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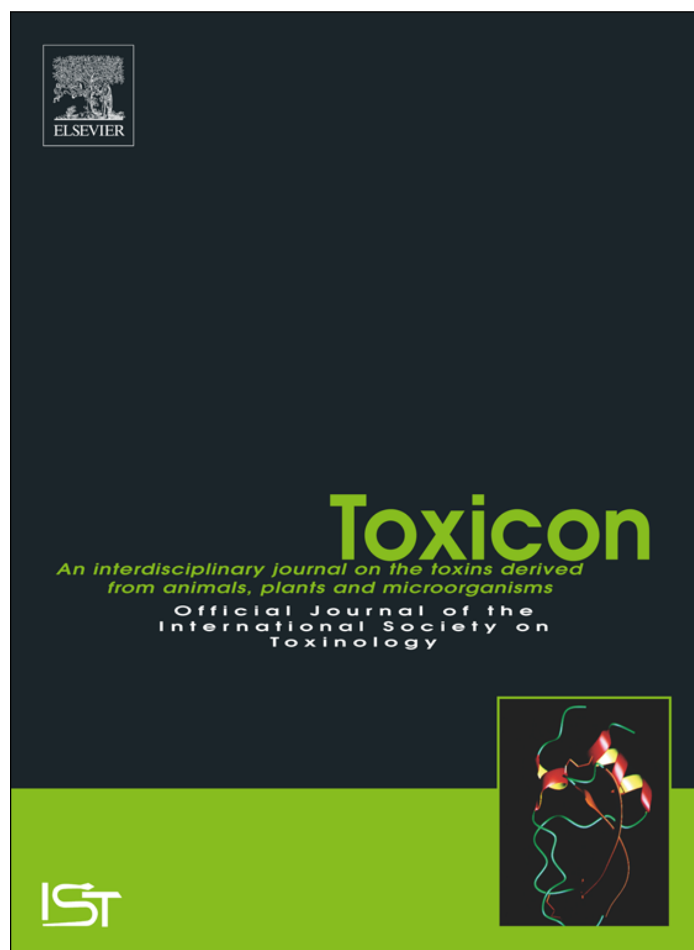

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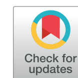
Toxicon 148 (2018) 202–212

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Assessment of the potential toxicological hazard of the Green Parrot Snake (*Leptophis ahaetulla marginatus*): Characterization of its venom and venom-delivery system



Matías N. Sánchez [a, b], Gladys P. Teibler [c], Carlos A. López [b], Stephen P. Mackessy [d], María E. Peichoto [a, b, *]

[a] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ministerio de Ciencia Tecnología e Innovación Productiva, Argentina
[b] Instituto Nacional de Medicina Tropical (INMeT), Ministerio de Salud de la Nación, Neuquén y Jujuy s/n, 3370, Puerto Iguazú, Argentina
[c] Facultad de Ciencias Veterinarias (FCV), Universidad Nacional del Nordeste (UNNE). Sargento Cabral 2139, 3400, Corrientes, Argentina
[d] School of Biological Sciences, University of Northern Colorado, 501 20th St., CB 92, Greeley, CO, 80639-0017, USA

ARTICLE INFO

Article history:
Received 10 March 2018
Received in revised form
11 April 2018
Accepted 25 April 2018
Available online 27 April 2018

Keywords:
Colubrid
Enzymes
Gland histology
Maxilla morphology
Rear-fanged snake
Toxins

ABSTRACT

Snakes are the major group of venomous vertebrates, and the rear-fanged snakes represent the vast majority of species and occur worldwide; however, relatively few studies have characterized their venoms and evaluated their potential hazards for humans. Herein we explore the protein composition and properties of the venom of the rear-fanged Green Parrot Snake, *Leptophis ahaetulla marginatus*, the most common snake found in the Iguazu National Park (Argentina), as well as the main features of its venom delivery system. This species has venom reminiscent of elapid venoms, composed mainly of components such as 3FTxs, CRiSPs and AChE, but it shows low toxicity toward mammals ($LD_{50} > 20$ μg/g mouse). The histology of its Duvernoy's venom gland is similar to that of other colubrids, with serous secretory cells arranged in densely packed secretory tubules. The posterior end of its maxilla exhibits 1–3 blade-shaped and slightly recurved fangs but without grooves. This study provides an initial analysis of the biological role of venom in *Leptophis*, with implications for potential symptoms that might be anticipated from bites by this species.

