

## Sex-based individual variation of snake venom proteome among eighteen *Bothrops jararaca* siblings

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### Abstract

Variation of venom proteome is relevant to basic research, to management of envenoming, and to studies on the evolution of poisonous snakes. In this study, we explored the venom proteomes of eighteen *Bothrops jararaca* specimens of a single litter born and raised in laboratory. Using electrophoretic techniques and various protocols for measuring the proteolytic activities of these venoms we have detected individual variability and highlighted sex-specific proteomic similarities and differences among sibling snakes. SDS-polyacrylamide gel electrophoresis under non-reducing conditions showed protein bands of ~100 kDa specific of male venoms. 2D-electrophoresis showed regions with varying spot complexity between pooled female and male venoms as well as spots that were gender specific. Gelatin zymography showed that female venoms contained proteinases of ~25 kDa absent from male venoms. Female venoms were more active than male venoms in degrading fibrinogen whereas on fibrin no significant differences were detected. Among various chromogenic peptide substrates tested, male venoms showed higher amidolytic activity than female venoms on D-Val-Leu-Lys-pNA and D-Phe-Pip-Arg-pNA. Taken together, these results show sex-based differences in the venom proteome of sibling snakes of a single litter raised under controlled conditions which seem to be genetically inherited and imposed by evolutionary forces.

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**Keywords:** Snake venom proteome; Sex-based variation; Metalloproteinase; Serine proteinase

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### 1. Introduction

Snake venoms are complex mixtures of hundreds of proteins and peptides that function to immobilize or to kill the prey as well as to assist in the digestion of the prey. The toxic effects exerted by snake venoms are complex because their different components have distinct actions and may also act in concert with other venom toxins in a synergistic fashion to enhance their activities. Venom variability has long been appreciated by investigators and it is a well

documented phenomenon. It occurs at several levels including interfamily, intergenus, interspecies, intersubspecies and intraspecies variation (Chippaux et al., 1991). Moreover, venom composition may be influenced by the geographical origin and habitat of the snake (Chippaux et al., 1991). Intraspecific venom variation occurs between individual specimens, and also in individual specimens, due to seasonal variation, diet, habitat, age, and sexual dimorphism (Chippaux et al., 1991).

Venom variation is relevant to both basic venom research as well as to the management of snake envenomation. In the former case, considerations of the variation in venom composition are relevant for the choice of snake donors that are used for venom production and in the latter

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case the selection of snake donors for antivenom production and subsequently the selection of anti-sera for therapeutic use (Chippaux et al., 1991; Theakston et al., 2003). Moreover, venom proteome variation is also an important issue of studies on the evolutionary aspects of venomous snakes.

At the level of intraspecific variation the individual contribution to the venom composition is important but the effects contributed by environmental conditions, age and feeding habits also influence the proteome picture exhibited by each specimen. Studies of captive-bred snakes indicated that the intraspecific variation in venom is genetically inherited rather than environmentally induced (Daltry et al., 1996a,b). However, according to Sasa (1999), microevolutionary forces other than selection for local prey should also be considered as a source of the high variation of venom components among populations.

Previous studies on the venoms from 21 individuals of *Echis carinatus* from a climatically, geographically and nutritionally homogeneous habitat showed both qualitative and quantitative variability among the venom electrophoretic profiles while enzymatic activity showed similarity in some cases (phosphodiesterase, 5'-nucleotidase and caseinase) and individual variation in phospholipase A2 and L-amino acid oxidase activities (Taborska, 1971). The venom analysis from over 100 specimens of *Calloselasma rhodostoma* using isoelectric focusing revealed significant geographic variation which was further correlated with variation in the biological activities of the venom (Daltry et al., 1996a). Venoms of 30 young from a single litter of *Bitis gabonica* were analyzed by electrophoresis and showed individual variation however no sex-based variation was noticed (Chippaux et al., 1982).

The pit viper *Bothrops jararaca* is of major clinical importance as a leading cause of snake bite in Brazil. Although *B. jararaca* venom has been the focus of extensive studies, the degree to which the venom proteome varies between individuals is poorly understood. Early studies by Schenberg and colleagues showed variability in the enzymatic content of *B. jararaca* individuals with reduction due to milking frequency (Schenberg et al., 1970). Recently, a multifaceted analysis of the *B. jararaca* venom proteome was conducted by 2D-electrophoresis and mass spectrometry analysis showing the subpopulations of toxins present in this venom (Serrano et al., 2005). At the individual level, the variation of *B. jararaca* venom composition was also evaluated by comparing the proteomes of six specimens by 2D-electrophoresis (Fox et al., 2002). The 2D-gel images showed notable differences between the proteomes of these individual snakes collected from geographically distinct regions in São Paulo State, Brazil (Fox et al., 2002).

