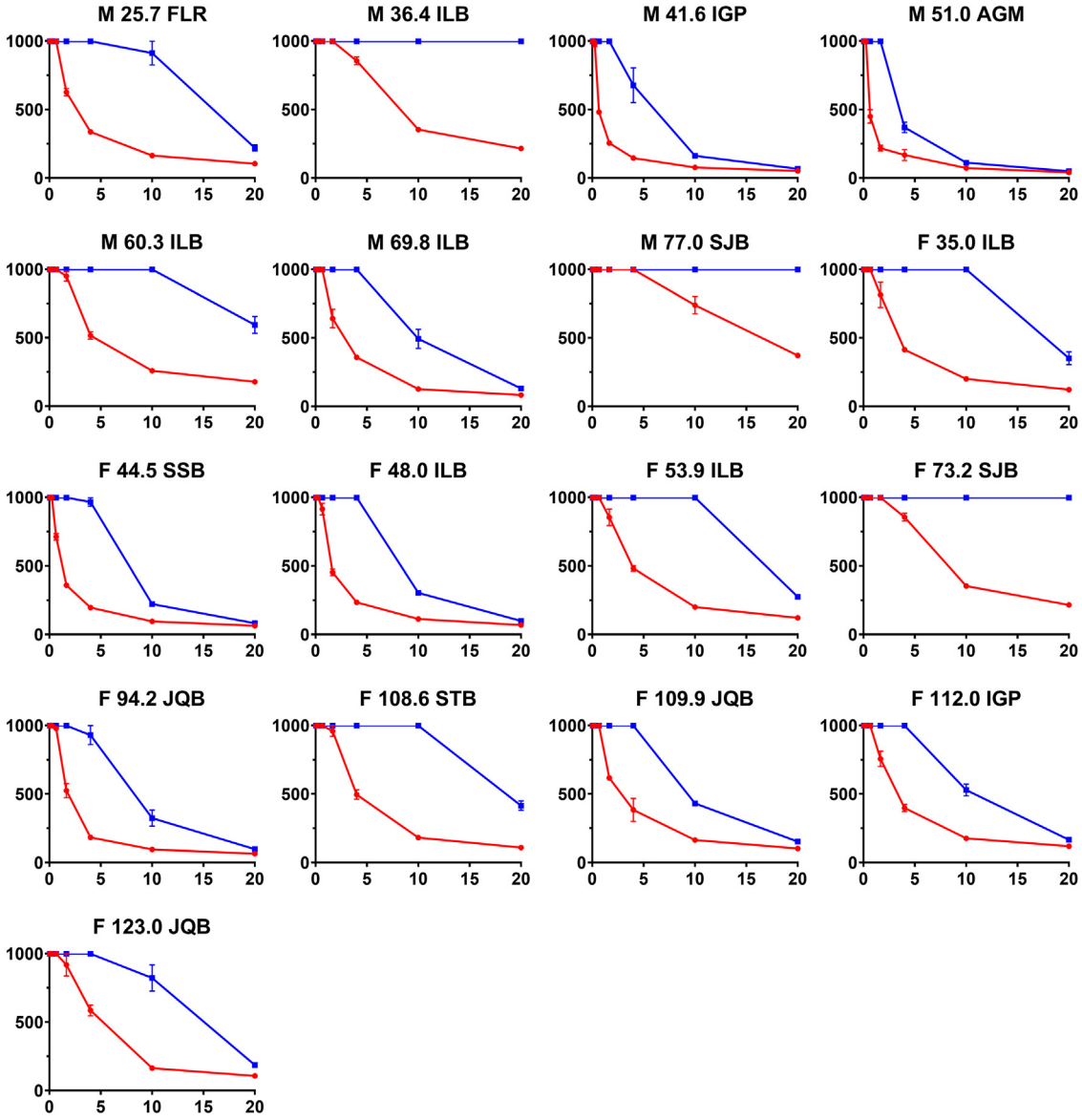

Fig. 3. Linear view of fibrinogen clotting time of *B. jararacussu* venoms from multiple sexes and ages (red line) and *B. jararacussu* venoms with antivenom (blue line) over eight different venom concentrations (20 μg/mL, 10 μg/mL, 4 μg/mL, 1.6 μg/mL, 0.66 μg/mL, 0.25 μg/mL, 0.125 μg/mL, and 0.05 μg/mL). Y axis represents clotting time (seconds) and X axis represents the venom concentration (μg/mL) All venoms tested in triplicate (N = 3). Positive control = 5.2 ± 0.08 s, Negative control venom = 999 s, Negative control antivenom = 999 s. Sample codes: M25.7FLR – Male, 25.7 cm, Florianópolis-SC; M36.4ILB – Male, 36.4 cm, Ilhabela-SP; M41.6IGP – Male, 41.6 cm, Iguape-SP; M51.0AGM – Male, 50.1 cm, Águas Mornas-SC; M60.3ILB – Male, 60.3 cm, Ilhabela -SP; M69.8ILB – Male, 69.8 cm, Ilhabela – SP, M77.0SJB – Male, 77.0 cm, São João da Barra-RJ; F35.0ILB – Female, 35.0 cm, Ilhabela-SP; F44.5SSB – Female, 44.5 cm, São Sebastião-SP; F48.0ILB – Female, 48.0 cm, Ilhabela-SP; F53.9ILB – Female, 53.9 cm, Ilhabela-SP; F73.2SJB – Female, 73.2 cm, São João da Barra-RJ; F94.2JQB – Female, 94.2 cm, Juquitiba-SP; F108.6STB – Female, 108.6 cm, Sete Barras-SP; F109.9JQB – Female, 109.9 cm, Juquitiba-SP; F112.0IGP – Female, 112.0 cm, Iguape-SP; F123.0JQB – Female, 123.0 cm, Juquitiba-SP.

more prominent on prothrombin (Student's *t*-test, Prothrombin: t = 4.243; p = 0.0006; Factor X t = 4.167; p = 0.0007).

3.5. Thromboelastographic tests

