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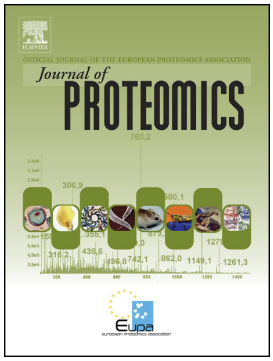

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Functional proteomic analyses of *Bothrops atrox* venom reveals phenotypes associated with habitat variation in the Amazon

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ABSTRACT

Venom variability is commonly reported for venomous snakes including *Bothrops atrox.* Here, we compared the composition of venoms from *B. atrox* snakes collected at Amazonian conserved habitats (*terra-firme* upland forest and *várzea*) and human modified areas (pasture and degraded areas). Venom samples were submitted to shotgun proteomic analysis as a whole or compared after fractionation by reversed-phase chromatography. Whole venom proteomes revealed a similar composition among the venoms with predominance of SVMPs, CTLs, and SVSPs and intermediate amounts of PLA_2s and LAAOs. However, when distribution of particular isoforms was analyzed by either method, the venom from *várzea* snakes showed a decrease in hemorrhagic SVMPs and an increase in SVSPs, and procoagulant SVMPs and PLA_2s. These differences were validated by experimental approaches including both enzymatic and *in vivo* assays, and indicated restrictions in respect to antivenom efficacy to variable components. Thus, proteomic analysis at the isoform level combined to *in silico* prediction of functional properties may indicate venom biological activity. These results also suggest that the prevalence of functionally distinct isoforms contributes to the variability of the venoms and could reflect the adaptation of *B. atrox* to distinct prey communities in different Amazon habitats.

Biological significance

In this report, we compared isoforms present in venoms from snakes collected at different Amazonian habitats. By means of a species venom gland transcriptome and the *in silico* functional prediction of each isoform, we were able to predict the principal venom activities *in vitro* and in animal models. We also showed remarkable differences in the venom pools from snakes collected at the floodplain (*várzea* habitat) compared to other habitats. Not only was this venom less hemorrhagic and more procoagulant, when compared to the venom pools from the other three habitats studied, but also this enhanced procoagulant activity was not efficiently neutralized by *Bothrops* antivenom. Thus, using a functional proteomic approach, we highlighted intraspecific differences in *B. atrox* venom that could impact both in the ecology of snakes but also in the treatment of snake bite patients in the region.

Highlights

- Variability in venom composition was detected in snakes collected at close but distinct habitats;
- Venom variability was noticeable at isoforms expressed rather than toxin groups;
- Variability in venom composition correlated to functional variability;
- Particular isoforms in floodplain venom compromised neutralization of coagulopathy

Key words

snake, venom, variability, *Bothrops atrox*, functional proteomics, antivenom

INTRODUCTION

Intraspecific variability in snake venom composition is a phenomenon reported for many species [1-4]. *B. atrox* venom variability is also extensively reported and has been related to both the ontogenetic development of this snake species and to its geographic distribution [5-9] suggesting that this variation may have functional significance. Determining if functional differences are present is particularly important because *B. atrox* has a wide distribution all over the Amazon [10], where it is the main cause of death due to snakebites [11]. However, it is still not clear if the variability in *B. atrox* venoms is correlated with the environment from which snakes are collected, nor if this variability induces changes in symptoms of the human envenomings, or in the treatment by serum therapy.

Recent advances in the "omics" methodologies had a great impact in biological sciences. For venomous animals, particularly snakes, venomics enlightened the knowledge of their ecology, evolution and significantly reflected in the design of antivenoms to treat snakebites [12]. Venomics was a term first applied to proteomic studies analyzing the composition of snake venoms [13], but gained a broader application including studies of genomes, transcriptomes, and proteomes of venoms from several animals [14]. The earlier studies related to venom variability used one-dimensional electrophoresis, chromatographic analysis or two-dimensional gel electrophoresis [15-20]. Later, with the use of venom fractionation by reversed-phase HPLC allied to mass spectrometry analysis, as well as with the emergence of high-throughput proteomics supported by transcriptomic and/or genomic databases [14], variability studies of snake venoms became more feasible. Two contrasting proteomic approaches, referred to as bottom-up and top-down, have been currently employed. In *shotgun proteomics*, a bottom-up approach, proteins are digested by endopeptidases and the peptides identified by liquid chromatography coupled to tandem mass spectrometry (MS/MS); in top-down, the protein identification is obtained directly from fragmentation of intact proteins, avoiding the digestion into peptides [21]. Although top-down proteomics is capable of identifying post-translational modification patterns [22], allowing protein identification at proteoform level [21], its application has mostly been limited to mixtures of small protein or peptides [14]. Indeed, for animal venoms, the use of top-down proteomics allied to bottom-up approach was applied to Elapid snakes' venoms [23], which are mostly composed by low molecular mass proteins. Recently, there has been a breakthrough in top-down snake venomics applied to the characterization of *Ophiophagus hannah* (king cobra) proteoform composition [24] and identification of previously undetected proteins, at both isoform and proteoform levels, in the venoms of *Dendroaspis angusticeps* (green mamba) and

Dendroaspis polylepis (black mamba) [25]; these studies could be a prelude to disseminated snake venomics using top-down analysis. On the other hand, shotgun proteomics provides a global profile of protein components in complex mixtures [26]. Additionally, it is the major high-throughput approach used in studies of animal venoms [14, 27] enlightening important biological issues such as inter genera [28], interspecies [29, 30], and intraspecies [1, 3, 31] venom variability. However, shotgun proteomics is usually restricted to the characterization of venom profile at protein-family level [28, 32], without significant advances in the identification of predominant isoforms, which could bring up functional insights. These are important issues since functional diversity is often observed within toxins sharing similar scaffolds [33] or, in opposition, similar functions may be shared by structurally different toxins [34]. Recently, some studies have focused on a "functional proteomic" approach [35, 36], while another has finally breached the protein family-level characterization barrier, paving the way for a broad quali-quantitative isoform-level venomics characterization [37]; these papers have enriched the discussion about the variability of snake venoms.

Snakes from genus *Bothrops* are particularly interesting for studies about variation due to their high levels of morphological diversity, natural history, and wide range of habitats [10]. In addition, *Bothrops* snakes are involved in most of the snakebites occurring in Brazil [38]. Envenomation often includes local effects as pain, bleeding, swelling, and myonecrosis at the bite site [39] as well as systemic reactions represented by coagulation disturbances, with bleeding in vital organs and consumptive coagulopathy [39]. In an orchestrated fashion, several toxin groups contribute to such disturbances: (i) snake venom serine proteinases (SVSPs) can act on a variety of components of the coagulation cascade, fibrinolysis, kallikrein-kinin system, and platelets [40] leading to hemostatic disorders; (ii) hemorrhagic snake venom metalloproteinases (SVMPs) degrade components of the extracellular matrix of vascular endothelium promoting hemorrhage [41]; (iii) phospholipases A_2 (PLA_2s), C-type lectins (CTLs), and non-hemorrhagic SVMPs, interfere in different ways to the formation of the hemostatic plug and platelet aggregation [42]. Regional ischemia due to impairment of blood supply caused by SVMPs and SVSPs and the direct action of myotoxic PLA_2s in the plasmatic membrane of muscle cells are key factors for the local damage [43].

B. atrox is the most common species in the Amazon [44]. The wide distribution of the species can be attributed to a large adaptability of this snake to different environments, possibly due to its generalist feeding habit [45]. As in other *Bothrops* snakes, *B. atrox* venom is complex and includes mostly different isoforms of PLA_2, SVSP, CTL, and a high prevalence of SVMP isoforms [1, 28]. *B. atrox* is responsible for most snakebites in the Amazon and its

venom has been investigated in several studies, which reported intraspecific variation related to ontogeny or geographical distribution [1, 6-8]. One factor that is often correlated to venom variability is the different composition of snake diet in different geographic regions [46]. However, it is still unclear if habitat variability in close geographical areas could influence venom variability such as the predominance of functionally distinct isoforms.

In this study, using two different proteomic approaches, we analyzed the variability in venoms of *B. atrox* snakes collected at the west of Pará State, Brazil, from distinct habitats present in Brazilian Amazon: *terra-firme* upland forest (forest), *várzea* (floodplain), and human modified habitats nearby a natural forest (degraded) or a distant pasture area (pasture). Our results indicate a differential distribution of functionally distinct isoforms in venoms collected from each location, particularly in snakes collected from the floodplain (*várzea*) habitat. The impact of such variability is discussed concerning the adaptation of *B. atrox* to different Amazon habitats and the possible implications for snakebite treatment.

EXPERIMENTAL SECTION

1. Snakes and venoms

B. atrox venom samples were obtained from 37 adult specimens, male and female, with sizes ranging from 71.2 to 124.5 cm captured from four locations in the vicinity of the cities of Santarém and Oriximiná, in the western region of the state of Pará, Brazil (Figure 1). These include 1) **FOREST** (n=10): an upland spot of dense tropical forest (*terra-firme*) within a protected area located in the municipality of Belterra known as Floresta Nacional do Tapajós (GPS S 03°03'59,03"/ W 54°58'8,94"); 2) **PASTURE** (n=10): this is a pasture area, previously upland forest, which is used for extensive cattle maintenance, located in the municipality of Oriximiná (GPS S 01°46'03,39"/ W 55°43'53,31"); 3) **DEGRADED** (n=8): This is a contiguous area to Floresta Nacional do Tapajós (GPS S 03° 10' 14.41"/W 54° 59' 23.13") recently degraded (20 to 30 years) due to human settlement and cattle maintenance; 4) **FLOODPLAIN** (n=9): a seasonally-flooded *várzea* habitat in the main course of the Amazon River in municipalities of Oriximiná (GPS S 01° 52' 24.42"/ W 55° 56' 01.85") and Santarém (GPS S 02° 18' 30.77"/W 54° 42' 48.28"). In order to avoid that interpopulational variations could be due to a few venoms showing strong individual variability, we adopted the following criteria: 1) the highest possible number of snakes milked to prepare each pool (10, 10, 8 and 9 for forest, pasture, degraded and floodplain areas, respectively); 2) equal proportions of individual venom were included in each pool; 3) only venom samples collected just after the capture of the snake were considered for the study; 4) only adult snakes were sampled, minimizing an undue effect of ontogenetic variation. Moreover, the

venoms were individually compared by RP-HPLC before preparing the pools and individual heterogeneity was considered to assure the pools were representative of each area (data not shown). Snakes were collected under SISBio license 32098-1. After collection, they were immediately transferred to the herpetarium of Faculdades Integradas do Tapajós, Santarém, Pará – Brazil, anesthetized by inhalation of controlled doses of CO_2 for venom extraction. Venom samples were individually freeze-dried and equal amounts of the individual venoms were added to prepare the venom pools according to the collection areas.