The objective of this investigation was to analyse venom samples from eighteen *B. jararaca* sibling snakes, which were born in a single litter at the Laboratory of Herpetology (Instituto Butantan) and kept under controlled laboratory

conditions, in order to evaluate sex-based differences in venom composition. Using electrophoretic techniques and various protocols for measuring the proteolytic activities of the individual venoms we have highlighted sex-specific proteomic similarities and differences among sibling snakes.

## 2. Materials and methods

### 2.1. Venoms

Venom samples were from eighteen *B. jararaca* snakes that were born in a single litter from a snake that was captured pregnant from the wild in the area of Ibiuna (São Paulo State, Brazil) and kept in the Laboratory of Herpetology of Instituto Butantan (São Paulo, Brazil) under controlled conditions. The siblings (eleven female and seven male snakes) were raised in the same laboratory and venom for this study was milked when the snakes were 36 months old.

### 2.2. Protein determination

Protein concentrations were determined using the Bradford reagent (Sigma) and bovine serum albumin (Sigma) as a standard, according to manufacture's instructions.

### 2.3. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to Laemmli (1970) using 12% polyacrylamide gels and staining with Coomassie blue R-250.

### 2.4. 2D-polyacrylamide gel electrophoresis

Venom samples (350 µg) were dissolved in 450 µl DeStreak rehydration solution (GE Healthcare) containing 0.5% immobilized pH gradient (IPG) buffer (GE Healthcare). First dimension IEF was carried out in an Ettan IPGphor Isoelectric Focusing System (GE Healthcare) as described by the manufacturer. Precast IPG strips (24 cm, pH 3–10 nonlinear) were employed for the first dimension separation at 20 °C using a three-phase electrophoresis program: 100 V for 5 min, 3500 V for 7 h, and 4000 V for 17 h. Prior to running the second dimension, the IPG strips were placed in the rehydration tray and the proteins in the strip were reduced and alkylated by sequential incubation in the following solutions: 0.05 M Tris-HCl, pH 8.8, 2% SDS; 30% glycerol, 6 M urea, 0.002% bromophenol blue (equilibration buffer-EB), 100 mg/mL DTT in EB; and then a solution of 250 mg/mL iodoacetamide in EB. They were directly applied to 12% SDS-polyacrylamide gels for second-dimension electrophoresis. The gels were fixed and then stained with silver.

### 2.5. Amidolytic activity

Amidolytic activity was determined on Bz-Arg-pNA (Merck), Tos-Gly-Pro-Arg-pNA (Medor), D-Val-Leu-Lys-pNA (Chromogenix Kabivitrum), D-Arg-Gly-Arg-pNA (Chromogenix Kabivitrum) and D-Phe-Pip-Arg-pNA (Chromogenix Kabivitrum) at 37 °C and different time intervals (10–30 min) in a system containing 0.45 ml of 0.1 M Tris-HCl buffer, pH 8.0 (buffer A), 0.45 ml of substrate solution and 0.01 ml of venom diluted in buffer A. Substrate assay concentrations were: Bz-Arg-pNA, 1mM; Tos-Gly-Pro-Arg-pNA, 0.5 mM; D-Val-Leu-Lys-pNA, 0.25 mM; D-Arg-Gly-Arg-pNA, 0.25 mM; D-Phe-Pip-Arg-pNA, 0.25 mM. Reactions were stopped by adding 0.09 ml of 30% acetic acid and release of *p*-nitroaniline was monitored at 405 nm. Amidolytic activities were calculated using a molar absorbance of 10200 for *p*-nitroaniline. Specific amidolytic activity is expressed as micromole of substrate hydrolysed per minute per mg protein.

### 2.6. Coagulant activity

Citrated human plasma was used to measure coagulant activity as described by [Henriques et al. \(1960\)](#). Briefly, 0.025 ml venom solution in at least three different concentrations were mixed with 0.1 ml of citrated human plasma (0.38 g of sodium citrate/100 ml of blood) at 37 °C and the clotting time was measured. Specific activity was defined as the venom protein concentration (in µg), which caused clotting in 60 s.