To further investigate the taxon-related differences related with the clotting velocity and size, we used the TEG analysis. All the representative venoms formed stable clots on human and toad plasma (Figs. 7 and 8), but the smaller individuals were faster in clotting (R) in comparison with larger individuals (Multiple Student's *t*-test, Human plasma = $p < 0.001$; Toad plasma = p = 0.011). Besides, they also reached the maximum thrombus generation faster (TMTRGG) (Multiple Student's *t*-test, Human plasma = $p < 0.001$; Toad plasma = p = 0.004), consistent with speed of action being the factor selected for. Otherwise, on human plasma, larger individuals had more thrombus formed (TGG) (Multiple Student's *t*-test, p = 0.017). On toad plasma, however, we did not observe differences regarding the strength of thrombus ultimately formed, suggestive of with the selection pressure operating upon the speed of action. Concerning the differences between plasmas, smaller individuals formed more thrombus on toad in comparison with human plasma (Multiple Student's *t*-test, p = 0.003).

4. Discussion

4.1. Synopsis

We investigated the procoagulant effects of *B. jararacussu* venom on plasma and fibrinogen, the ontogenetic differences in

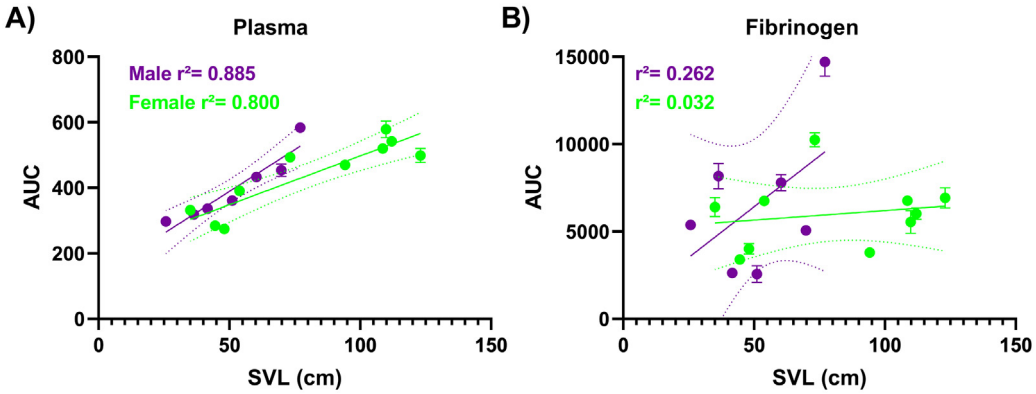

Fig. 4. Linear regressions for comparison of the relative procoagulant potency of *B. jararacussu* venoms from multiple sexes and sizes upon plasma (A) and fibrinogen (B). The potency was evaluated by the AUCs (area under the curves). Lower values represent more procoagulant potency. Values of r^2 greater than 0.7 were considered as a strong relation between coagulotoxicity and size. The analyses were separated by sex.