2. Animals

Swiss mice of both sexes (18 - 20 g body weight) were obtained from Instituto Butantan animal house for use in LD_{50} and other functional assays. The Ethical Committee for Animal Research of the Instituto Butantan (CEUAIB 13710-14) approved all the procedures used during the experiments with animals.

3. Antivenom

For tests of antivenom effectiveness, the *Bothrops* antivenom (SAB) produced at Instituto Butantan, São Paulo, Brazil, was used. The antivenom (batch number 1305077, expiry date May 2016) was raised in horses immunized with a mixture of *Bothrops jararaca* (50%), *Bothrops neuwiedi* (12.5%), *Bothrops alternatus* (12.5%), *Bothrops moojeni* (12.5%), and *Bothrops jararacussu* (12.5%) snake venoms. The final preparation consisted of soluble IgG $F(ab')_2$ fragments with tested neutralizing capacity of 5 mg reference venom/mL antivenom (according to manufacturer´s information).

4. Venom fractionation by reversed-phase chromatography

The pools of *B. atrox* venoms were fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC) according to previously described protocol [28]. Briefly, 5 mg of crude lyophilized venom were dissolved in 250 µL of 0.1% trifluoroacetic acid (TFA) and applied to a Vydac C18 column (250 mm X 4.6 mm, 10 µm particle size) coupled to a Shimadzu LC 20 - AT HPLC system. Mobile phases used were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). Proteins were gradient-eluted at 2 mL/min (5% B for 5 min, 5-15% B over 10 min, 15-45% B over 60 min, 45-70% B over 10 min, 70-100% over 5 min, and 100% B over 10 min). Separation was monitored at 214 nm, fractions were collected manually and dried in a vacuum centrifuge concentrator Savant SpeedVac (ThermoFisher Scientific, USA).

5. Proteomic characterization by mass spectrometry

Technical duplicates of each venom (50 µg) or chromatographic fractions (10 µg) were reduced and alkylated before treatment with trypsin solution (0.2 µg/µL), as previously described [47]. The tryptic digests were desalted with in-house made columns packed with Poros R2 resin (Life Technologies, USA) and submitted to reversed-phase nanochromatography coupled to nanoelectrospray high resolution mass spectrometry for identification. Each digest was analyzed in technical triplicate in the mass spectrometer. Initially, 4 µL of desalted tryptic peptides were applied to a 2-cm long (100 µm internal diameter) trap column packed with 5 µm, 200 Å Magic C18 AQ matrix (Michrom Bioresources, USA) at 2 µL/min followed by separation on a 30-cm long (75 µm internal diameter) column that was packed with the same matrix directly on a self-pack 15 µm PicoFrit empty column (New Objective, USA) at 200 nL/min. Chromatography was carried out on an EASY-nLC II instrument (Thermo Scientific, USA). Mobile phase A consisted of 0.1% (v/v) formic acid in water while mobile phase B consisted of 0.1% (v/v) formic acid in acetonitrile and gradient conditions were as follows: 2 to 40% B during 162 min; up to 80% B in 4 min, maintaining at this concentration for 2 min more. The eluted peptides were directly introduced to an LTQ XL/Orbitrap mass spectrometer (Thermo, USA) for analysis. Spray voltage of 1.9 kV, a capillary temperature of 200 °C and a tube lens voltage of 100 V were used for ionization. MS1 spectra were acquired using the orbitrap analyzer (300 to 1,700 *m/z*) at a 60,000 resolution (for *m/z* 445.1200) and for each spectrum, the 7 most intense ions were submitted to CID fragmentation (minimum signal required of 10,000; isolation width of 2; normalized collision energy of 35.0; activation Q of 0.25 and activation time of 30 ms) followed by MS2 acquisition on the linear trap analyzer. The monoisotopic precursor selection feature was enabled and the dynamic exclusion option was activated for the following parameters: repeat count = 1; repeat duration = 30 s; exclusion list size = 500; exclusion duration = 45 s and exclusion mass width = 10 ppm. The charge rejection option was set for unitary and unassigned charge states.

Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by pXtract version 2.0 (http://www.pfindstudio.com/software/pXtract/index.html). All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1) and X! Tandem [(The GPM, thegpm.org; version CYCLONE (2010.12.01.1)]. Mascot was set up to search a database containing 10,364 entries comprised of forward and reverse sequences of: (i) 152 entries from transcriptomic data generated from the analyses of venom glands from five adult specimens of *B. atrox* captured at Tapajós National Forest (n=3) and at pasture area in the municipality of Oriximiná (n=2), in west Pará State, Brazil (Gene Bank SRA SRP056745),

sequences available under accession numbers JAV01810.1 to JAV01961.1; (ii) 4,915 entries of proteome of *Escherichia coli* 0139:H28 (strain E24377A / ETEC) (Proteome ID UP000001122) obtained from Uniprot (July 05, 2014); (iii) 115 entries from the common Repository of Adventitious Proteins (cRAP) available at ftp://ftp.thegpm.org/fasta/cRAP. The *E. coli* and cRAP databases were used to improve the calculation of false discovery rate (FDR). The search assumed the digestion enzyme to be trypsin. X! Tandem was set up to search a subset of the above described database, also assuming trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10 ppm. Carbamidomethylation of cysteine was specified in Mascot and X! Tandem as a fixed modification. Dethiomethylation of methionine, deamidation of asparagine and glutamine, and carbamidomethylation of aspartic acid, glutamic acid, histidine, lysine and the n-terminus were specified in Mascot as variable modifications. Dethiomethylation of methionine, glu->pyro-Glu of the N-terminus, ammonia-loss of the N-terminus, gln->pyro-Glu of the N-terminus, deamidation of asparagine and glutamine, and carbamidomethylation of aspartic acid, glutamic acid, histidine, lysine and the N-terminus were specified in X! Tandem as variable modifications.

Scaffold (version Scaffold_4.6.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 99.0% probability by the Scaffold local FDR algorithm. Peptide identifications were also required to exceed specific database search engine thresholds. Mascot identifications required ion scores greater than both the associated identity scores and 40. X! Tandem identifications required –Log (E-value) scores greater than 2.0. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [48].

Quantitative values for identifications at isoform level and protein class level for whole venom analyses (Supplementary table 1) were expressed as *exclusive unique spectrum counts* corresponding to the number of unique spectra attributed to a single protein, where a unique spectrum should identify a different charged state and/or a different modified form of amino acid sequence. However, quantitative values for the chromatographic fractions (Supplementary table 2), where there was no intersample comparison, the *exclusive spectrum count* criteria was used, which also computes spectra from the same peptide (same amino acid sequence) with different allowed modifications. Finally, in order to predict the functional property of selected isoforms (described below), complete amino acid sequences (deduced

from the transcriptome) that were identified in the pools of venom were blasted against the NCBI database and the function of the isoforms was inferred according to the protein present in the databank, with experimentally tested function, that presented the highest identity matches with *B. atrox* isoforms (Supplementary table 3).

Complete worksheets of proteomic data listing the statistics for the identifications of the proteins of the four venom pools (Supplementary Table 4) and each fraction isolated by RP-chromatography (Supplementary Table 5) are included.

6. Isoforms distribution

Whole venoms were compared according to isoforms composition by hierarchical clustering of observations using as variables the mean of normalized exclusive unique spectrum counts of each isoform accessed by shotgun mass spectrometry present in venoms of each area. For these tests, we selected the isoforms of the most relevant protein classes concerning venom function (SVMPs, SVSPs, PLA2s and CTLs) which were detected with 10 or more *Exclusive Unique Spectra Counts* in at least one venom pool in the replicates of each pool. The Clustering analysis was performed using R statistical software version 3.2.3 (http://www.R-project.org). The set of dissimilarities were normalized by z-score and used to compute the Pearson correlation coefficient and distance matrix with the functions “cor” and “dist”, such matrices were used as parameters to the hierarchical clustering based on the package and function “hclust”. There was employed the agglomerative method with complete linkage linked by the minimum Euclidean distance between an item in one cluster and another one in the other cluster (nearest neighbor). Only isoforms of CTL, PLA2, SVSP and SVSP with more than 10 spectral counts for any venom were considered, corresponding to 37 protein observations between 8 variables. Based on the normalized observations we used principal component analysis (PCA) to find which linear combinations of protein quantitation values would explain most of the variability of the data for the different assays. The studies were carried out by “princomp” [49] and plotted with “biplot” [50] packages in R statistical software.

7. Functional tests

7.1. Enzymatic assays

SVMP, SVSP and PLA_2 activities were carried out as described [51]. For SVMP assays, we used venom samples (1µg) incubated with FRET (Fluorescence Resonance Energy Transfer) substrate Abz-AGLA-EDDnp (GenOne Biotechnologies) and the enzymatic reactions were

monitored in a SpectraMax® M2 fluorimeter (Molecular Devices) with excitation at 320 nm and emission at 420 nm, at 37 °C in kinetic mode over 10 min with read range of 1 min. The results were expressed in RFU/min/µg.

The SVSP activity was determined using the chromogenic synthetic substrate benzoyl-arginyl-p-nitroanilide (L-BAPNA) (Sigma-Aldrich®) incubated with venom samples (5µg) at 37 °C for 40 min. Hydrolysis was measured spectrophotometrically at 405 nm and activity was expressed as absorbance at 405 nm/min/mg of venom.

The PLA_2 activity of venom samples (5µg) was assayed using the synthetic substrate 4-nitro-3- [octanoyloxy] benzoic acid (Enzo® Life Sciences) at a final concentration of 320 µM. The plates were incubated for 40 min at 37 °C and hydrolysis values were determined according to the absorbance at 425 nm and expressed as absorbance/min/mg of venom. The results represent the mean ± SD of three independent experiments.

7.2. Bioassays

For the hemorrhagic activity, venom samples containing 10 µg in 50 µL of PBS were injected intradermally into the dorsum skin of groups of 5 mice. Control groups included mice injected only with PBS or with the same amount of *B. jararaca* venom. Three hours after injection, the mice were euthanized in a CO_2 chamber and immediately after, the skin of the dorsum was removed and the hemorrhagic spot measured as the product of the longest diameter and the one perpendicular to this. Results were expressed in cm^2, as mean ± SD of three independent experiments.

The coagulant activity of venom pools was assessed in citrated human plasma from healthy donors as described previously [52] with modifications. Venom samples were diluted to different concentrations in 25 µL of PBS, incubated with 100 µL of plasma at 37 °C and the clotting time measured in a coagulometer (Diagnostica Stago, START 4). Three independent experiments were performed in triplicate and results were expressed as mean ± SD of the 9 obtained values.