### 2.7. Caseinolytic activity

*N,N*-dimethylated casein (Sigma) was used as a substrate in a system containing 0.4 ml buffer solution (0.1 M Tris-HCl buffer, pH 8.8, 0.01 M CaCl<sub>2</sub>), 0.1 ml venom solution and 0.5 ml 2% casein solution (solubilized in the same buffer), for 30 min, at 37 °C. The reaction was stopped by adding 1 ml 5% trichloroacetic acid, the mixture was centrifuged at 14,000 rpm for 15 min and absorbance at 280 nm was measured. One unit of activity was defined as the amount of venom yielding an increase in O.D. of 1.0 per min at 280 nm. Specific activity was expressed as units/mg protein.

### 2.8. Fibrinolytic activity

Hundred micrograms human fibrinogen (Kabi Diagnostica) was incubated with individual venoms (5 µg) in 0.05 M Tris-HCl buffer, pH 8.0, at 37 °C for 15 min. Reaction was stopped by adding SDS-electrophoresis sample buffer ([Laemmli, 1970](#)). The hydrolysis profile was followed by SDS-PAGE (12% SDS-polyacrylamide gel).

### 2.9. Fibrinolytic activity

The fibrin clot (100 µg) was prepared from human fibrinogen using human thrombin (Roche) following the procedure of [Willis and Tu \(1988\)](#). The individual venoms (4 µg) were added to the clot and incubated at 37 °C for 1 h. Reaction was stopped by adding SDS-electrophoresis sample buffer ([Laemmli, 1970](#)). The hydrolysis profile was followed by SDS-PAGE (12% SDS-polyacrylamide gel).

### 2.10. Gelatinolytic activity

Gelatinolytic activity was visualized by zymography. Gelatin (Bio-Bras) was co-polymerized (1 mg/ml) with 12% w/v acrylamide, 0.3% bisacrylamide, 0.1% SDS, 1% TEMED, 0.1% ammonium persulfate and 0.375 M Tris-HCl (pH 8.8) to make the gelatin/acrylamide gel. Samples of 25 µg of individual venoms were loaded to the gel and after electrophoresis, the gel was incubated for 30 min at room temperature on a rotary shaker in 0.05 M Tris-HCl, pH 7.4, containing 2.5% Triton X-100 to remove traces of SDS. The gel was washed with deionized water to remove excess Triton X-100 and then incubated in zymography incubation buffer (0.05 M Tris-HCl, pH 8.0, 0.15 M NaCl, 0.01 M CaCl<sub>2</sub>, 0.02% CHAPS) at 37 °C for 12 h. The gel was stained with Coomassie blue R-250 and destained. Gelatin digestion was identified as clear zones of lysis against a blue background.

## 3. Results and discussion

### 3.1. Comparison of individual venom samples by SDS-polyacrylamide gel electrophoresis

In order to get a thorough understanding of a venom proteome, both reducing and non-reducing SDS-PAGE are best used as complementary approaches to understand the nature of venom complexity. The analysis of individual venom samples from the single litter of *B. jararaca* snakes by SDS-PAGE under reducing conditions showed variation of protein bands among the eighteen siblings irrespective of the gender of the animal ([Fig. 1\(A\)](#)). Female venoms showed a more homogenous electrophoretic profile than male venoms. Four main groups of components with molecular masses of ~50, ~20, ~14 and ~10 kDa were detected in both female and male venoms. However, components of ~50 kDa were weakly stained in male venoms indicating a lower abundance of these toxins in these venoms. On the other hand, some male venoms (3, 8, 13, 15 m) showed bands of ~45–48 kDa which were nearly absent in other venoms (12, 23 and 27 m). When these venoms were pooled according to the gender the main difference observed in the electrophoretic profile was the presence of bands of ~70 and 35–40 kDa more intense in