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1. Introduction

Many advanced snakes (Caenophidia) inject venom to subdue their prey using a sophisticated venom-delivery system that shows considerable variation between taxa. Snake venoms are a trophic adaptation and constitute an evolutionary shift that allowed the transition from constriction to envenomation, i.e., from mechanical to chemical subjugation (Calvete et al., 2009; Mackessy and Saviola, 2016; Savitzky, 1980). Specialized venom glands are closely associated with modified teeth – the fangs – adapted for venom conduction (Fry et al., 2006; Kardong, 1982; Vonk et al., 2008). According to Weinstein (2016), snakes can be divided into two large groups, based on venom delivery systems: front-fanged colubroid snake, including viperids, elapids, and atractaspine lamprophiids, and non-front-fanged colubroid snakes (here termed rear-fanged snakes), including "colubrids", a loosely related collection of families/subfamilies with enlarged and modified rear maxillary teeth (opisthoglyphous) or unmodified maxillary teeth (aglyphous). These posterior maxillary teeth, quite variable in their morphology, may or may not be enlarged, grooved, bladed or enlarged/paired. The different structural and functional features of venom-delivery systems suggest that they represent different biological solutions to the common environmental challenges of prey capture or/and defense (Mackessy, 2010).

Rear-fanged snakes can inhabit a tremendous diversity of ecological habitats (terrestrial, arboreal and aquatic environments, tropical/temperate climates, low to high elevations, etc.), with a variety of diets and habits, and many produce venoms with very diverse biochemical properties (Junqueira-de-Azevedo et al., 2016; Mackessy, 2002). Many of these snakes possess a specialized oral gland known as the Duvernoy's venom gland (DVG; Saviola et al. (2014)), which is located in the temporal region and produces a

* Corresponding author. Instituto Nacional de Medicina Tropical (INMeT), Ministerio de Salud de la Nación, Neuquén y Jujuy s/n, 3370 Puerto Iguazú, Argentina.
E-mail addresses: mepeichoto@yahoo.com.ar, mepeichoto@conicet.gov.ar (M.E. Peichoto).

https://doi.org/10.1016/j.toxicon.2018.04.027
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venom containing a simple to complex mixture of biologically active compounds, some of which can exhibit prey-specific toxicity (Heyborne and Mackessy, 2013; Mackessy, 2010; Mackessy and Saviola, 2016; Oliveira et al., 2016; Zelanis et al., 2010). Although the DVG is homologous with the venom glands of families Viperidae, Elapidae and Atractaspididae, it is anatomically and functionally distinct (Arikan et al., 2005). Duvernoy's venom glands typically lack any significant storage capacity and usually have no direct striated muscle insertion to pressurize the fundus of the gland. As a consequence, these glands constitute a low-pressure venom injection system (Kardong and Lavín-Murcio, 1993).

As a trophic adaptation, the primary function of venom is to facilitate prey capture, and it is known that composition is closely associated with the snake's diet (Calvete et al., 2009; Daltry et al., 1996; Mackessy, 1988; Pawlak et al., 2009). Although the number of published works investigating the complexity of venoms from front-fanged snakes is quite high to date, the composition and biochemical properties of rear-fanged snake venoms remain relatively poor studied, mainly because of the exceedingly small quantities of raw material that is possible to obtain for investigation (Hill and Mackessy, 2000; Mackessy, 2002; Weldon and Mackessy, 2010). Despite this disadvantage, rear-fanged snake venoms constitute a largely untapped source of bioactive compounds and potential drug leads (King, 2011; Koh et al., 2006; Saviola et al., 2014). For this reason, and because there is no toxicological information about many genera of colubrid snakes, investigation of these venoms has accelerated somewhat in the last several years (Junqueira-de-Azevedo et al., 2016).

Leptophis ahaetulla marginatus, the Southern Green Parrot Snake, is a rear-fanged neotropical snake that occurs from southeastern Bolivia to western and southern Brazil, through Paraguay to northeastern Argentina and Uruguay (López et al., 2003). It is a diurnal and arboreal snake, characterized by its colorful appearance, and it is commonly seen in shrubbery and trees (Albuquerque et al., 2007). It feeds mainly on hylid frogs, lizards and young birds (Clegg, 2015), and it shows stereotypic aggressive behavior when threatened (Fig. 1). Although it is the most common snake found in the Iguazu National Park (López et al., 2016), an international tourist destination in Argentina that is visited by more than a million people per year, there has not been a study of the medical importance of this species.