For determination of myotoxic activity, venom samples of 100 µg in 50 µL of PBS were injected intramuscularly into the gastrocnemius muscle of mice. After 3 h, the blood was collected and the sera were assayed for creatine-kinase activity with a commercial kit CK-UV (Bioclin), according to the manufacturer's instructions. Groups of 5 animals were tested and compared to controls groups, with animals injected only with PBS or 50 µg of venom from *B. jararacussu*. Results were expressed as mean ± SD of three independent experiments.

The lethal toxicity of venoms was determined by median lethal dose (LD_{50}) estimated by Probit analysis [53]. Five concentrations of each venom (14.4; 36.0; 90.0; 225.0 and 562.6

μg/mL of PBS pH 7.4) were assayed. Doses of 0.5 mL were injected intraperitoneally into groups of 5 mice/dose and the survival time was recorded for 48 h. A control group was injected only with PBS.

7.3. Reactivity with antivenoms

To determine the antibody titers, plates coated with 1 μg of each pool of venom were incubated with serial dilutions of SAB followed by incubation with anti-horse IgG labeled with peroxidase as described [28]. The reactions were developed with ortho-phenylenediamine/H_2O_2 as the enzyme substrate, and the products were detected at 490 nm. The reactions were performed in triplicate in three independent experiments. The results of the antivenom titration were expressed as mean ± SD of the nine absorbance values.

For neutralization assays of the main toxic activities of venoms, Swiss mice were used as animal model in the neutralization tests of hemorrhagic and lethal activities. For neutralization of hemorrhagic activity, 10 μg of the *B. atrox* venoms were incubated with SAB at ratios of 1, 2 or 4 times the volume required to neutralize the same amounts of the reference venom (1 mL/5 mg venom). The mixtures were incubated (37 °C for 30 min) and 50 μL injected intradermally into the dorsum of the mice (5/group) for the determination of the hemorrhagic activity as described above. The results represent the mean of values obtained in 3 independent experiments, expressed as % neutralization taking as 100% activity the values obtained after injection with venom incubated with PBS. For neutralization of the lethal activity, five LD_{50} doses of the venoms: forest (245 μg), degraded (285 μg), pasture (205 μg) or floodplain (135 μg) were incubated (37 °C for 30 min) with SAB at ratios of 1, 2 or 4 times the potency reference value. Next, 500 μL of the mixtures were injected by intraperitoneal route in groups of 5 mice. The control groups were injected only with PBS or venom incubated with PBS. The results represent the values obtained in 5 different mice and are expressed as the % neutralization considering the number of dead/total animals after 48 hours. For the neutralization of the coagulant samples containing two minimum coagulant doses of each venom (forest - 32 μg/mL; pasture - 35 μg/mL; degraded - 29 μg/mL; floodplain 26 μg/mL) venoms were incubated with several dilutions of SAB for 30 min 37 °C. The mixture was added to 100 μL of citrated human plasma from healthy donors and clotting times were recorded as described above. Neutralization was expressed as effective dose (ED), defined as the antivenom/venom ratio at which clotting time was increased three-fold when compared to clotting time of plasma incubated only with venom.

8. Statistical analysis

Analysis of Variance (ANOVA) One-Way followed by Tukey post-test (for multiple comparisons) were employed and the level of significance was set at $p \leq 0.05$.

RESULTS

1. Composition of venoms from *B. atrox* snakes collected at different habitats

Shotgun proteomics was our first approach to evaluate the variability of *B. atrox* venom according to the habitats. This is a powerful method to access the overall composition of snake venoms; however, due to the great number of isoforms present in each protein group, it increases the redundancy in the total spectral counts obtained for some protein classes, particularly for the ones with a greater number of isoforms. To overcome this limitation, the distribution of the protein groups in each pool of venom was based on the *exclusive unique spectrum counts*, as defined in the methods section. Venoms extracted a few hours after the capture of the snakes were pooled according to their habitats in four groups: forest, pasture, degraded, and floodplain. These pooled venoms were processed and subjected to shotgun mass spectrometry. As shown in figures 2 and 3, the analysis revealed the presence of 11 protein families: SVMP (snake venom metalloproteinase - classes P-I, P-II and P-III), CTL (C-type lectin), SVSP (snake venom serine proteinase), LAAO (L-amino acid oxidase), PLA_2 (phospholipase A_2), CRISP (cystein-rich secretory protein), PDE (phosphodiesterase 1), NUC (nucleotidase), VEGF (vascular endothelial growth factor), NGF (nerve growth factor), and HYAL (hyaluronidase). The overall abundance of toxin groups in venoms from the different habitats was similar. There was a predominance of SVMPs in the four pools (47-54%), followed by CTLs (10-13%) and SVSPs (10-14%), with intermediate amounts of LAAOs (9%) and PLA_2s (6-8%). The minor components included CRISP, NUC, PDE, VEGF, NGF, and HYAL that together accounted for 9-12% of the proteomes. However, some small differences in percentage distribution of each protein group were observed among the venoms. Snakes collected at the degraded area exhibited a higher abundance of class P-III SVMP in their venoms while the ones from pasture area had a higher class P-I SVMP content. Regarding SVSPs, floodplain and pasture snakes displayed a higher percentage compared to the pools from the other three habitats. PLA_2 composition displayed an incremental abundance within the four compared habitats (forest < pasture < degraded < floodplain). Finally, floodplain pooled snake venoms had a higher VEGF content.

As a complementary approach, venom pools were also compared according to their chromatographic profiles (RP-HPLC) followed by identification of protein composition of peaks with variable areas, using a previously standardized proteomic analyses of each fraction contained in different samples of *B. atrox* venoms from snakes of different origins (Supplementary figure 1/Data in Brief article). The comparison of the chromatographic profiles (Figure 4) showed broad similarities but also fine-scale differences among venoms from snakes collected at the different areas, in accordance with the previous shotgun data (Figures 2 and 3). The simplest chromatographic profile corresponded to the venom of snakes collected at the forest with the predominance of peaks 21 and 23, eluted after 80 min of chromatography (Figure 4), which corresponded mostly to SVMPs (Figure 5). Venoms from snakes collected at the pasture presented a similar chromatographic profile. However, two peaks, 5 and 10, were higher than the corresponding ones isolated from venoms of forest snakes (Figure 4). Peaks 5 and 10 included predominantly PLA_2s and SVSPs, respectively (Figure 5). In venoms of snakes from degraded area, we observed an increase of peak 3 (Figure 4), which corresponded mostly to PLA_2s (Figure 5). Venoms from snakes collected at the floodplain presented the most complex chromatogram (Figure 4). It was observed clear reduction of peak 21, constituted mostly by class P-I SVMPs, besides an enlargement of peaks 3, 5 and 9, which contained mainly PLA_2s, peak 10, containing mostly SVSPs, and peak 20 composed of a mixture of CTLs, SVMPs, SVSPs and other proteins (Figure 5). Generally, these findings were in agreement with the shotgun approach. However, there were two discrepancies between the shotgun (Figures 2 and 3) and RP-HPLC (Figures 4 and 5) proteomic approaches. In the former, a higher VEGF content was found in floodplain pooled venom; VEGF was not detected in the RP-HPLC pipeline, probably because it was eluted as a minor peak not selected for MS/MS identification. On the other hand, peak 20 (mostly constituted of CTLs and PI SVMPs) was higher in floodplain (Figures 4 and 5) while this observation was opposite in shotgun analysis (Figures 2 and 3).

We decided to evaluate the overall coverage of our proteomic approaches for the different venom samples when compared to the total number of protein entries (152) present in the transcriptomics-derived database and available at Gen Bank under accession numbers JAV01810.1 to JAV01961.1. The shotgun approach was able to detect 86 entries (56.6%), while the pre-fractionation (RP-HPLC) detected 106 entries (69.7%). Eight proteins (4 CTLs, 1 SVMP P-I, 2 SVSPs, and 1 VEGF) were only detected in the former, while 27 (14 CTLs, 2 SVMPs P-I, 1 SVMP P-II, 6 SVMPs P-III, and 4 SVSPs) were only detected in the latter experimental setup.

Thirty-nine entries (13 BPPs, 13 CTLs, 3 KUNs, 1 SVMP P-I, 1 SVMP P-II, 2 SVMPs P-III, 2 SVSPs, 3 VEGFs, and 1 WAP) were not detected by either approach.

These two approaches give us a good picture of the quantitative variability of each protein class in the four pools of venom. However, each protein class may contain isoforms that differ considerably in their biological function. Thus, to evaluate if proteomics data could allow any inference for predicting venom activity, the variability among these pools of venom was compared at the isoform level, with the quantitation values (in *Exclusive Unique Spectrum Count* – EUSC, normalized by Z-score) originated from the shotgun analysis, by Spearman correlation tests and Principal Component analysis. For these tests, we selected the isoforms of the most relevant protein classes concerning venom function (SVMPs, SVSPs, PLA_2s and CTLs) which were detected with 10 or more EUSCs in at least one replicate of each pool (Supplementary table 3). Furthermore, each isoform was functionally annotated according to known toxins displaying the highest sequence identity and experimentally defined function (Supplementary table 3). The differential abundance of each isoform is shown as a heatmap constructed using the correlation matrix (Figure 6A). The clustering of venoms based on differential abundance showed higher similarity between venoms from snakes collected at the forest or degraded area followed by venom from floodplain snakes; venoms from snakes collected at the pasture were the most dissimilar (Figure 6A). A distinct cluster of isoforms preferentially expressed was noted in venoms from pasture snakes (Figure 6A, cluster 1) consisting of a mixture of all toxin groups including CTLs, PLA_2s, SVSPs, and SVMPs. Venoms from snakes collected at the floodplain, particularly replicate 2, were distinguished by the presence of a larger number of SVSP isoforms (Figure 6A, cluster 2) and a drastic reduction of two SVMP isoforms (Figure 6A, cluster 1a). Venoms from snakes collected at the degraded area differed mostly by the presence of the isoforms grouped in cluster 3, composed majorly of SVMPs, while venoms from forest snakes distinguished from the previous ones by concentrating most of the highly expressed isoforms in one cluster that included mostly class PIII SVMPs (Figure 6A, cluster 4). Principal component analysis (Figure 6B) corroborated the venom clustering described above and also showed SVSPs as components with most prominent loadings in venoms from floodplain snakes, class PIII SVMPs in venoms from forest and degraded area snakes, and a mixture of CTLs, SVMPs, SVSPs, and PLA_2s with important loading in venoms from snakes collected at the pasture.