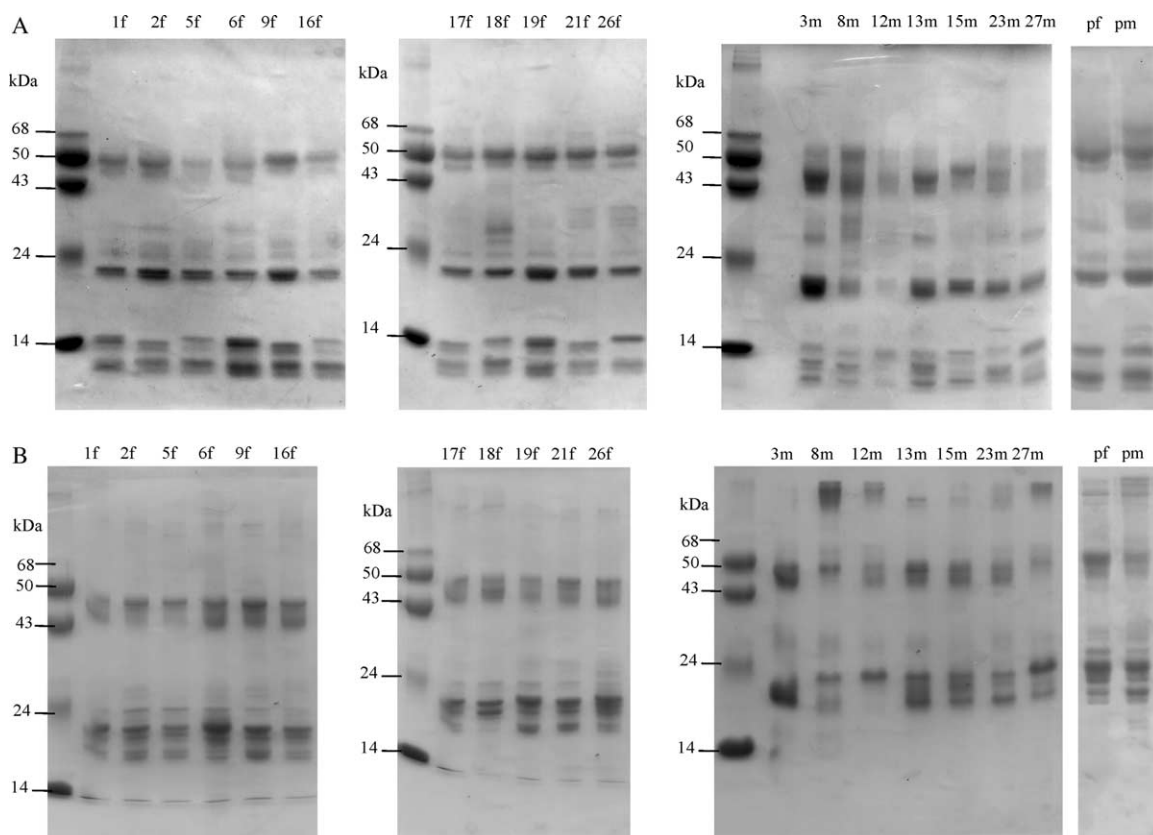


Fig. 1. SDS-PAGE of individual *B. jararaca* venom samples from sibling snakes (f, female; m, male; pf, pool of female venoms; pm, pool of male venoms). Venom samples (25  $\mu$ g) were submitted to electrophoresis under reducing (A) or non-reducing (B) conditions. Gels were stained with Coomassie blue. Numbers at the left indicate the mobility of molecular mass markers.

the pool of male venoms than in female venoms (Fig. 1(A)). Individual variability was also observed when the venom samples were run under non-reducing conditions (Fig. 1(B)). From the comparison of the reducing and non-reducing gels there is loss of apparent complexity with the non-reducing gels. Components of  $\sim$ 14 kDa and below were absent both in male and female non-reduced venom samples indicating that these bands stem from proteins in oligomeric state or they originate from the processing of venom protein precursors which are not clearly visualized in non-reducing gels. Interestingly, five male venoms showed bands of  $\sim$ 100 kDa with individual varying intensity which were absent in female venoms indicating a clear sex-based difference between the 18 sibling snakes. These protein bands may contain non-processed precursors of metalloproteinases or dimeric metalloproteinases of the P-III class (Fox and Serrano, 2005). Moreover, the analysis of pooled female and male venoms under non-reducing conditions confirmed the presence of a higher amount of high molecular mass components in the male venoms (Fig. 1(B)).

### 3.2. Comparison of pooled male and female venoms by 2D-PAGE

For analysis by 2D-PAGE the venom samples from the single snake litter (11 female specimens, 7 male specimens) were pooled according to the snake gender. The 2D-PAGE images of pooled male and female venoms are shown in Fig. 2. Venoms from both genders had protein dispersed across the pI and molecular mass range of the gels. The general profiles of these venoms were similar to the profile reported in previous works (Fox et al., 2002; Serrano et al., 2005) using *B. jararaca* pooled venom from Instituto Butantan (Sao Paulo, Brazil) which is prepared with venom samples from  $\sim$ 200 snakes. The visual analysis of the gels indicated that there are differences between the observed 2D-PAGE profiles of male and female pooled venoms. A close comparative analysis of the gels showed that there are regions within the gels (see circled outlines labelled I–V) that are in fact quite similar, as would be expected with venoms from the same species. However,