Similarly, there is little information regarding the morphological-histochemical characteristics of the DVG of the genus *Leptophis* (Pough et al., 1978; Taub, 1967), and the composition and biological role(s) of its venom remains wholly unknown. Moreover, there is some confusion about the means by which snakes of this genus may envenomate their prey or potentially produce envenomation in humans, because some specialists consider them as “opisthoglyphous” (Barrio-Amorós and Ortiz, 2015; Ruíz, 2014) while others as “aglyphous” with enlarged teeth on the posterior maxillae (rear-fanged) but without any modification (Giraudo, 2014; Jackson and Fritts, 1995). In this study we conducted a morphological study of the maxillary teeth and a histological examination of the DVG of *L. a. marginatus* and present analyses of the protein composition and properties of its venom. Besides shedding light on the biological role of this secretion, this study aims to provide relevant information that will reveal any potential hazards for humans that are bitten by this species.

2. Materials and methods

2.1. Gland histology and histochemistry

Freshly dead specimens of *L. a. marginatus*, obtained from the serpentarium of the National Institute of Tropical Medicine (INMeT, Argentina) were used to analyze the histological characteristics of its DVG. Glands were removed, fixed in phosphate-buffered 10% formaldehyde (pH 7.2), and embedded in paraffin for classical histological techniques. Then, 5 μm sections were stained with hematoxylin-eosin (H&E) for general histology and with periodic acid-Schiff (PAS) stain for histochemical evaluation.

Fig. 1. A specimen of the Green Parrot Snake *Leptophis ahaetulla marginatus*. Note the stereotypic aggressive stance, characterized by a wide gape and tendency to strike when threatened. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.2. Structure of maxillae and fangs

Maxillae and the maxillary teeth were studied by scanning electron microscopy (SEM). In order to prepare the samples, soft tissues were removed manually, maxillae cleaned and then mounted on a stub with double-sided tape. Samples were then critical-point dried, coated with a thin layer of gold (Denton Vacuum Desk II) and examined using a JEOL 5800LV scanning electron microscope at an acceleration voltage of 15 kV.

2.3. Biochemical and toxicological analysis of *L. a. marginatus* DVG secretion

2.3.1. Extraction of DVG secretion

Adult specimens of *L. a. marginatus* were captured in the Iguazu National Park (Misiones, Argentina), in accordance with authorization from the National Park Administration (APN N° 335/13), and transported to the INMeT serpentarium. To collect venom, capillary tubes were placed over the enlarged rear fangs (Fig. 2A), and then lyophilized and stored at −20 °C (Ferlan et al., 1983). Saliva was collected from the oral cavity using a micropipette. When required, the venom was freshly dissolved in 50 mM Tris-HCl buffer (pH 7.4) and the protein content was determined by fluorometry using a Qubit 2.0 (Life Technologies, USA).

2.3.2. Gel electrophoresis

The protein profile of *L. a. marginatus* venom was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels (Laemmli, 1970) under reducing (with 2-mercaptoethanol) and non-reducing (without 2-mercaptoethanol) conditions, and then silver-stained (Blum et al., 1987). Each lane was loaded with 2.5 μg of protein.

2.3.3. Quantitative enzyme assays

Collagenolytic activity was determined as reported previously

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(Antunes et al., 2010). One unit of enzymatic activity toward collagen was defined as the amount of protein that causes an increase of 0.003 units of absorbance per min at 540 nm and the specific activity was expressed as U/mg protein. Activity toward casein was determined by a previously reported method (Quintana et al., 2017). The amount of protein that causes an increase of 0.005 units of absorbance per min at 450 nm was defined as one unit of enzymatic activity and the specific activity was expressed as U/mg protein. Acetylcholinesterase (AChE) activity was assayed according to Ellman et al. (1961) and activity was expressed as micromoles of product formed per minute per milligram of protein. Phospholipase A_2 (PLA_2) and hyaluronidase activities were evaluated as previously described by Antunes et al. (2010) and Sánchez et al. (2014), respectively. All enzyme assays were performed in triplicate. Negative controls were also performed in triplicate.

2.3.4. Fibrinogenolytic and fibrinolytic activity

Specific cleavage of fibrinogen by *L. a. marginatus* venom was determined by SDS-PAGE using 12% polyacrylamide gels, as described elsewhere (Peichoto et al., 2007). One hundred and fifty microliters of 2 mg/mL human fibrinogen dissolved in 50 mM Tris-HCl buffer (pH 7.4) were incubated with venom (50:1 mass ratio) at 37 °C. At various time intervals, aliquots were withdrawn from the digestion mixture, and then denatured and reduced by boiling for 10 min with denaturing solution (4% SDS, 20% glycerol and 20% 2-mercaptoethanol) for gel electrophoresis. For the fibrinolytic activity, fibrinogen aliquots (20 μL) were coagulated by adding bovine thrombin (5 U/mL, final concentration, Sigma) prior to the addition of venom (50:1 ratio). At different time intervals, 20 μL of denaturing solution were added to the reaction mixture and then processed as above.