Next we evaluated the putative function of isoforms responsible for venom variability, according to the function experimentally assessed for toxins sharing high sequence identity (Supplementary table 3) with its respective *B. atrox* isoform. The most important functional

differences were observed in venoms from floodplain snakes. The isoforms BATXSVMPIII28 and BATXSVMPI4 showed lower abundance in venoms from snakes collected at the floodplain (Figure 6A, cluster 4). The isoform BATXSVMPI4 presents sequence identity higher than 95% with Atroxlysin I, a hemorrhagic PI SVMP from *B. atrox* venom [54]. The isoform BATXSVMPIII28 presents sequence identity of 96% with jararhagin, isolated from *B. jararaca* venom [55], which is a versatile toxin with multiple activities [56], and was classified in that study as hemorrhagic SVMP (MHD ~1µg/mouse). The protein corresponding to isoform 28 was recently isolated in our laboratory and named batroxrhagin, a P-III class SVMP displaying hemorrhagic and fibrinolytic activities which is able to inhibit the collagen-induced platelet-aggregation [57]. Thus, considering the biological activities of these two isoforms, we can predict that venoms from snakes collected at the floodplain would be less hemorrhagic. The other remarkable aspect was the presence of a larger number of SVSPs in the floodplain snake venom. Most SVSPs described to date act as thrombin-like enzymes, cleaving fibrinogen to fibrin. However, unlike thrombin, the fibrinogen cleavage by these enzymes results in friable clots, easily dissolved by the fibrinolytic system, causing *in vivo* an anticoagulant effect by depletion of fibrinogen [40]. Batroxobin is a thrombin-like enzyme isolated from *B. atrox* venom [58], presenting anti-coagulant activity by specific cleavage of the alpha-chain of fibrinogen, releasing fibrinopeptide A. The isoform BATXSVSP2 was the most abundant SVSP in venoms from snakes collected at the floodplain and is 94% identical to batroxobin. Another isoform with high load-value in venoms from floodplain snakes was BATXSVMPIII21. This is a PIII class SVMP with 82% identity with berythractivase, a non-hemorrhagic PIII SVMP from *B. erythromelas* venom, described as a prothrombin activator [59], supporting the prediction that venoms from floodplain snakes would be less hemorrhagic and more procoagulant.

Venoms from snakes collected at the forest and degraded areas were very similar with high abundance of the hemorrhagic isoforms BATXSVMPIII28 and BATXSVMPI4. The components that showed different abundances in these venoms were mostly PIII class SVMPs with sequence identity of approximately 80% with HF3 [60] or Atrolysin-A [61], which present high hemorrhagic activities (at nanogram level, in murine model) predicting that these venoms would be distinguished by the hemorrhagic activity.

Venoms from snakes collected at the pasture area were the most dissimilar according to the overall composition. However, regarding the biological activity of each isoform, they also showed a high abundance of the hemorrhagic isoforms BATXSVMPIII28 and BATXSVMPI4 and the isoforms that were differentially abundant in these venoms corresponded to a mixture of SVSPs, CTLs, PLA_2s, and SVMPs (classes PII and PIII). These isoforms were divided in different

biological functions with no hints to a particular functional characteristic for this pool of venoms. The most evident particularity of venoms from pasture snakes was the presence of the isoform BATXSVMPI6, not detected in the venoms from the other habitats (Supplementary table 1), which showed 90% identity with BmooMPα1, isolated from *B. moojeni* venom. This SVMP is not hemorrhagic but displays fibrino(gen)olytic activity [62].

Taken together, the functional inferences for the proteomic data suggested a less hemorrhagic profile for floodplain venom, while the venoms from forest, pasture, and degraded areas would be comparatively more hemorrhagic. On the other hand, the great variety of anticoagulants and thrombin-like SVSP and CTL isoforms could contribute to both procoagulant and anticoagulant activities of all four venoms; this would probably be mainly the case for the significant procoagulant activity of the floodplain venom, which had a higher amount of thrombin-like isoforms. Finally, due to the similarity in the distribution of the myotoxic isoforms in the venoms, no significant differences in myotoxicity could be predicted.

3.2. Functional tests for comparison of venom pools at experimental conditions

Our next approach was to biologically validate the functional differences predicted by proteomics of the snake venoms collected at the different habitats. For this purpose, we assayed each of the four venom pools not only for their SVMP, SVSP, and PLA_2 catalytic activities (using synthetic substrates), but also for their hemorrhagic, coagulant, myotoxic and lethal activities.

The SVMP activity was assessed with a FRET substrate (Abz-AGLA-EDDnp) and the hemorrhagic action of the venoms was measured at dorsal skin of mice, 3 h after the intradermal inoculation of equal amounts of the venoms. As shown in figure 7A, hemorrhagic and catalytic activities of venoms from snakes collected at forest, pasture or degraded areas were similar. However, venom from floodplain snakes presented a SVMP catalytic activity significantly lower and induced a hemorrhagic lesion significantly smaller compared to the other venoms. This result was predicted by our functional proteomics analysis, where a low amount of hemorrhagic isoforms of SVMPs was detected in floodplain venom.

We also compared the SVSP catalytic activity and coagulant activity of the different pools of venoms. The serine protease activity assay was performed using the synthetic chromogenic substrate benzoyl-arginyl-p-nitroanilide (L-BAPNA) and procoagulant activity was evaluated in citrated plasma from healthy donors in terms of minimum coagulant dose (MCD). Figure 7B shows that venoms from snakes collected at forest, pasture or degraded areas had similar SVSP catalytic and procoagulant activities. Once more, differences were observed in

venom from snakes collected at the floodplain that presented higher SVSP catalytic activity and was more procoagulant than the other venoms. Moreover, higher serine proteinase catalytic activity and lower minimum coagulant dose values, when compared to the other venoms, confirmed the higher content of SVSPs and procoagulant SVMPs found for the floodplain venom by proteomics.

The next functional test was to evaluate the PLA_2 catalytic and myotoxic activities of these venoms. The specific chromogenic substrate 4-nitro-3-[octanoyloxy] benzoic acid (NOBA) was used for measuring PLA_2 activity and serum levels of creatine-kinase in mice injected with the venoms was used to compare myotoxicity. The results showed that the four venoms were able to induce myotoxicity in mice at comparable levels. However, regarding PLA_2 catalytic activity, the venom from snakes collected at pasture showed a lower activity against the substrate used (Figure 7C). Differences in distribution of PLA_2s among the venoms were more easily observed when a chromatographic fractionation preceded the MS analysis. Figures 4 and 5 show that at least four fractions containing PLA_2s were variable among the venoms. Peaks 3, 8 and 9 predominated in venoms from snakes collected at floodplain and degraded area, while peak 5 predominated in venoms from snakes collected at the pasture, which is almost devoid of PLA_2s eluted at peak 9. In a separate study, we isolated four of the six isoforms of PLA_2s detected in the proteomes and preliminary results show that peak 3 contains mostly K49 PLA_2 non-catalytic homologues, peak 5 contains an acidic PLA_2 with low catalytic activity, while most of the catalytic activity was detected in the isoform eluted in peaks 8 and 9 (unpublished data). Consequently, this difference in specific activity of venoms from pasture snakes could be explained by a higher content of a low catalytic isoform (contained in peak 5) and a lower abundance of the most catalytic isoform present in peak 8.

Considering the clinical manifestations of envenomation by *B. atrox* as well as the impact that the variation in hemorrhagic and coagulant activities could have for the venom toxicity, we also evaluated the lethal activity of these venoms, measured as LD_{50}. Figure 7D shows that there was no statistical difference in the LD_{50} values for the pooled snake venoms for the different areas, mostly due to the large confidence interval resulting from the small number of animals allowed for the experiments. This observation was surprising due to the less hemorrhagic profile of the floodplain venom and the importance of hemorrhagic activity to the lethality of *Bothrops* venoms [63, 64]. In a recent study, mice injected with 4 LD_{50} of *B. asper* venom died with several alterations such as hemorrhage, plasmatic extravasation, hemoconcentration, and coagulopathy, resulting in hypovolemia and cardiovascular collapse, which ultimately caused death. These effects were assigned to a combined action of SVMPs

and yet unidentified components [63]. In our study, floodplain venom was less hemorrhagic (Figure 7A), but was more procoagulant (Figure 7B) with lower LD_{50} value (Figure 7D). SVMPs acting as prothrombin activators or thrombin-like SVSPs, more abundant in this venom, could exacerbate their toxicity to a severe coagulopathy also associated with lethality. Although our proteomics data have shown the presence of pro-coagulant SVMPs in the four pools analyzed, these components have not yet been isolated and characterized from *B. atrox* venom. Thus, we have to consider that the lethality of *B. atrox* venom is a phenomenon not yet fully understood where hemorrhagic SVMPs play a central (but not exclusive) role, with the eventual involvement of pro-coagulant components.

3.3. Reactivity with *Bothrops* antivenom

After the evaluation of the major toxic activities, our next approach was to analyze the reactivity of the venoms from snakes collected at the four areas with *Bothrops* commercial antivenom (SAB), currently employed in the treatment of patients envenomated by *Bothrops* snakes. First, we evaluated SAB antibody titers relatively to the venoms from snakes collected in the four areas, in comparison to *B. jararaca* venom, the major antigen in the antivenom production. As shown in figure 8A, SAB titration curves were very similar against all venoms and showed slightly higher absorbance values in the fourth and fifth dilutions against *B. jararaca* venom. Antibody titers were obtained at the same dilution of 1,024,000 against all venoms. The small difference reported may be related to a lower reactivity of some components found in *B. atrox* venom, particularly from the floodplain (slightly lower values at fourth and fifth dilutions). The good reactivity of the SAB with *B. atrox* venoms had already been shown by antivenomic analysis [1], and by serum neutralization experiments [28]. Next, we evaluated SAB neutralizing efficiency against the hemorrhagic, coagulant, and lethal activities of the venoms. Neutralization protocols were carried out by pre-incubation of a constant amount of venom with increasing antivenom to venom ratios, with the minimum potency equal to the one referred by the manufacturer (1 mL SAB neutralizes 5 mg of the reference venom).

To assess the neutralization of coagulant activity, we used a constant amount of venom corresponding to two minimum coagulant doses of each venom incubated with several dilutions of antivenom. As shown in figure 8B, venoms from snakes collected at the forest, pasture or degraded areas had their coagulant activity neutralized by SAB in a similar fashion, with ED values close to 100 µL antivenom/mg venom (degraded area = 100.9 µL antivenom/mg venom; forest = 104.0 µL antivenom/mg venom; pasture = 89.7 µL antivenom/mg venom).