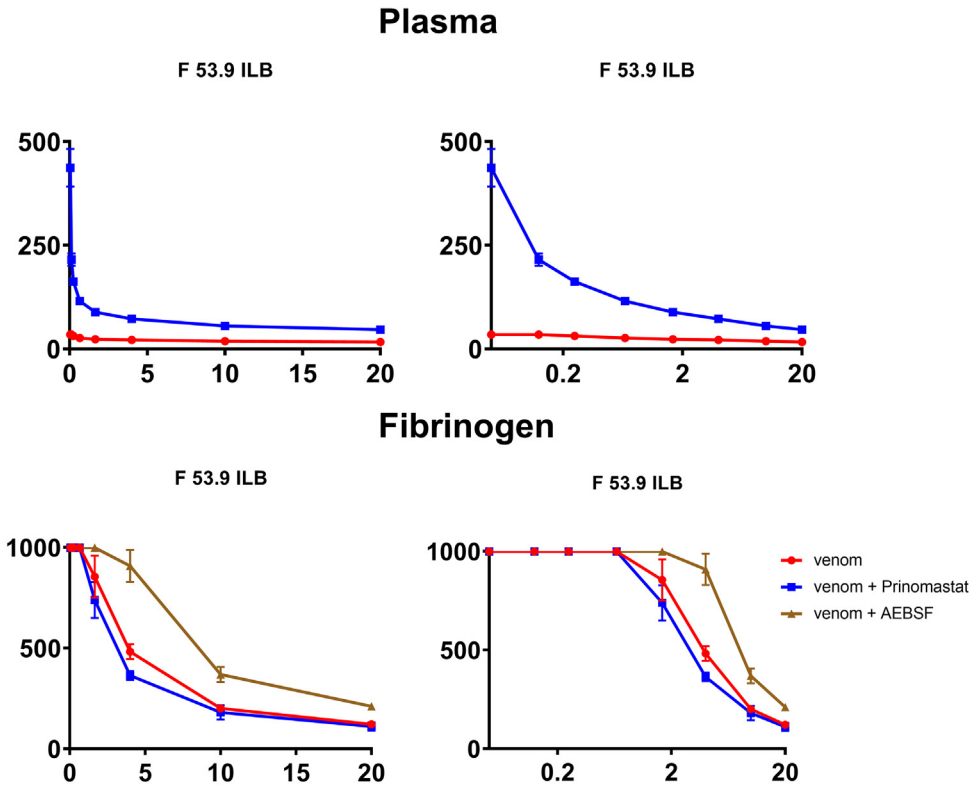

Fig. 5. Linear (left) and Log (right) view of plasma and fibrinogen clotting times of *B. jararacussu* venom (red line) and *B. jararacussu* venom with Prinomastat (blue line) or AEBSF (brown line) over eight different venom concentrations (20 μg/mL, 10 μg/mL, 4 μg/mL, 1.6 μg/mL, 0.66 μg/mL, 0.25 μg/mL, 0.125 μg/mL, and 0.05 μg/mL). Y axis represents clotting time (seconds), and X axis represents the venom concentration (μg/mL) All venoms tested in triplicate (N = 3). Negative control Prinomastat on plasma = 500.5 ± 21.8 s, Negative control Prinomastat on fibrinogen = 999 s, Negative control AEBSF on fibrinogen = 999 s. Sample code: F53.9ILB – Female, 53.9 cm, Ilhabela-SP.

venom potency, and antivenom neutralisation. Our results revealed that small snakes are more potent in plasma clotting than larger, suggesting that this is a trait evolving under strong evolutionary selection pressure. It was shown that the smaller snakes form clots faster than larger snakes in both human and toad plasma, which ratifies our previous results and also highlights the efficacy on prey capture, consistent with amphibians and mammals being significant parts of *B. jararacussu* diet (Andrade and Abe, 1999; Martins et al., 2002). The differential coagulotoxicity resulted in more antivenom being needed for an equal mass of venom from small snakes relative to larger snakes.