2.3.5. Toxicity assays

Venom of *L. a. marginatus* was evaluated for lethality and myotoxicity using male CF-1 mice weighing 18–20 g. The animals were supplied by the Animal House of the School of Medicine of the University of Northeastern Argentina (UNNE). All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by the Ethical Committee for the Use of Animals of College of Veterinarian Sciences (UNNE) according to protocol Nº 070/17.

2.3.5.1. Lethal toxicity. Briefly, groups of three animals were inoculated intraperitoneally with doses from 0 to 20 μg/g in 100 μL of total volume and monitored with periodic observations for 24 h post injection (Weldon and Mackessy, 2010).

2.3.5.2. Myotoxicity. Animals were injected in the right gastrocnemius muscle with 50 μg protein dissolved in 100 μL of 50 mM Tris-HCl buffer (pH 7.4). Control mice were injected with only vehicle under identical conditions. After 1, 3, 6, 9 and 12 h following injection, animals were sacrificed and muscles were dissected. For histological analysis, small samples of muscle were collected and fixed in phosphate-buffered 10% formaldehyde (pH 7.2) for 24–48 h. Thereafter, the samples were dehydrated in an ethanol ascending series, cleared in xylol, and embedded in liquid paraffin. Sections of 5 μm thickness were cut, stained with H&E and examined with a Zeiss Primo Star microscope equipped with a digital camera AxioCam ERc 5s.

2.3.6. Cross-reactivity with elapid and viperid antivenoms used in Argentina

In order to determine the presence of components cross-reacting with anti-*Bothrops* and anti-*Micrurus* horse serums produced by the Instituto Nacional de Producción de Biológicos (INPB) from Argentina, a Western blot analysis was performed. Proteins separated by SDS-PAGE were transferred to 0.2 μm nitrocellulose