However, pooled venom from snakes collected at the floodplain had a higher ED value (346.1 µL antivenom/mg venom), and the complete neutralization of the coagulant activity was not achieved up to the proportion of 1,600 µL antivenom/mg venom. These observations suggest that the *in vitro* procoagulant activity of the *B. atrox* venom, particularly from the floodplain, may be related to low antigenic toxins, which would be weakly recognized by the antivenom, or to particular isoforms to this pool of venoms that are not immunologically cross-reactive with those used in immunization for the production of antivenoms.

To evaluate the neutralization of hemorrhage, venom doses of 10 µg were pre-incubated with SAB and injected in the dorsum skin of mice at the following ratios: 1 mL SAB/5 mg venom (1:5), 2 mL SAB/5 mg venom (2:5), and 4 mL SAB/5 mg venom (4:5). As shown in figure 8C, hemorrhage induced by the four venom pools was partially neutralized by SAB at 1:5 antivenom to venom ratio but completely neutralized when using a 4:5 ratio. At the intermediate proportion (2:5 antivenom to venom ratio) only floodplain venom hemorrhagic activity was completely neutralized, probably due to its intrinsic lower hemorrhagic activity. These findings confirm the SAB efficiency in the neutralization of the hemorrhage induced by *B. atrox* venoms, as previously reported [28].

Considering the implications of the differences reported above we also assessed the neutralization of venom lethality. Five LD_{50} doses of each venom (forest = 245 µg; degraded area = 285 µg, pasture = 205 µg; floodplain = 135 µg) were incubated with SAB at the same ratios as described for hemorrhagic activity. The results of lethality neutralization, represented in figure 8C, indicate that SAB efficiently neutralized the lethality induced by all venoms at a 4:5 antivenom (mL) to venom (mg) ratio. Venom from snakes collected at the floodplain was slightly easier to neutralize and reduction of lethality was already observed at lower ratios (1:5 and 2:5) when compared to the other venoms.

4. Discussion

The region chosen for this study, west of Pará State in the Brazilian Amazon, covers a vast area marked by a diversity of habitats that houses a high diversity of snakes [65]. In recent decades, there has been significant environmental changes in this region with devastation of large forests and the advance of the soybean crops, mainly due to road pavement and agribusiness development [66]. The modification of the landscape may interfere with the balance of local ecosystems, altering the equilibrium of the snake communities. This could favor an increase in the number of snakebites in a region, now inhabited by human beings involved in agriculture, leading to higher incidence of human envenomation events. Thus, a deeper knowledge on the variability in *B. atrox* venom composition obtained from snakes living in different habitats from west Pará could help to establish possible relationships between the functional diversity of the venoms and the environment where the snakes inhabit. In addition, this knowledge could also scientifically support the efficacy of existing *Bothrops* antivenoms in the region.

Published venom proteomic profiles of *B. atrox* snakes collected at different areas are similar to the pattern previously described for *B. atrox* venoms of the Brazilian Amazon [1, 28]. Two phenotypes, geographically differentiated, were described for *B. atrox* venoms: adult specimens from Venezuela and Colombia present venoms with higher proportion of P-I class SVMPs and PLA_2s in [7], while towards Brazilian Amazon venoms present a phenotype rich in P-III class SVMP throughout their lifetime [1]. Our results corroborate the previous reports indicating that P-III class SVMPs are the most abundant components in the venoms from snakes collected at the four different areas, with lower amounts of P-I class SVMPs and PLA_2s; these low-abundance components were used as specific markers for each population [1]. However, the most important differences described in this study concerns to the distribution of particular isoforms in each pool of venom and the inference of functional activities for these characterized isoforms.

Our proteomic analyses followed two strategies: (i) shotgun proteomics, consisting of tryptic digestion of the whole venoms followed by identification by nanoLC coupled to high resolution mass spectrometry and (ii) previous fractionation of the whole venoms by RP-HPLC, and identification of variable fractions according to a protocol as used above. Shotgun proteomics generates global profiles of protein components in complex mixtures [26] and is considered an appropriate approach for comparative studies since it allows simultaneous analysis of different samples that ran under the same conditions [28]. Interestingly, using both

approaches, the final results were comparable indicating the pool of venoms from floodplain snakes as the most dissimilar with the predominance of pro coagulant SVSP isoforms. In our study we found that shotgun proteomics combined with hierarchical clustering of unique spectral counts was the best approach to identify the variability markers at isoform level, especially when the identification of the proteins is supported by a transcriptomic database of the same or related species. In recent years, proteomics analyses based on genomic data, *the proteogenomic* approach, allowed the identification of novel proteins in cone snail venom [67] while the *proteotranscriptomic* approach has already been used in studies about variability in snake venoms [31, 36, 68]. On the other hand, venom proteomes are also frequently reported by previous fractionation of venoms prior LC-MS/MS that, together with the top-down approach, result in the most complete proteomic coverage [27, 69], as has recently been done for *Bothrops jararaca* venom´s proteopeptidome determination [37]. Nevertheless, each approach has advantages and disadvantages that may limit their use in certain types of studies and best results are achieved in comparisons of phenotypically distinct venoms using both approaches. In this study, our samples were obtained from snakes living in nearby areas and only subtle differences were expected. Therefore, we improved the toxin identification at the isoform level with functions inferred by sequence identity to proteins with known biological activities. Mass spectrometry has already been used in the study of the composition of snake venoms. However, only a few studies had attempted the functional classification of identified toxins [35, 36, 70]. Proteomic approaches based in toxin categories and functional classification were used to analyze *Naja naja atra* and *Agkistrodon halys* venoms and revealed the high abundance of cardio/neurotoxins in the cobra venom and significant amount of haemotoxins and metalloproteinases in the viper venom [70]. Recently, the venom of *Ophiophagus hannah* was subjected to proteomics showing the total composition of the venom and their subtypes of toxins, which allowed the establishment of toxinological correlations with the bioactivities evaluated [36]. Functional proteomic analysis was also used to study the geographical variability of *Ophiophagus hannah* venoms in Southeast Asia, focusing mainly on the identification, isolation and characterization of some PLA_2s, three-finger toxins (3FTx) and Kunitz-type inhibtors, differentially expressed in the venoms from Malaysia, Indonesia and China [35]. Recently, the concept of toxicovenomics has been introduced based on studies with *Dendroaspis angusticeps* [71], *Naja melanoleuca* [72] and *Micrurus spp* [73]venoms. The generated data represented a breakthrough in functional venomics [74]. Thus, using similar approaches we were able to identify important differences in *B. atrox* venoms from snakes collected at different habitats in the same geographical area.

In whole venom analysis, there was an indication of higher abundance of SVSPs in venoms from pasture and floodplain snakes. As expected, the chromatographic profiles were also relatively similar; however, the complexity was increased in venoms from pasture and floodplain snakes, with a greater number of peaks with considerable height. In terms of relative abundance, the most different fractions in the chromatograms were composed mainly by P-I SVMPs, acid D-49 and basic K-49 PLA_2s, SVSPs, and CTLs. The functional classification of these proteins showed the variability among hemorrhagic SVMPs, as Atroxlysin-I, acid anticoagulant or basic myotoxic K-49 PLA_2s, thrombin-like SVSPs, and anticoagulant CTLs. Thus, the predictable phenotype was that the pooled venom from snakes collected at floodplain was less hemorrhagic and more procoagulant, while only small functional differences were predicted for venoms of the remaining three studied habitats. The differences in venom composition could be interpreted as differences in diet type or availability in each environment. *B. atrox* snakes have a generalist diet, composed of arthropods, frogs, lizards, birds, and small mammals [45, 75, 76]. In fact, in preliminary observations of stomach contents of snakes collected at the forest, remains of amphibians, mammals and birds were observed, while the presence of prey remains could not be detected in the stomach contents of a few specimens collected in the floodplain (H. M. Chalkidis, unpublished). Differences in prey abundance were noticed during our field trips for collecting floodplain snakes. A wide variety of prey was observed during the dry season, but we did not notice the presence of prey during the flood season, suggesting that during this period, food would be scarce, and the venom should be more effective to a wide range of different or even unusual possibilities.

Considering the SVMPs, the most abundant component in all venom pools, the action of the hemorrhagic SVMPs is associated to the degradation of basement membrane, directly related to the catalytic activity of these toxins. However, P-III SVMPs are much more hemorrhagic than P-I SVMPs [41]. P-III SVMPs are the predominant components of *B. atrox* venoms [1, 28], and the most antigenic toxins of the *Bothrops* venoms [28], which explains the efficacy of the commercial antivenoms in the treatment of the systemic effects during the envenomation by *Bothrops* snakes. Recently, we isolated functionally distinct SVMP isoforms from *B. neuwiedi* venom, each one affecting different targets in the hemostatic system of birds, small rodents, and humans [77]. According to these data, some multifunctional isoforms were assumed as "housekeeping" proteins, responsible for the general effect of the venom, while selective isoforms would modulate the effectiveness of the venom to different prey and predators, contributing for adaptation of the snake. Thus, the presence of several isoforms, particularly of P-III SVMPs would contribute to their adaptability within different habitats with

different prey communities. In this case, the smaller hemorrhagic activity of the floodplain venom was related to a lower expression of batroxrhagin [57], that according to the functions of its homologous SVMP, jararhagin, would be a typical multifunctional housekeeping toxin. In contrast, other isoforms would be more expressed in the venom enhancing the prey possibilities in the floodplain area subjected to remarkable seasonal environmental changes. Floodplain is a *várzea* region that alternates periods of drought and flood throughout the year and seasonal variations may interfere on variety as well as availability of prey for floodplain snakes. The reduction of hemorrhagic SVMPs in venom from floodplain snakes was likely compensated by the increase of pro/anticoagulant toxins: CTLs (peak 20 - figure 4), SVSPs (peak 10 – figure 4) and anticoagulant PLA_2s (peak 9 – figure 4).

Although there are only a few studies describing CTLs isolated from *B. atrox* venom [78, 79], our results suggest a correlation between the varieties of CTL isoforms with functional diversity, significantly contributing to the hemostatic disturbances observed in the envenomation by *B. atrox*. In addition, thrombin-like SVSPs cleave fibrinogen at a unique position, resulting in formation of abnormal fibrin clots, quickly degraded by the fibrinolytic system [42], resulting in their anticoagulant effects. Other SVSPs, such as PA-BJ isolated from *B. jararaca* venom, can directly activate platelets by cleavage of PAR [80]. Still, PLA_2s may also exert anticoagulant effects such as inhibition of platelet-aggregation and inhibition of key steps in the blood coagulation cascade [81]. These three toxin classes are predominant in the venom of snakes collected from the floodplain habitat and certainly contribute not only to the more intense procoagulant activity observed *in vitro* in floodplain venom (Figure 7B) but also to the imbalance in hemostasis very common in the envenomation by *B. atrox*.