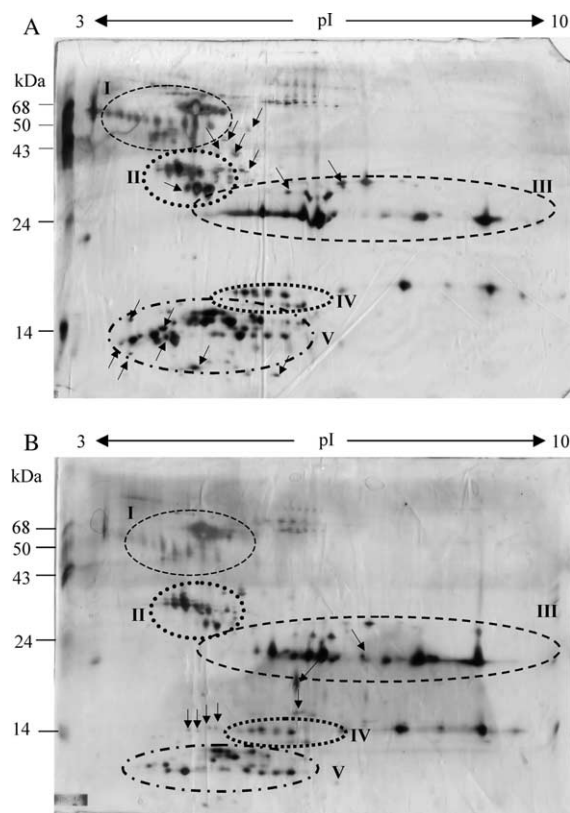


Fig. 2. 2D-PAGE of *B. jararaca* pooled female and male sibling venoms. Pooled venom (350  $\mu$ g) from eleven female snakes (A) or seven male snakes (B) was applied to 3–10 IPG strips (24 cm) followed by electrophoresis on 12% SDS-polyacrylamide gels. Gels were stained with silver. Circled outlines indicate gel regions that were chosen for comparison between the venoms. Arrows indicate spots seen only in female (A) or male (B) pooled venom. Numbers at the left indicate the mobility of molecular mass markers.

within those regions of the gels one could detect differences both in terms of the number of protein spots in the region as well as their intensities (Fig. 2). With exception of region III, which showed a similar profile on both 2D-gels, the comparison of the other regions showed a higher complexity in terms of overall number of protein spots as well as their intensities on the female venom 2D gel (Fig. 2). Moreover, within those regions as well as in other gel regions mainly in the acidic part of the gel it was possible to observe spots that occurred either only in male or in female venoms indicating their gender-specificity in these sibling snakes. In a previous work, by isoelectric focusing Daltry and colleagues (1996a) noticed an intense band in female venoms that was absent from male venoms among 106 *C. rhodostoma* specimens from different locations. Our 2D-electrophoresis analysis revealed hitherto unrecorded sex-based differences among specimens of a single snake litter.

### 3.3. Comparison of proteolytic activities of individual venoms

The main feature of *B. jararaca* venom is its strong proteolytic activity. It contains several proteolytic enzymes that belong to either the family S1A of serine proteinases (Serrano and Maroun, 2005) or to the Reprolysin subfamily of the M12 family of metalloproteinases (Fox and Serrano, 2005). These enzymes are the main players of the envenomation by this snake. Considering the individual proteome variation among the venoms of this litter as well as the sex-based differences observed by both SDS-PAGE and 2D-PAGE we decided to compare the individual venom samples by measuring their proteolytic activities on protein and peptide substrates. At first we determined the plasma coagulant activity of individual venoms, in order to verify differences in the content of serine proteinases with the ability of converting fibrinogen into fibrin previously described in *B. jararaca* venom (Nishida et al., 1994; Serrano et al., 1998, 2000). However, no differences in potency between male and female venoms were detected by measuring the coagulant activity on plasma. The individual venom specific activities on plasma ranged between 0.96 and 1.69 U/mg for the female venoms and between 0.93 and 1.66 U/mg for the male venoms (not shown).

Fibrinogen can be cleaved by snake venom serine proteinases to yield fibrin or it can be degraded by metalloproteinases (Swenson and Markland, 2005). The incubation of fibrinogen with the individual snake venom samples showed variation in the hydrolysis profile among the venoms and in most cases the hydrolysis products were of small size and were not visualized on the gel (Fig. 3(A)). Ten female venoms completely degraded alpha chain while beta and gamma chains were only partially degraded (Fig. 3(A)). In general, male venoms were less active on fibrinogen than female venoms. Differently from the female venoms, four male venoms only partially hydrolysed alpha chain and one of them (venom 3 m) generated three polypeptide products that were detected by SDS-PAGE. Interestingly, despite the differences observed among male and female venoms on the hydrolysis of fibrinogen, the incubation of the individual venoms with fibrin showed similar degradation profiles with the alpha chain being extensively degraded by all venoms (Fig. 3(B)). These data indicate a similar content of enzymes that are active on fibrin in the sibling venoms irrespective of the specimen gender and contrast with the differences in the content of proteinases active on fibrinogen and casein noticed in this work between female and male venoms.