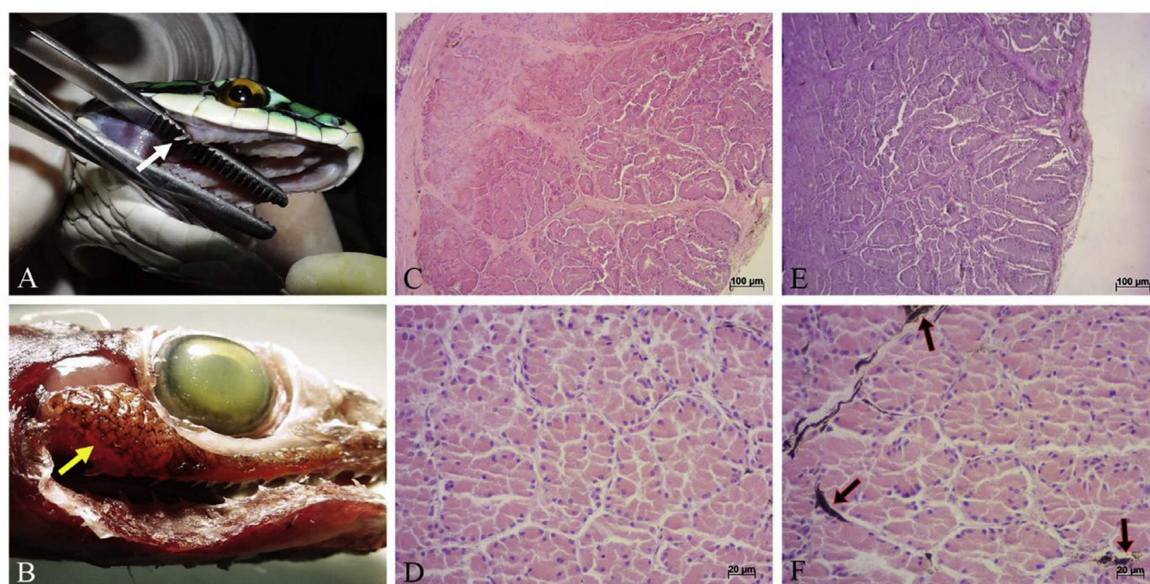

Fig. 2. Rear fang and Duvernoy's venom gland of *L. a. marginatus*. **A.** View of enlarged tooth at rear of maxilla (white arrow). **B.** Lateral view showing the position of the venom gland and the extensive pigmentation (yellow arrow). **C.** Cross-section of the venom gland (H&E staining) showing lobes defined by connective tissue septa. **D.** Micrograph showing details of the secretory tubules constituted by columnar cells with basal round nuclei. **E.** PAS histochemical staining of a venom gland section showing lobes and tubules consisting mainly of serous secretory cells. **F.** Micrograph showing the melanin deposits that may act as UV shielding of the gland (arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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membranes in a tank transfer system (Hoeffer mini VE, Amersham Biosciences) at 25 V for 1.5 h. Membranes were then blocked with 5% nonfat dry milk and incubated with bivalent or tetravalent anti-*Bothrops* or anti-*Micrurus* serum (kindly donated by the INPB) diluted 1:500, and subsequently with 1:10,000 peroxidase-conjugated anti-horse IgG (Sigma A9292). The reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma D5637) as reported elsewhere (Antunes et al., 2010).

2.3.7. Reverse-phase high performance liquid chromatography (RP-HPLC)

Venom was dissolved in buffer A (0.1% trifluoroacetic acid, TFA, in ultrapure water) and then injected onto an Innoval C18 column (5 μm, 100 Å, 4.6 × 250 mm, Agela Technologies), previously equilibrated in the same buffer. The sample was eluted with 0–100% increasing linear gradient of solvent B (0.1% TFA/90% acetonitrile) over 135 min at a flow rate of 1 mL/min using a YL9100 HPLC System (YL Instruments, Korea). The elution profile was monitored at 215 and 280 nm. One mL fractions were collected, dried and then electrophoresed on 12% polyacrylamide gels. Gels were stained with silver (Blum et al., 1987) or Coomassie G-250 (Candiano et al., 2004).

2.3.8. Mass spectrometry

Venoms were also analyzed using a Bruker Microflex LRF MALDI-TOF mass spectrometer operating in linear mode at the Proteomics and Metabolomics Facility at Colorado State University (Fort Collins, CO). Approximately 1.0 μg protein was dissolved in 1.0 μL 50% acetonitrile (ACN) containing 0.1% TFA, mixed with 1.0 μL sinapinic acid matrix (10 mg/mL, dissolved in the same solvent), and spotted onto target plates, dried and followed by a brief 5% isopropanol wash (2.5 μL) to remove salts. Spectra were acquired in the mass range of 2.0–40 kDa and toxins were tentatively identified based on mass similarity with known colubrid toxins (Modahl et al., 2016).

For protein identification of the main peaks in the RP chromatogram, target bands were excised from SDS-PAGE gels (15%) stained with Coomassie Brilliant Blue G 250. Protein digestion and mass spectrometry analysis were performed at the Proteomics Core Facility CEQUIBIEM, at the University of Buenos Aires/CONICET as follows: excised protein bands were sequentially washed with 50 mM ammonium bicarbonate (AB), 25 mM AB, 50% ACN, and 100% ACN; reduced and alkylated with 10 mM dithiothreitol (DTT) and 20 mM iodoacetamide (IAA), and in-gel digested with 100 ng trypsin (Promega V5111) in 25 mM AB overnight at 37 °C. Peptides were recovered by elution with 50% ACN/0.5% TFA, including brief sonication, and then further concentrated by speed-vacuum drying. Samples were resuspended in 15 μL of water containing 0.1% formic acid (FA). Digests were analyzed by nanoLC-MS/MS in a nanoHPLC EASY-nLC 1000 (Thermo Scientific) coupled to a QExactive Mass Spectrometer. A C18 precolumn (Acclaim PepMap 3 μm, 100[a], 75 μm × 20 mm) was used to desalt peptides, and then a 75-min gradient of H_2O:ACN at a flow of 33 nL/min was used with a C18 2 mm Easy Spray column x 150 mm. Data-dependent MS2 method was used to fragment the top 12 peaks in each cycle. The raw data from mass spectrometry analysis were processed using the Proteome Discoverer, version 1.4 (Thermo Scientific) software for database searching with the SEQUEST search algorithm. The search was performed against a Uniprot database generated using 'snake' and 'venom' as keywords as well as trypsin as the enzyme used. Precursor mass tolerance was set to 10 ppm and product ion tolerance to 0.05 Da. Static modification was set to carbamidomethylation of Cys, and dynamic modifications were set to oxidation of Met and N-terminal acetylation. Protein hits were filtered for high confidence peptide matches with a maximum protein and peptide false discovery rate of 1% calculated by employing a reverse database strategy.