PLA_2s were present in all the analyzed venoms (Figure 3), but at low proportions (*ca.* 6%) in venoms from snakes collected at forest, pasture, and degraded areas. Venom from snakes collected at the floodplain had a slightly higher (*ca.* 8%) PLA_2 content. Moreover, three fractions containing PLA_2 molecules were increased in floodplain venom chromatograms (Figures 4 and 5). Some PLA_2s have already been isolated from *B. atrox* venom. $BaPLA_2I$, a non-enzymatic K-49, and $BaPLA_2III$, a catalytic D-49 PLA_2 were isolated from *B. atrox* venom and both induced myonecrosis, edema, and mast cell degranulation [82]. Recently, three other PLA_2s have been isolated from the same venom, Batx-I, a non-catalytic K-49, Batx-II a catalytically active D-49, and $BaPLA_2$, a myotoxic PLA_2, all able to induce inflammatory effects [83]. Unfortunately, none of these proteins has its complete amino acid sequence deposited in databanks; hence, we could not correlate these activities with the variable fractions detected in the floodplain venom.

Considering the significant differences in hemorrhagic and coagulant activities observed particularly in the venom from snakes collected at the floodplain, we decided to evaluate if this functional variability could impair the efficiency of the antivenom used in the treatment of envenomation by *B. atrox*. Similar antibody titers were assessed when the commercial antivenom was assayed against venoms from snakes collected at the four areas. In spite of that, in the neutralization tests the antivenom showed different efficacy towards the pooled venom from floodplain snakes. The commercial antivenom was not able to neutralize the coagulant activity of floodplain snake venom. Since the commercial antivenom was not able to neutralize the coagulant activity of floodplain venom, although has neutralized completely hemorrhagic and lethal activities of all pools of venoms. Recently, it was reported that hemorrhagic SVMPs are the components mainly responsible for *B. asper* venom-induced lethality [63]. Thus, the lower amounts of hemorrhagic SVMPs in venom from floodplain snakes could easily explain the efficient neutralization of floodplain venom-induced hemorrhage and lethality. In our samples, hemorrhagic SVMPs are probably playing a central role in the lethality induced by the venoms, as the antivenom neutralized the lethality of all venoms at the same ratios. However, these data bring out important concerns about the neutralization of procoagulant activity of venom from floodplain snakes. Our data suggest that in the floodplain venoms, thrombin-like SVSPs, acidic PLA_2s, CTLs, and eventually procoagulant SVMPs contribute significantly to coagulant activity, but not for the lethality induced by the venom. In addition, although there are studies showing a positive correlation between the neutralization of the coagulant and lethal activities of the venoms from other *Bothrops* snakes [63, 84], our findings indicate that this correlation might not hold true depending on the snake population studied . Despite that, our data evidenced that procoagulant components in venom from snakes inhabiting the floodplain area are not well neutralized by the commercial antivenom. Unfortunately, the database used in this study was composed of five transcriptomes originated from *B. atrox* snakes collected only at forest and pasture areas. Possibly, using a transcriptomic database derived from venom glands from snakes collected at the floodplain habitat (which is currently being carried-out by our group), we should be able to find these (new) putative protein structures that account for the observed procoagulant activity of floodplain venoms.

CONCLUSIONS

The use of a functional proteomic approach allowed us to identify a variety of isoforms in the main protein families found in the venoms. Furthermore, a distinct phenotype (less

hemorrhagic but more procoagulant) was established for the venom pool from snakes collected at the floodplain area. This suggests that the functional proteomic approach described in this study may be a useful option for further studies aimed at analyzing the variability of venoms from closely related snakes, especially at intraspecific level. The largest differences were found in the venom of snakes collected in floodplain, extremely dynamic scenery, suggesting a possible role for environment in the modulation of the variability of *B. atrox* venoms. These issues are still under investigation by our group, through evaluation of the variability at individual level, within each habitat, and by characterization of the main variable fractions of the venoms; we expect these new data should contribute to understanding the environmental and individual aspects of the variability of *B. atrox* venom. Despite the differences reported, the SAB (*Bothrops* antivenom) showed a good reactivity with all venoms and neutralized their main toxic activities, suggesting that the functional diversity observed may not compromise the effectiveness of serum therapy in the region. However, each accident is caused by a particular snake and lack of neutralization of individual venom activities may occur, indicating the need for a constant surveillance of antivenom efficacy and a special attention from the clinicians when antivenoms are not readily efficient in neutralizing venom effects.

5. AGKNOWLEDGEMENTS

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LEGENDS FOR FIGURES

Figure 1 - Areas used for snake collection. Brazilian map (white) highlighting the Amazon region (light gray) and Pará State (dark gray). The amplification of the square located in the west of Pará denotes the location of the areas of snake collection: pasture (red) at the

north of the Amazon River, municipality of Oriximiná; forest (yellow) and degraded area (purple) at Belterra municipality, south of the Amazon River; floodplain region in Santarém and Oriximiná municipalities.

Figure 2 - Proteomic profiles of *B. atrox* venoms from four habitats. The proteomes are represented by the total number of *exclusive unique spectrum count*s of all identified protein entries associated to each protein family. The following toxin families were identified: CTL (C-type lectin); CRISP (cysteine-rich secretory protein); NUC (ecto-5'-nucleotidase); HYAL (hyaluronidase); LAAO (L-amino acid oxidase); NGF (nerve growth factor); PDE (phosphodiesterase 1); PLA_2 (phospholipase A_2); SVMP-I, -II and –III (snake venom metalloproteinases - classes P-I, P-II and P-III); SVSP (snake venom serine proteinase), and VEGF (vascular endothelial growth factor). Data represent the mean and standard deviation of duplicate technical samples analyses.

Figure 3 - Proteomic profiles of B. atrox venoms from four habitats. The proteomes are represented by the percentage of exclusive unique spectrum counts of all identified protein entries associated to each protein family. The following toxin families were identified: CTL (C-type lectin); CRISP (cysteine-rich secretory protein); NUC (ecto-5'-nucleotidase); HYAL (hyaluronidase); LAAO (L-amino acid oxidase); NGF (nerve growth factor); PDE (phosphodiesterase 1); PLA2 (phospholipase A2); SVMP-I, -II and –III (snake venom metalloproteinases - classes P-I, P-II and P-III); SVSP (snake venom serine proteinase), and VEGF (vascular endothelial growth factor).

Figure 4 - Chromatographic separation profiles of *B. atrox* venoms from four habitats. For the chromatographic analysis, 5 mg of crude *B. atrox* venom of snakes from each area were applied to a Vydac C-18 column (4.6 x 250 mm, 10-µm particle size). Mobile phases used were 0.1% TFA in water (A) or 0.1% TFA in acetonitrile (B). Proteins were gradient-eluted at 2 mL/min (5% B for 5 min, 5-15% B over 10 min, 15-45% B over 60 min, 45-70% B over 10 min, 70-100% over 5 min, and 100% B over 10 min). Separation was monitored at 214 nm.

Figure 5 - Percentual contribution of protein classes present in chromatographic peaks obtained from pooled venoms reversed-phase separation. One representative of each of the 26 peaks depicted in figure 4 was digested with trypsin and submitted to LC-MS/MS for protein identification. The total number of *exclusive spectrum count*s associated to each protein entry was summed to generate the appropriate protein family count. These numbers

were then plotted as a relative percentual family contribution per chromatographic fraction. The following toxin families were identified: CTL (C-type lectin); CRISP (cysteine-rich secretory protein); NUC (ecto-5'-nucleotidase); LAAO (L-amino acid oxidase); NGF (nerve growth factor); PDE (phosphodiesterase 1); PLA_2 (phospholipase A_2); SVMP-I, -II and –III (snake venom metalloproteinases - classes P-I, P-II and P-III) and SVSP (snake venom serine proteinase).

Figure 6 - Hierarchical clustering and principal component analysis (PCA) of isoforms' expression levels in the different venom samples. A: Heat map presentation of a hierarchical cluster of CTL, SVMP, SVSP and PLA_2 isoforms detected with 10 or more *exclusive unique spectra counts* (EUSC) in at least one replicate of each venom pool: forest (F1 and F2), pasture (P1 and P2), degraded area (D1 and D2), and floodplain (V1 and V2). EUSC values were normalized, mean-centered and standardized. The green color represents low and red color represents high expression levels. **B:** Principal component analysis in a 2D graph of DIM1 and DIM2, which explains 74.4% of the variance. Vectors that are close together are highly correlated in terms of the observed proteome for each replicates while vectors that are orthogonal are poorly correlated. PC1 correlates well with changes between the pasture and other samples as the projection of the tips of the arrows on PC1 axis indicate.

Figure 7 - Experimental validation of functional proteomics predictions. (**A**) **SVMP activities**: for hemorrhagic activity, 10 µg of each pool of venoms were injected in the dorsum of mice, the hemorrhagic lesions measured three hours after injection and the results were expressed in cm^2; for the catalytic activity, 1 µg of each venom was incubated with 200 µM of FRET substrate Abz - AGLA – EDDnp and the SVMP activity was assessed using a fluorimeter in kinetic mode, at λ_{EM} 420 and λ_{EX} 320 nm; the results were expressed as RFU/min/µg of venom. (**B**) **SVSP and coagulant activities:** for catalytic activity, 5 µg of the venoms were incubated with 500 µM of chromogenic synthetic substrate (L-BAPNA) for 40 min, the absorbance was monitored spectrophotometrically at 405 nm and the results were expressed in Abs/min/mg of venom. The coagulant activity was assessed in citrated human plasma, incubating 100 µL of plasma with venom samples diluted for different concentrations in 25 µL of PBS. The clotting time was measured in a coagulometer and the results were express in µg of venom/mL of assay. **(C) PLA_2 and myotoxic activities:** For the PLA_2 activity assay, 5 µg of each venom pool were incubated with 320 µM of chromogenic synthetic substrate (NOBA), the absorbance was monitored at 405 nm and the results were expressed in Abs/min/mg of venom. The myotoxic activity was assessed in the serum of mice, after the injection (gastrocnemius muscle) of 100

µg of venom pool dissolved in 50 µL of PBS. After 3 h, the mice were bled, via ophthalmic plexus, and creatine-kinase activity was measured using a commercial kit CK-UV (Bioclin). **(D) Lethal activity:** determined by median lethal dose (LD_{50}) estimated with Probit analysis. Five serial dilutions of each venom were carried out in 500 µL of PBS, injected intraperitoneally in mice and survival time of each animal was recorded for 48 h. All results listed above represent the mean +/- SD of three independent experiments done in triplicate. The tests involving animals represent three independent experiments with n=5. Differences were considered significant if $p \leq 0.05$. (*) significant differences in Floodplain venom; (#) significant differences in Pasture venom.