4.2. Differential coagulotoxicity biochemistry

It has been previously reported that SVMP in *Bothrops* venoms activate prothrombin and Factor X (Sartim et al., 2016; Sousa et al., 2018). In this study, the fast-clotting snakes activated both cofactors more strongly than the slow-clotting ones, with Factor X being activated much stronger than prothrombin. It is important to emphazise that the speed of clotting was related to the snakes' size, as stated by our previous results. Interestingly, there was a virtual disappearance of prothrombin activation in slow-clotting snakes. Thus, there was a differential variation between Factor X

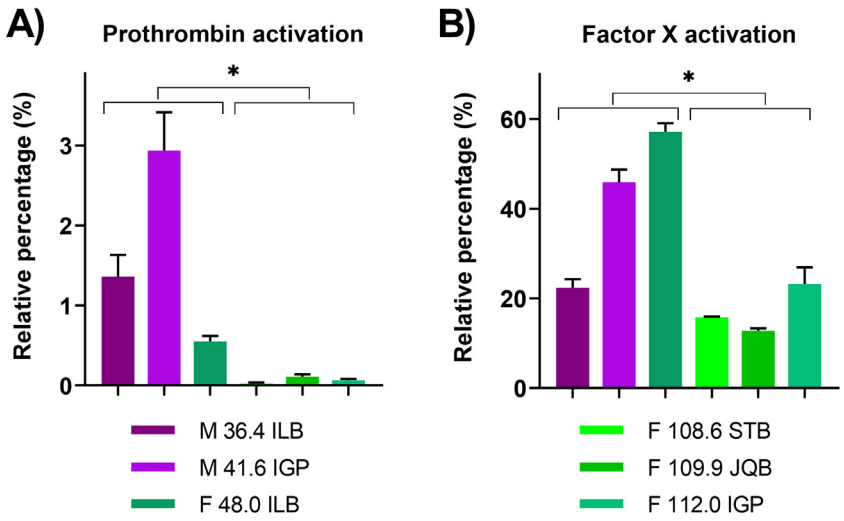

Fig. 6. The relative percentage of zymogens prothrombin and Factor X activation assay of the three fastest/poorly neutralised and three slowest/well neutralised *B. jararacussu* venoms on plasma curves (Fig. 1). In both tests, we observed a significant difference in the factor activation activity, where neonates activated more the factors than adults. The Student's *t*-test was used to compare neonates and adults. Prothrombin: ($t = 4.243$; $p = 0.0006$). Factor X ($t = 4.167$; $p = 0.0007$). * represents statistical significance ($p < 0.05$). Sample codes: F48.0ILB – Female, 48.0 cm, Ilhabela-SP; M41.6IGP – Male, 41.6 cm, Iguape-SP; M36.4ILB – Male, 36.4 cm, Ilhabela-SP; F109.9JQB – Female, 109.9 cm, Juquitiba-SP; F112.0IGP – Female, 112.0 cm, Iguape-SP; F108.6STB – Female, 108.6 cm, Sete Barras-SP.

and prothrombin, which suggests that some coagulotoxic enzymes decrease proportionally greater than others during this ontogenetic transition (Fig. 6).

Recently, we have reported a comprehensive characterization of the composition of the venom samples used in this study by transcriptomic and proteomic approaches (Freitas-de-Sousa et al., 2020). In general, *B. jararacussu* venom presented a high diversity of PIII-class metalloproteinases and most of the isoforms are new sequences yet to be functionally characterized (Correa-Netto et al., 2010). The differential expression during ontogeny appeared to be on a locus-specific level rather than a protein-family level and particular isoforms were predominantly present in venoms of small snakes, particularly the isoform SVMPIII002 that was approximately 4 times more abundant in venoms of small snakes and other 6 isoforms that were almost absent in venoms of larger snakes. Interestingly, the most abundant of these isoforms, BJSUSVMPIII002, presented similarity with Berythractivase, a non-hemorrhagic SVMP able to activate prothrombin (Silva et al., 2003), corroborating our hypothesis that venoms of small snakes are more coagulant due to the presence of particular isoforms of SVMPs and ratifying our results, where the samples with slower clotting-times and larger sizes have less prothrombin and Factor X activation activity than the smaller ones (Fig. 6).