The most remarkable difference between male and female venoms in terms of proteolytic enzymes was detected by gelatin zymography. For this purpose, venom samples were prepared for the SDS-PAGE in the absence of a reducing agent to retain the enzymatic activity of the gelatinolytic enzymes. In Fig. 3(C) is seen the gelatin hydrolysis profile by the individual venoms. In general,



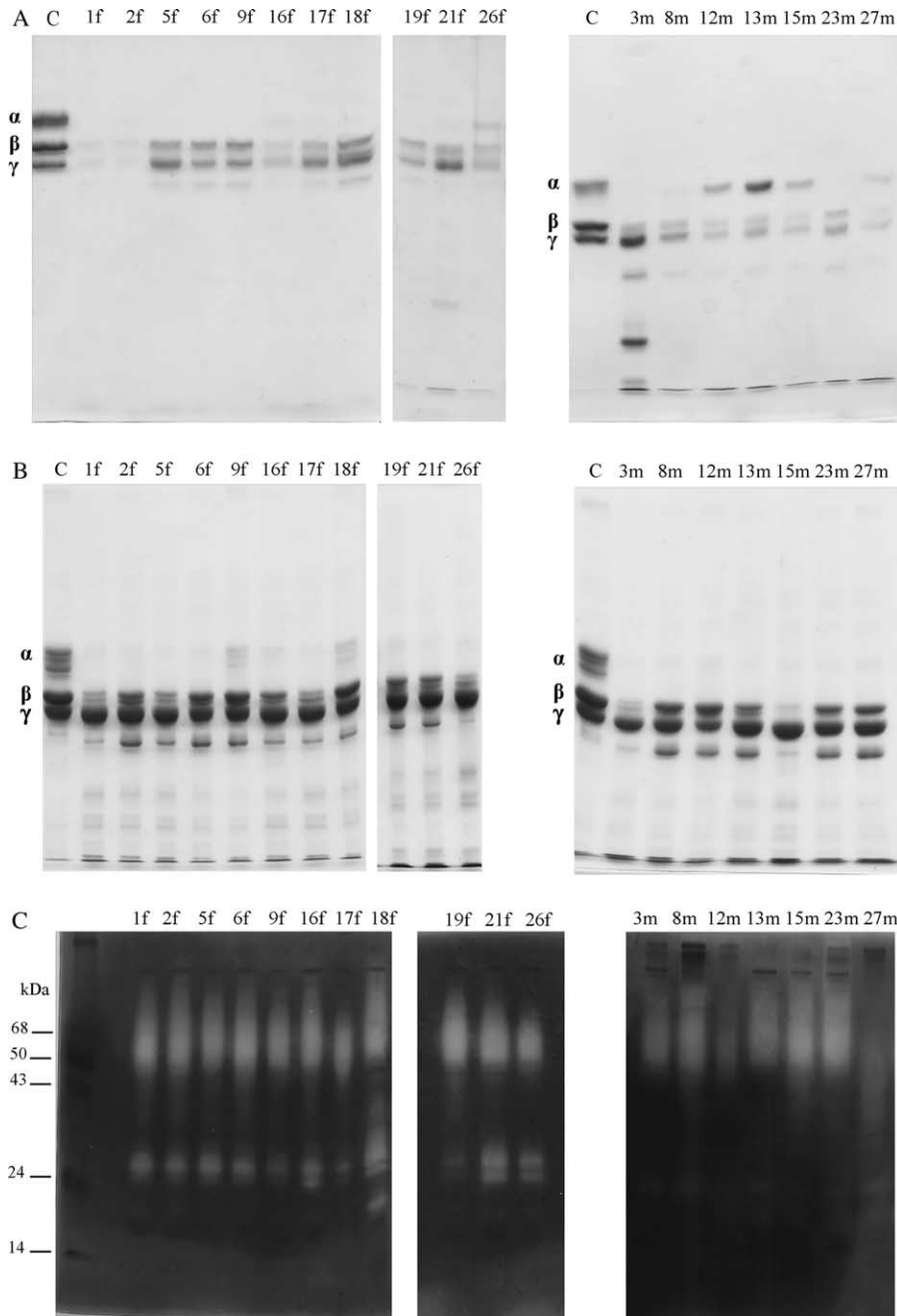


Fig. 3. Fibrinogenolytic, fibrinolytic and gelatinolytic activities of individual *B. jararaca* venoms (f, female; m, male). Reducing SDS-PAGE of fibrinogen (A) and fibrin (B) incubated with individual venoms; lane C, controls of fibrinogen and fibrin. (C) Gelatin zymography. Numbers at the left indicate the mobility of molecular mass markers.