2.4. Statistical analyses

Where appropriate, values were expressed as the mean ± standard deviation (SD) and statistical comparisons were done using one-way analysis of variance (ANOVA) followed by Dunnett's test. All data analyses were performed using the Infostat software, with a value of $p < 0.05$ indicating statistical significance.

3. Results

3.1. Histology and histochemistry of DVG

Macroscopically, when DVGs from *L. a. marginatus* were removed, they showed a high level of dark pigmentation (Fig. 2B). Histologically, the glands are delimited by a capsule of connective tissue from which several septa penetrate the glandular body, forming lobes (Fig. 2C). Each lobe in turn is constituted by tubules that comprises columnar secretory cells with basal round nuclei, and the tubules possess small lumina into which the secretion passes (Fig. 2D) until it reaches a common duct to the base of the enlarged maxillary teeth. Cells from the secretory tubules reacted negatively to PAS staining, indicating the serous nature of the gland (Fig. 2E). Some melanin deposits were observed microscopically inside the gland (Fig. 2F).

3.2. Morphology of maxillae and fangs

By SEM analysis it was confirmed that the maxillae of *L. a. marginatus* possess recurved teeth that allow for a better grip on prey; none showed surface recesses, grooves or other specialized features (Fig. 3A). Posterior to these, at least one (but up to three) enlarged tooth (30–60% longer than the front teeth) was observed (n = 5), lacking grooves but exhibiting a prominent posterior ridge, giving the tooth a blade-like appearance (Fig. 3B–D).

3.3. Electrophoretic profile

The electrophoretic profile of the venom from *L. a. marginatus* showed a broad distribution of proteins with molecular weights mainly in the range of <10.0–30.0 kDa (Fig. 4). This profile was considerably different from that obtained with saliva from *L. a. marginatus*, in which the major protein bands observed were in the 50.0–70.0 kDa range.

3.4. Enzyme assays

The most notable enzymatic activities of the venom of *L. a. marginatus* are shown in Table 1; all activities were very low except acetylcholinesterase. The fibrinogen digest revealed a rapid hydrolysis of the Aα-chain of fibrinogen, and after 60 min there appeared to be some degradation of the Bβ-chain (Fig. 5A). The presence or absence of calcium did not affect the degradation profile of fibrinogen. For the fibrin digestion assay, only the α-monomer was affected (Fig. 5B). The γ-chain was resistant in both assays, even when the incubation time was prolonged to 24 h (Fig. 5A and B). There was no detectable PLA_2 or hyaluronidase activities.

3.5. Lethal toxicity

Venom of *L. a. marginatus* showed no lethal effects in mice at a dose level of up to 20 μg/g. However, all injected animals showed an

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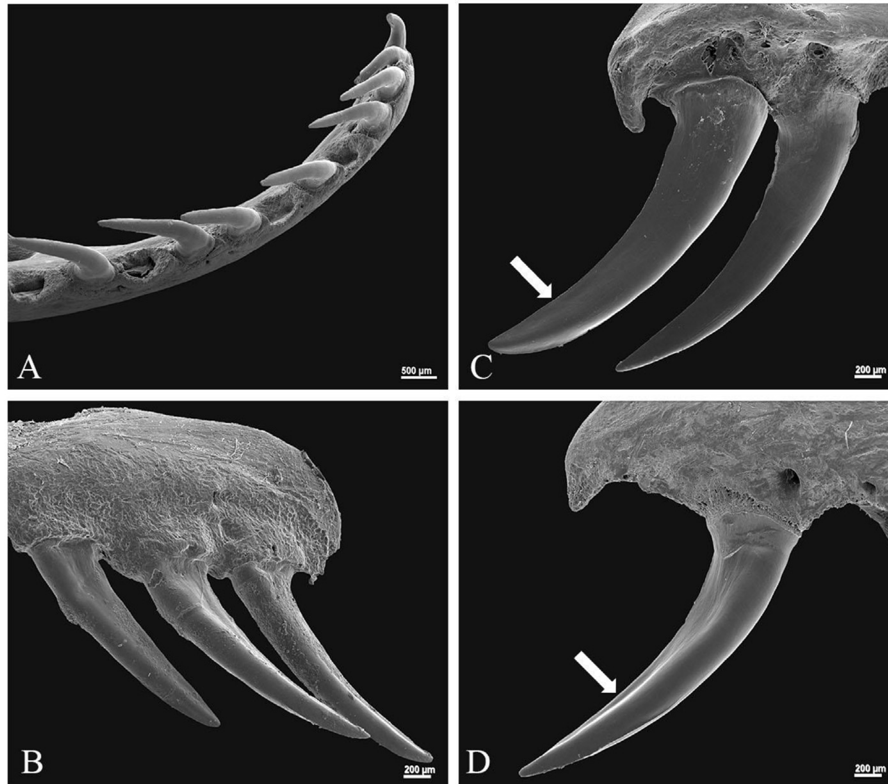