Comparing the differences between clotting factors activation, we found greater activation of Factor X than prothrombin (Fig. 6). In particular, the F48.0ILB, the fastest venom evaluated here, also had the greater Factor X activation and lower prothrombin activation between the fast-clotting individuals. This suggests that the Factor X activation is the most prominent feature of the coagulotoxicity promoted by *B. jararacussu* and may be related to the novel SVMP isoforms present in venoms of smaller snakes (Freitas-de-Sousa et al., 2020). Furtado et al. (1991) showed an ontogenetic shift on coagulotoxicity promoted by *B. jararacussu* venoms. However, they did not demonstrate activation of prothrombin and Factor X in the coagulant activity of *B. jararacussu* adults. In contrast, our results showed that, despite being less potent than the small snakes, the larger individuals still have high procoagulant activity regarding Factor X activation. Previous work indicated that *B. jararacussu* venom induced a high level of thrombin generation, evidenced by the thrombin generation tests (TGT) (Duarte et al., 2019). This finding would be consistent with *B. jararacussu* venom having components that activate Factor X and/or prothrombin. The lack of activity in the Furtado et al. (1991) study may be due to the lack of inclusion of calcium, which has been shown to be a critical cofactor for Factor X activation (Sousa et al., 2018).

Although *B. jararacussu* venoms showed low potency on fibrinogen clotting in comparison with the plasma (Figs. 3 and 4), potency on fibrinogen clotting was still evident, which was in accordance with the pseudo-procoagulant manner founded in other *Bothrops* species (Sousa et al., 2018). In addition, we did not find a pattern that relates size and coagulation potency on the fibrinogen action, indicating that the pseudo-procoagulant pathway on fibrinogen does not undergo ontogenetic shifts. This could be explained by our previous findings showing that the abundance of SVSPs in the same samples of *B. jararacussu* venom was not altered proportionally to the growth of the snakes as well as no significative differences being observed in SVSP activity among the venoms (Freitas-de-Sousa et al., 2020). As the action on fibrinogen is a basal state, it might not be subject to considerable selective pressure due to not being the most important pathway for prey immobilization in comparison with the procoagulant action on plasma (Debono et al., 2019b).

4.3. Coagulotoxicity and prey capture

Sousa et al. (2018) investigated the geographical venom variability of *B. atrox* species and observed a trade-off in the speed and strength of the clot, where the quicker clotting resulted in weaker clots. However, on *B. jararacussu*, we did not observe this trade-off for amphibian plasma but did observe it for mammalian plasma. That is, venoms from small and fast-clotting snakes acted quickly but also formed clots as strong as the venom from large and slow-clotting snakes for amphibian plasma but produced faster yet weaker clots in mammalian plasma.

The coagulant activity is related to the ontogenetic dietary shift in the *Bothrops* genus, whereby the procoagulant activity is a prominent feature in prey immobilisation by small snakes in this genus (Bernal et al., 2020; López-Lozano et al., 2002; Monteiro et al., 2020). In addition, for other generalist species from *Bothrops* genus, a shift in the toxicity from amphibians to mice, following the ontogenetic shift, was observed (Andrade et al., 1996; Andrade