there appears to be a similar gelatinolytic profile by the female venoms. Likewise, male venoms displayed comparable activity on gelatin. The regions with the most clearing of the gel by all venoms are associated with the P-III class of snake venom metalloproteinases showing molecular masses of ~50 kDa and above as would be expected for

the gelatinolytic metalloproteinases (Fox and Serrano, 2005). However, additionally to these clearing regions the female venoms showed gelatinolytic activity at ~25 kDa, which was not displayed by the male venoms. These data indicate the presence in the female venoms of metalloproteinases of the P-I class active on gelatin that are virtually

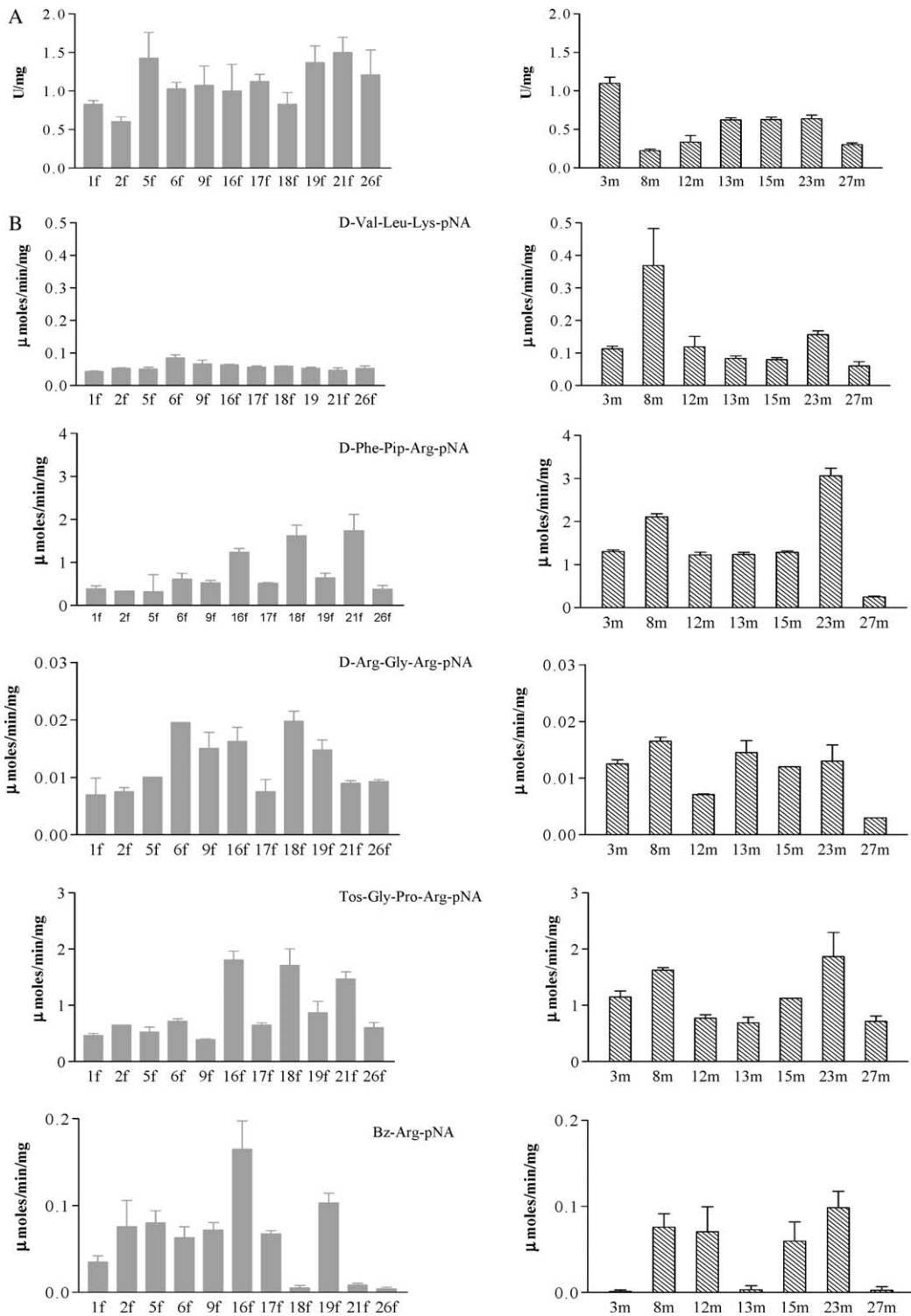


Fig. 4. Caseinolytic (A) and amidolytic (B) activities of individual *B. jararaca* venoms (gray bars, f, female; hatched bars, m, male) on substrates D-Val-Leu-Lys-pNA, D-Phe-Pip-Arg-pNA, D-Arg-Gly-Arg-pNA, Tos-Gly-Pro-Arg-pNA and Bz-Arg-pNA. Assays were performed as described under Section 2.

absent in the male venoms of this litter. Interestingly, a close inspection of the zymogram indicated the presence of bands significantly stained with Coomassie blue at ~100 kDa on the male venom lanes that resemble the bands seen in the SDS-PAGE of male venoms under non-reducing conditions (Figs. 1(B) and 3(C)). These data confirmed the presence of components of high molecular mass in the male venoms, which are not active on gelatin.

We also determined the proteolytic activity of the individual venom samples on casein, a broadly used substrate that can be degraded by both serine proteinases and metalloproteinases from snake venoms. Besides individual variation among the samples, the specific activity on casein by the sibling venoms showed a higher activity by the female venoms indicating the presence of more active caseinolytic enzymes in these venoms (Fig. 4(A)).

The determination of the amidolytic activity on peptide *p*-nitroanilides containing Arg or Lys at position P1 by the individual venoms was performed in order to detect differences in the serine proteinase proteome of the sibling venoms. Individual variation of the amidolytic activity was detected on both female and male venoms (Fig. 4(B)). Among five substrates tested, D-Val-Leu-Lys-pNA and D-Phe-Pip-Arg-pNA, commonly used for measuring the activity of plasmin and thrombin, respectively, were cleaved with higher specific activity by male venoms whereas on peptides D-Arg-Gly-Arg-pNA, Tos-Gly-Pro-Arg-pNA, and Bz-Arg-pNA no sex-based difference of amidolytic activity was detected (Fig. 4(B)). Taken together, these data indicate that the hydrolytic activity towards these peptide *p*-nitroanilides is strongly influenced by the peptide moiety adjacent to the scissile bond and that the content and specificity of the serine proteinases varies between male and female venoms of this litter.