Fig. 3. SEM micrographs of *L. a. marginatus* maxilla; ventral view showing shorter anterior and enlarged rear teeth (**A**). One-three maxillary teeth are enlarged (B–D), and note the absence of grooves along the rear fangs on both labial (**B**) and lingual (**C, D**) sides. The ridge (arrows) on the posterior surface gives the enlarged maxillary teeth a blade-like shape.

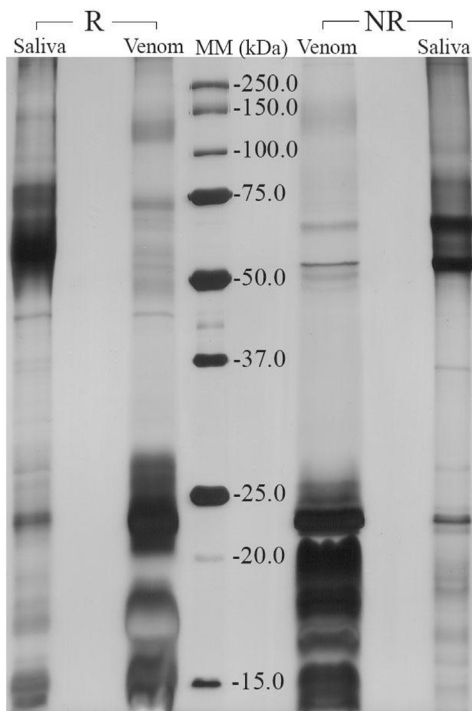

Fig. 4. Electrophoretic profiles of venom and saliva from *L. a. marginatus* in SDS-PAGE (12%). The gel was silver-stained. Note that lower mass proteins dominate in the venom, whereas higher mass proteins are more abundant in the saliva. R: reducing conditions. NR: non-reducing conditions. MM: molecular mass markers.

abnormal behavior characterized by an unusual apathy and a rapid rate of breathing (tachypnea), with marked contraction of the intercostal muscles. Initially, all of the animals became anorexic but returned to normal by 24 h post-injection.

3.6. Myotoxicity

When venom from *L. a. marginatus* was locally injected into mouse gastrocnemius muscle, the macroscopic appearance of the treated muscle was only slightly different from the control. No evidence of macroscopic hemorrhage was observed. In addition, the histopathological analysis revealed only a slight myonecrotic activity with limited edema and minimal inflammatory reaction (Table 2 and Fig. 6).

3.7. Cross-reaction with elapid and viperid antivenoms used in Argentina

By Western blotting, a very weak cross-reaction was observed between venom from *L. a. marginatus* and either bivalent or tetravalent anti-*Bothrops* sera (Fig. 7) or anti-*Micrurus* serum. With the

Table 1
Specific activities of *L. a. marginatus* venom toward several substrates.

Enzymatic activity	Value
Collagenolytic (U/mg protein)	0.00076 ± 0.00011
Caseinolytic (U/mg protein)	0.024 ± 0.001
Acetylcholinesterase (µmol/min/mg protein)	10.55 ± 0.41

All values are mean values of triplicate assays ± standard deviation.

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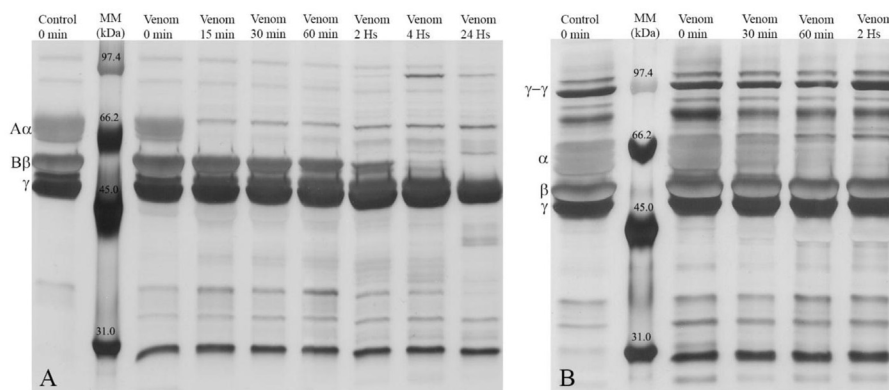