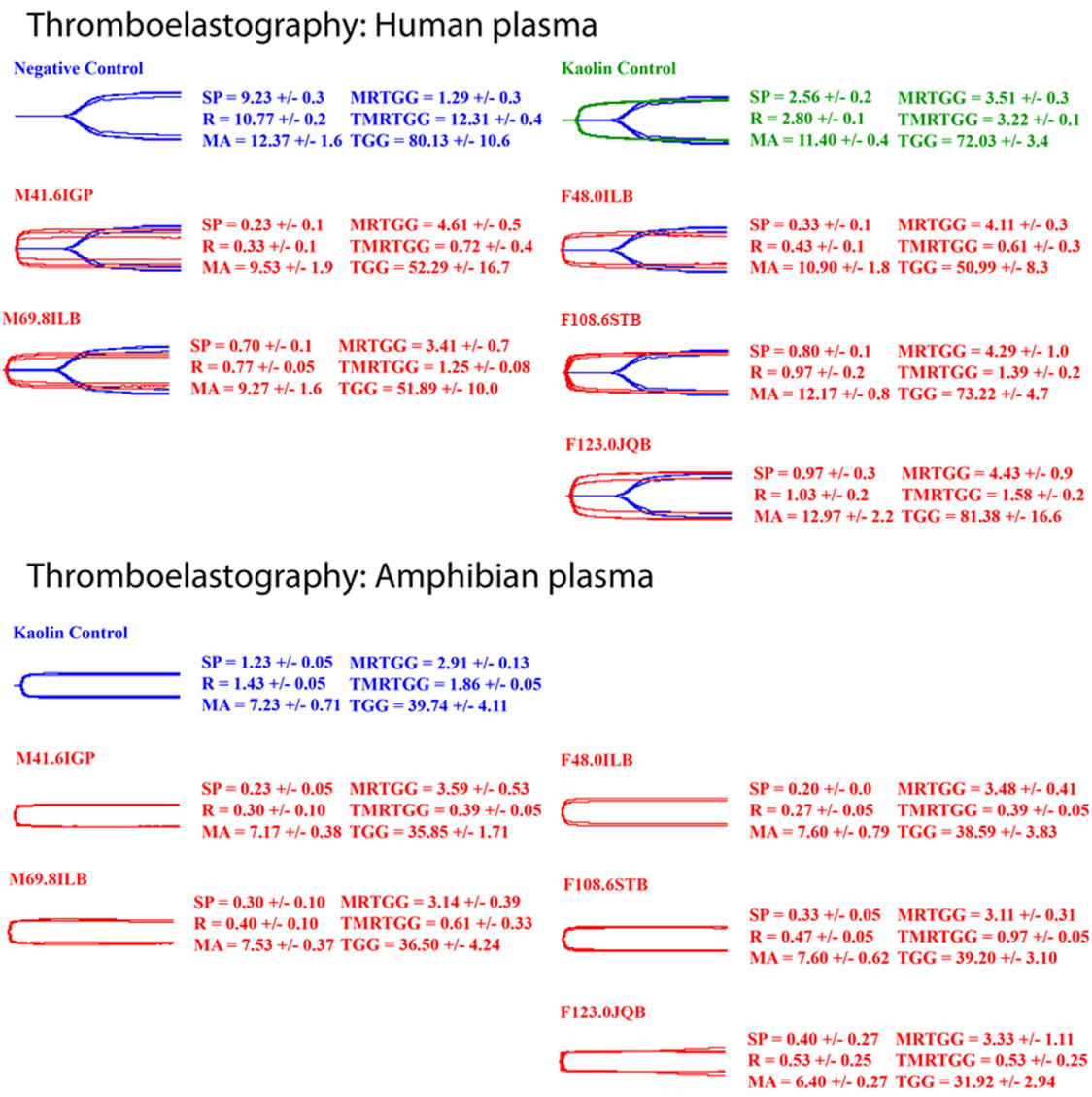

Fig. 7. Comparative thromboelastography on human and toad plasma. SP = split point—the time at clot formation starts; R = time to reach 2 mm amplitude; MA = maximum amplitude – strength of the clot. MTRGG = maximum rate of thrombus generation. TMTRGG = time to the maximum rate of thrombus generation. TGG = total thrombus generated. Sample codes: M41.6IGP – Male, 41.6 cm, Iguape-SP; F48.0ILB – Female, 48.0 cm, Ilhabela-SP; M69.8ILB – Male, 69.8 cm, Ilhabela-SP; F108.6STB – Female, 108.6 cm, Sete Barras; F123.0JQB – Female, 123.0 cm, Juquitiba-SP.

and Abe, 1999; Martins et al., 2002). The higher toxicity on neonates could also be related to prey immobilisation, whereas the digestive role could be more prominent for adults, allowing for larger prey to be eaten (Andrade and Abe, 1999). Moreover, the selective pressures that underlay the coagulotoxic shift evidenced here act on the speed of the clotting. In agreement, two recent studies that observed the ontogenetic variation in venom composition reported the highest procoagulant activity in venoms from small *B. jararacussu* snakes with a greater abundance of SVMPs, while venoms of large snakes were found to be richer in myotoxic PLA_2 toxins favoring a digestive role of these venoms (da Silva-Aguiar et al., 2020; Freitas-de-Sousa et al., 2020).

As *Bothrops* venoms have evolved potent procoagulation as a novel trait relative to other pit vipers from the Americas, the direct action upon fibrinogen is a trait that is at low-levels in the background and reflects the evolutionary history of the venom but does not play a significant role in prey subjugation (Sousa et al., 2018) (Table 2).

4.4. Coagulotoxicity and clinical medicine

While the antivenom was highly effective against all the *B. jararacussu* sizes, proportionally more would be required for an equal mass of small snakes' venom versus bigger snakes due to the greater procoagulant potency of small snakes. In contrast, for the pseudo-procoagulant action upon fibrinogen, we observed no evident differences in the venom activity or potency or AV neutralization.