Figure 8 - Reactivity with *Bothrops* antivenom and neutralization of the coagulant, hemorrhagic, and lethal activities. (**A)** - For antibody titration, plates coated with whole venoms were incubated with growing dilutions of SAB followed by incubation with anti-horse IgG labeled with peroxidase (1:2,000). The reactions were developed using ortho-phenylenediamine/H_2O_2 as enzyme substrate and the products detected at 490 nm. Experiments were carried out in triplicate in three independent experiments, and results were expressed as mean ± SD of the nine absorbance values. (**B)** - For neutralization of coagulant activity, a constant amount of venom (two minimum coagulant doses) was incubated with several dilutions of antivenom, the mixture added to 100 µL of bovine plasma and clotting times recorded using a coagulometer. Neutralization was expressed as effective dose (ED), defined as the antivenom to venom ratio at which clotting time was increased three-fold when compared to clotting time of plasma incubated with venom alone. **C** - For neutralization of hemorrhagic activity, doses of 10 µg were injected in the dorsum skin of mice pre-incubated with SAB at the following ratios: 1 mL SAB/5 mg venom (1:5), 2 mL SAB/5 mg venom (2:5) and 4 mL SAB/5 mg venom (4:5) in a total volume of 50 µL and injected intradermally in the dorsum of groups of 5 mice. The results show % neutralization considering as 100% activity the values obtained after injection of venom incubated with PBS only. **(D)** For the lethality neutralization assays, 5 LD_{50} of each venom (forest - 171 µg; pasture - 123 µg; degraded - 196 µg; floodplain - 81 µg) were incubated with SAB at the following ratios: 1 mL SAB/5 mg venom (1:5), 2 mL SAB/5 mg venom (2:5), and 4 mL SAB/5 mg venom (4:5) in a total volume of 500 µL, and injected intraperitoneally in groups of 5 mice. The lethality was recorded over a period of 48 h. The results were expressed as % neutralization considering the number of alive/total mice after this period.

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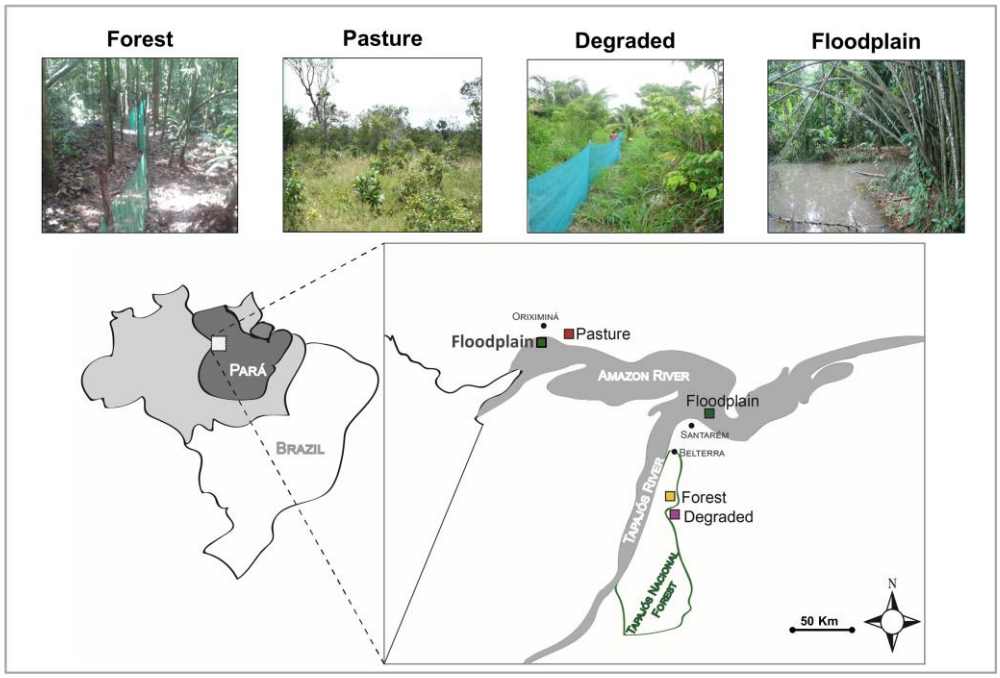
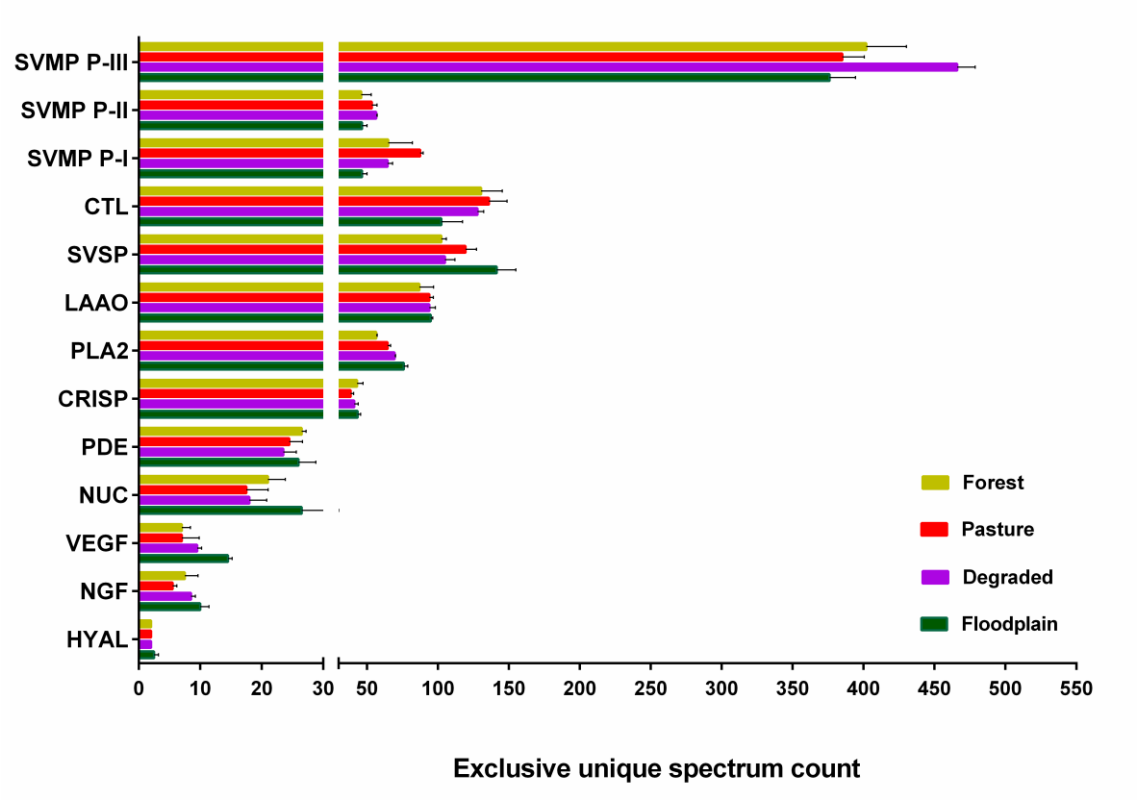


Figure 1



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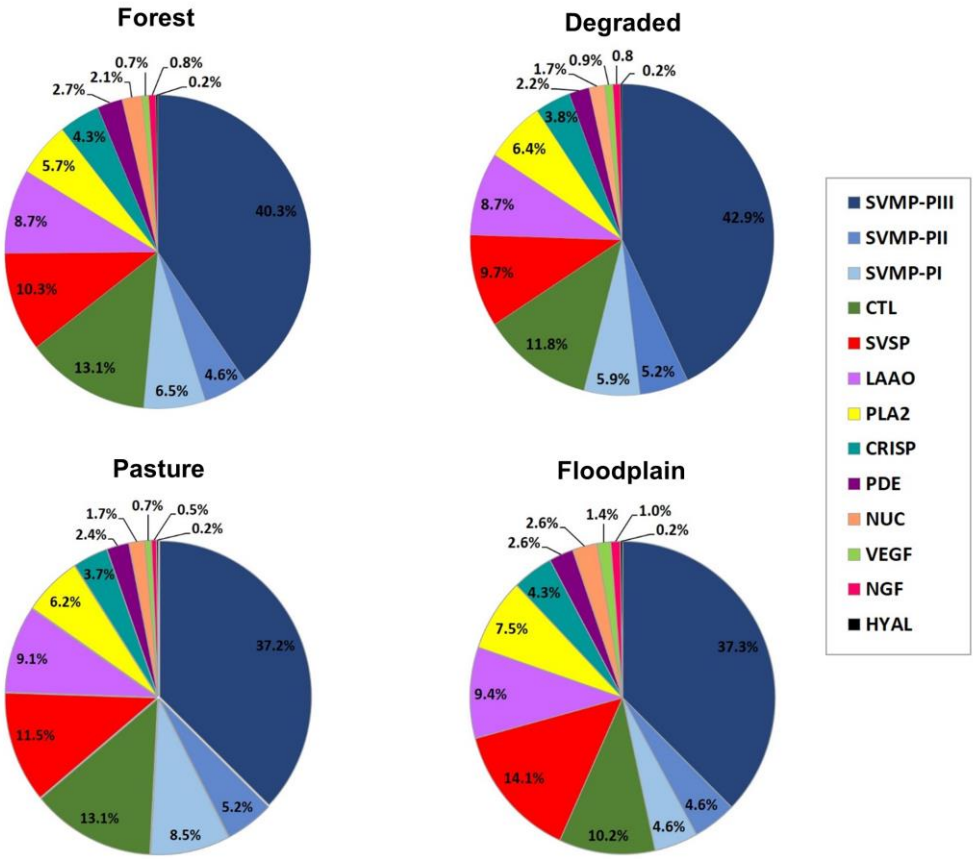