The proteome variability among eighteen sibling venoms of a single snake litter showed here by various protocols cannot be attributed to variation of environmental conditions, age or diet of the specimens analysed since these were born and raised under strictly homogeneous laboratory conditions. Instead, the diversity observed in the venom of these siblings seems to be genetically inherited and imposed by evolutionary forces. One important aspect of the intraspecific variability of snake venoms is the variability of specific venom components, another well documented phenomenon. Metalloproteinases, serine proteinases and phospholipases A2 are among the most abundant enzymes found in snake venoms (Nakashima et al., 1993; Bjarnason and Fox, 1995; Fox and Serrano, 2005; Serrano and Maroun, 2005; Kini, 2005). Diverse isoforms of these snake venom enzyme families have been identified which vary in their biophysical and biochemical properties. These enzymes have diversified amino acid sequences and display a variety of physiological activities. Comparisons of cDNA sequences encoding serine proteinases originated from mRNA isolated from single specimens of *Trimeresurus flavoviridis*, *T. gramineus* (Deshimaru et al., 1996) and *B. jararaca* (Saguchi et al., 2005) and a comparison of

genomic sequences for phospholipases A2 from *T. flavoviridis* (Nakashima et al., 1993) supported the hypothesis that accelerated evolution is universal in multiple isozyme families isolated from snake venom glands. Moreover, the analysis of toxin clusters encoded by a cDNA library of the venom glands of *B. insularis* showed that the metalloproteinases were the far most diversified and expressed group of toxins in this venom gland (Junqueira-de-Azevedo and Ho, 2002). In another example, Zupunski and colleagues (2003) analyzed various nucleotide sequences encoding snake venom serine proteinase inhibitors of the Kunitz/BPTI family and concluded that positive Darwinian selection was operating on the highly conserved BPTI fold. Therefore, the evolutionary effects on various alleles coding for isoforms of the same component is a relevant aspect of venom diversity.

Taken together, our results highlight the variability of the venom proteome of eighteen sibling specimens and show sex-based differences between venoms of a single snake litter raised under controlled laboratory conditions.

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