The coagulotoxicity of *B. jararacussu* small snakes were less neutralised than in larger individuals, similarly to other *Bothrops* venoms (Antunes et al., 2010). Furthermore, Antunes et al. (2010) showed that venom from *B. jararaca* newborns was less recognized by the antivenom despite *B. jararaca* composing the higher percentage of the venom mixture than other species used to produce the SAB. These variations are due to the higher concentration of the procoagulant toxins in the small snake's venoms relative to larger ones, but that the antivenom is ultimately

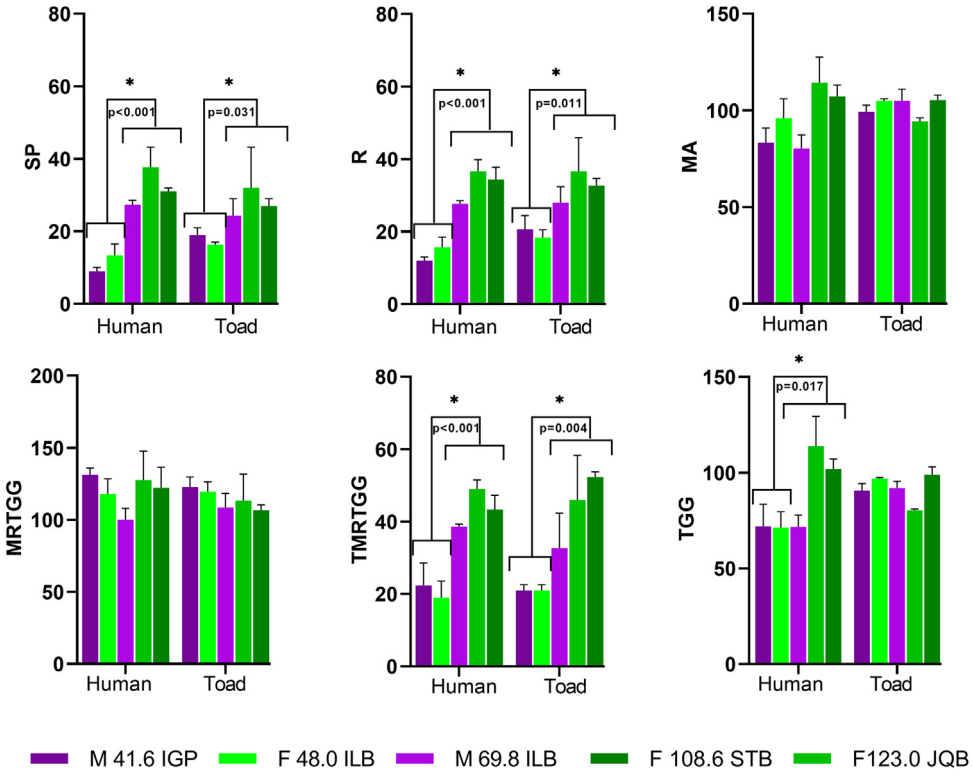

Fig. 8. Relative percentage of the thromboelastographic parameters relative to the positive control (Kaolin) of small (purple) and large (green) individuals on human and toad plasma. SP = split point—the time at clot formation starts; R = time to reach 2 mm amplitude; MA = maximum amplitude – strength of the clot. MTRGG = maximum rate of thrombus generation. TMTRGG = time to the maximum rate of thrombus generation. TGG = total thrombus generated. Multiple Student's t-tests were performed to ascertain the differences between smaller and larger snakes within plasmas. * represents statistical significance between small and large individuals within plasmas ($p < 0.05$). Sample codes: M41.6IGP – Male, 41.6 cm, Iguape-SP; F48.0ILB – Female, 48.0 cm, Ilhabela-SP; M69.8ILB – Male, 69.8 cm, Ilhabela-SP; F108.6STB – Female, 108.6 cm, Sete Barras; F123.0JQB – Female, 123.0 cm, Juquitiba-SP.

effective in neutralizing these life-threatening effects. However, this observation is offset by the venom amount injected by small snakes being much lower than by larger snakes, thus compensating for the differences in antivenom potency.