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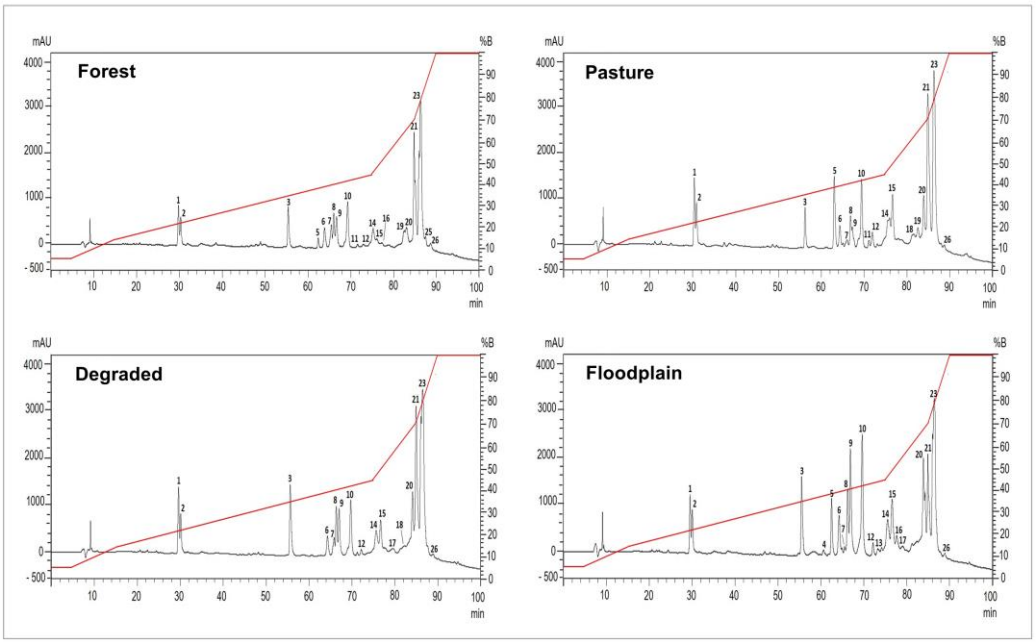

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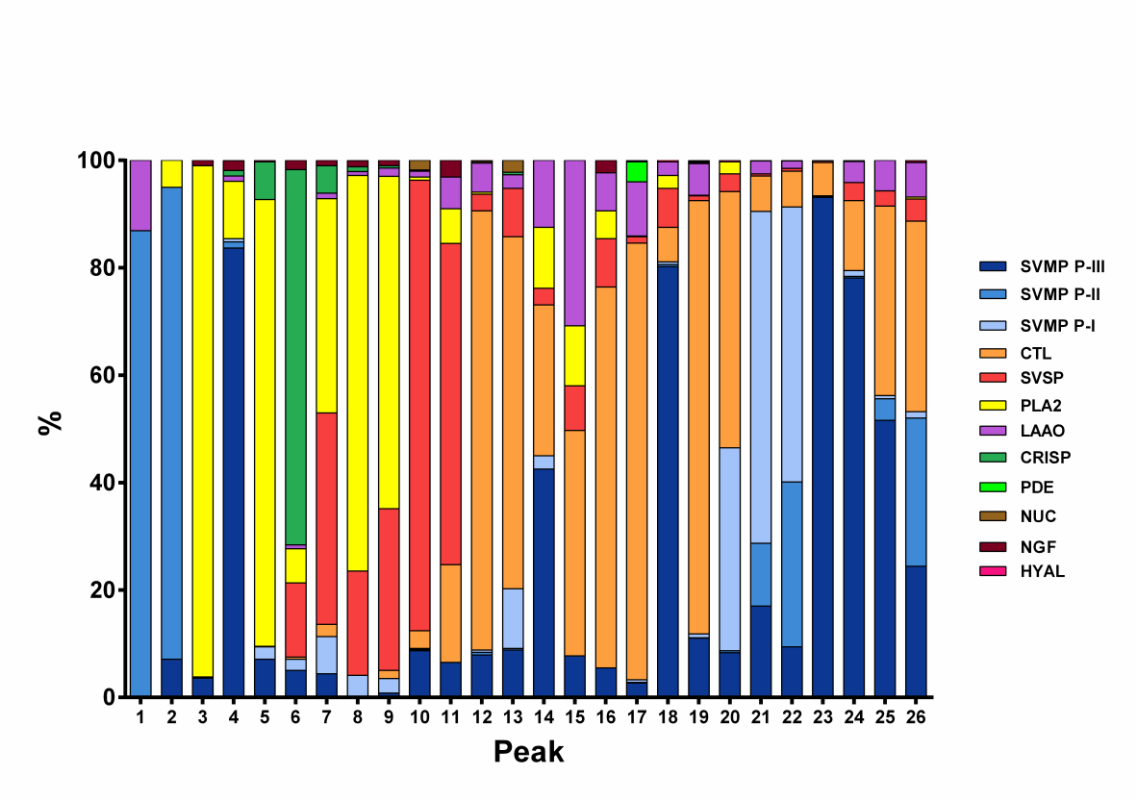

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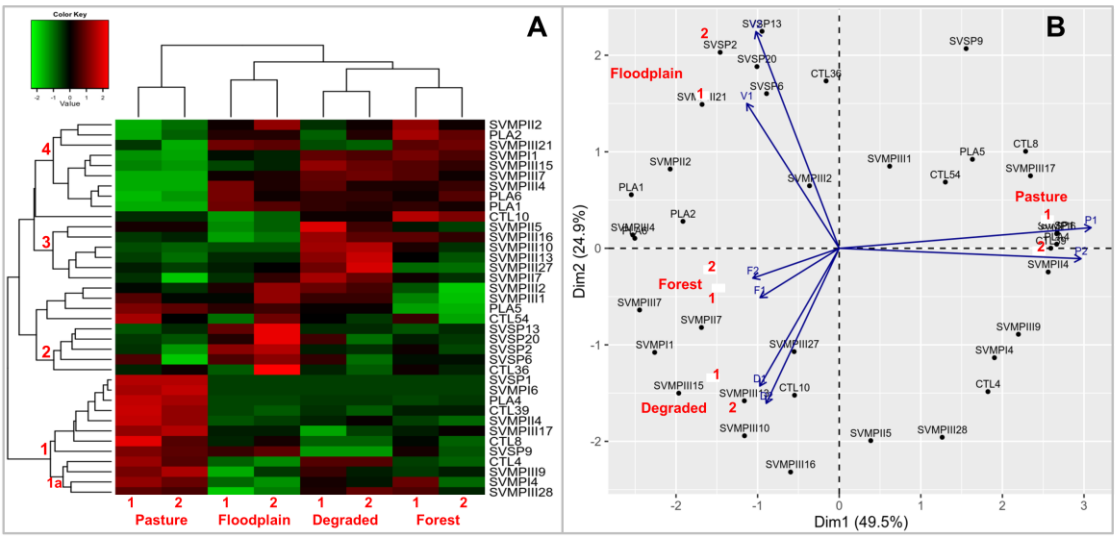

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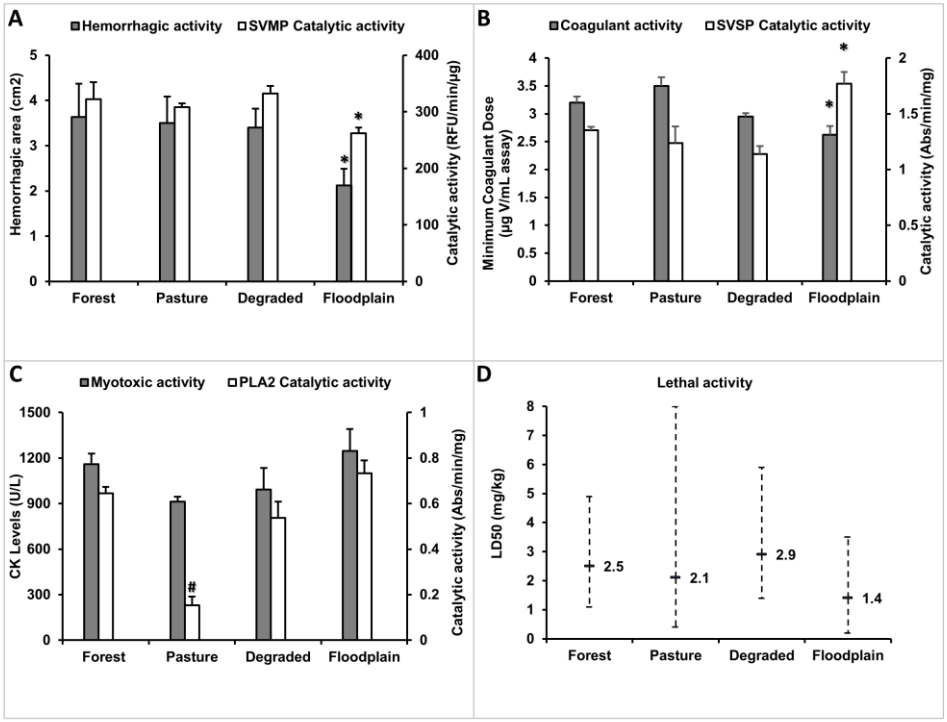

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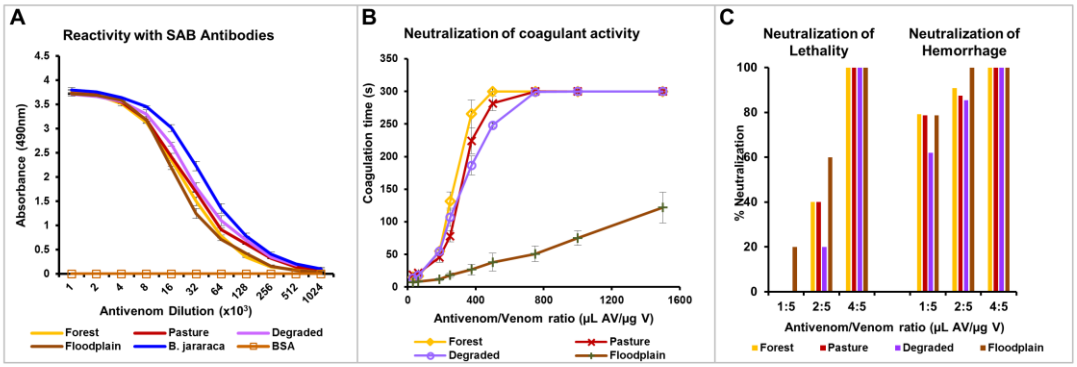

Figure 8

Conflict of interest

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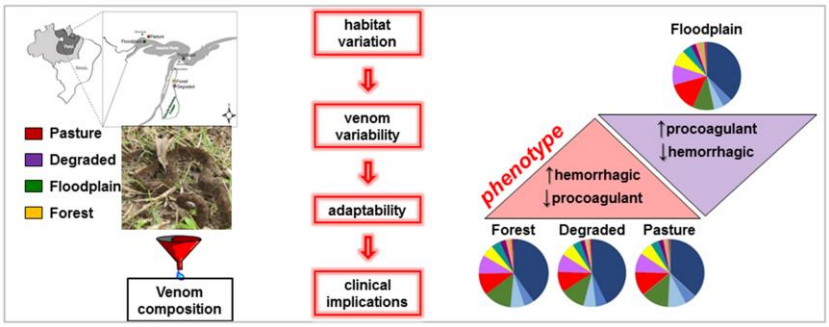

Graphical abstract