Interestingly, males were on average more potently procoagulant than females, and this was reflected in the same mass of female venom being better neutralized by antivenom than that of male venoms (Fig. 2). This dichotomy also occurs in *B. moojeni* (Amorim et al., 2018). As female *B. jararacussu* are much larger than males, the procoagulant trait appears to have a strong ontogenetic signal that carries through to adulthood and is linked to size rather than sexual maturity. Although the antivenom production is undertaken using snakes of different sizes and sexes, the female adults, due to their massive venom yields, have greater relative quantities in the venom pool (Amorim et al., 2018; Sousa et al., 2013). Thus, as large females have proportionally less of the procoagulant toxins in their venom (Freitas-de-Sousa et al., 2020), the antivenom neutralisation patterns would reflect this. However, the antivenom would be effective in a dose-dependent manner.

Prey are immobilized by the fast induction of massive strokes, which has been reported also in human bite victims including for very small paediatric patients and some adults exceptions (Silva de Oliveira et al., 2017). However, this is rare in human envenomations because in this case the venom is diluted into a much larger blood volume, resulting in the activation of clotting factors forming microthrombi rather than large clots, and the microthrombi are too small to block blood vessels. Instead, consumptive coagulopathy results, whereby the patient's blood is depleted of coagulation factors and thus unable to clot, leading to systemic hemorrhage. Thus, the pseudo-procoagulant trait of clotting fibrinogen into weak, short-lived fibrin clots, while not being relevant from a prey capture perspective, is relevant from the perspective of human medicine due to its contribution to the depletion of the clotting factor fibrinogen. Thus, the demonstration of antivenom efficacy for pseudo-procoagulant toxicity is important data for use in monitoring patient recovery, as fibrinogen may take 18–24 h to replenish. Therefore, while all fibrinogenolytic toxins may be well-neutralised quickly by the antivenom, the slow production of endogenous fibrinogen may mask the neutralisation of the responsible toxins.

Regarding the inhibitors, the SVMP inhibitor showed a prominent shift in plasma coagulation, demonstrating an SVMP-driven pathway for the potent Factor X activation (Fig. 5). Prinomastat is a well-documented inhibitor (Howes et al., 2007; Preciado and Pereañez, 2018). Some studies suggest the use of inhibitors as an adjunct treatment for snake envenomation treatment, where their action will delay the toxicity and ensure the survival of envenomated people, especially in the field where it is difficult to access antivenom quickly due to the refrigeration requirement of some antivenoms and thus the limited supply in remote areas with intermittent power supplies (Albulescu et al., 2019; Girish and Kemparaju, 2011; Lewin et al., 2016). By demonstrating neutralisation of the coagulotoxicity on plasma when the inhibitor was used, this study adds to the body of knowledge supporting the use of Prinomastat as a field-deployable first-aid measure (Chowdhury et al., 2021; Seneci et al., 2021). While Prinomastat passed clinical trials Phase I and II, we would emphasise however our results are *in vitro* data, and that *in vivo* animal studies and human clinical trials regarding efficacy are necessary before Prinomastat can be used in real-world settings for snakebite firstaid. It is important also to note that, while Prinomastat delays coagulotoxicity, it is not a substitute for the

standard protocol (AV administration); instead, it should be considered from a remote medicine perspective in that it may help stabilize the patient while being transported to a hospital to receive antivenom treatment that will not only neutralize procoagulant effects but also other coagulopathic effects (e.g. the fibrinogenolytic effects are not neutralized by Prinomastat) and other pathophysiological actions such as myotoxicity. Thus, Prinomastat should be considered an important resource as an adjunct treatment, especially to remote regions, to ensure access to standard therapy in time, but not as a replacement for the extremely effective SAB antivenom.

4.5. Conclusion

In this study, we demonstrated that *B. jararacussu* venom had both procoagulant and pseudo-procoagulant functions, which is in agreement with the coagulopathic effects produced in human envenomation by *Bothrops* snakes (Monteiro et al., 2020; Sousa et al., 2018). Also, the ontogenetic shift in coagulotoxic effects was illustrated in our study, regarding the plasma clotting on different taxa and the clotting factors activation by the venoms. These results are consistent with clinical reports, whereby coagulopathy occurs more frequently in envenomation by neonates (Bernal et al., 2020; Milani et al., 1997; Ribeiro and Jorge, 1990). Altogether, these different lines of evidence highlight the necessity of studies that use functional approaches to deduce potency and neutralisation patterns. Such studies that compare intraspecies venom variation in this manner are particularly important because this venom feature can result in diverse clinical outcomes for envenomation accidents.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2021.05.005.

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