Fig. 5. Effect of *L. a. marginatus* venom on human fibrinogen (**A**) and fibrin (**B**). The assays were evaluated by SDS-PAGE (12%) under reducing conditions. Controls were incubated in absence of the venom. Gels were silver stained. In the left figure (**A**) the polypeptide chains of human fibrinogen are indicated: Aα (63 kDa), Bβ (56 kDa), and γ (47 kDa), whereas in the right figure (**B**) the chains of human fibrin are indicated: γ-γ dimer, α-monomer, β-monomer, and γ-monomer. *In vitro* fibrin clots are unstable in the absence of Factor XIII, so digests were sampled only for 2 h to evaluate venom enzyme-catalyzed activity. MM: molecular mass markers.

latter, only a very weak band at ~50 kDa was apparent under non-reducing conditions (data not shown).

3.8. RP-HPLC and mass spectrometry

MALDI-TOF mass spectrometry also revealed a limited diversity of peptide/protein components of *Leptophis* venom of 2–40 kDa (Fig. 8). Masses of three proteins (6618, 7529 and 8772 Da) are consistent with three-finger toxins (3FTx) from rear-fanged snake venoms, while two proteins (13,230 and 13,437 Da) have masses commonly observed for C-type lectins (CTL) in rear-fanged snake venoms. The final protein, with a mass of 24,646 Da, is a cysteine-rich secretory protein (CRiSP), a component that is nearly ubiquitous among snake venoms.

Venom from *L. a. marginatus* was fractionated into four major protein peaks by RP-HPLC (Fig. 9). SDS-PAGE-separated protein bands were excised, in-gel digested with trypsin and the resulting peptides were analyzed by LC-MS/MS. The most intense protein bands present in peaks 1, 2 and 3 were visualized in the range of 15–20 kDa (SDS-PAGE insert 1, Fig. 9), and tryptic peptides were matched by SEQUEST to an internal sequence, TCPTAGPDER, from a three-finger toxin of *Boiga irregularis* venom (Uniprot accession N° A0A0B8RSZ2). Tryptic peptides of the 23 kDa protein present in peak 4 (insert 2, Fig. 9) was matched by SEQUEST to an internal sequence, WYSEAASNAER, from a cysteine-rich secretory protein of *Pantherophis guttatus* (Uniprot accession N° A0A098LYK0).

4. Discussion

Venomous animals and their toxins constitute a unique source of molecules with interesting biological activities, and there is growing interest in them for therapeutic potential (Escoubas et al., 2008; Takacs and Nathan, 2014). Snakes are the major group of venomous vertebrates, and the rear-fanged snakes represent the vast majority of species around the world; however, only a few studies have been dedicated to characterize their venoms (Junqueira-de-Azevedo et al., 2016). Herein, we provide information about the biochemical and toxicological properties of the venom from the Green Parrot Snake *Leptophis ahaetulla marginatus* and its venom delivery system.

Like *Philodryas olfersii* and *Chironius bicarinatus*, *L. a. marginatus* is a diurnal arboreal snake (Giraudo, 2014). The two former species are also similar in their aggressive behavior and in prey consumed (Carreira et al., 2005); however, *C. bicarinatus* does not have any enlarged rear maxillary teeth (Giraudo, 2014), whereas *L. a. marginatus* exhibits 1–3 blade shaped and slightly recurved fangs at the posterior end of the maxillae. Taking into account that the anterior teeth of *L. a. marginatus* already have an appropriate morphology to facilitate prey retention, we suggest a major role of the rear fangs is in the delivery of venom into prey tissues, facilitating rapid prey subjugation.

The histology of the DVG of *L. a. marginatus* is similar to that described for DVGs from other colubrids such as *Philodryas olfersii*, *Alsophis portoricensis*, *Clelia plumbea* and *Boiga irregularis* (Renner and de Sabóia-Morais, 2000; Serapicos, 2006; Taub, 1967; Weldon and Mackessy, 2010; Zalisko and Kardong, 1992). We observed that the gland has significant levels of pigmentation (melanin); Pough et al. (1978) suggested that the presence of melanin pigments on the gland surface protected it from solar UV radiation and acted as a shield that prevents photo-detoxification of venom components. In addition, those authors concluded that, among the colubrids studied in their work, *L. ahaetulla* was the species with the highest density of melanin pigments associated with the DVG. This condition may be relevant considering that *L. a. marginatus* is a diurnal arboreal snake and that exposure to UV radiation is quite high during daylight hours.

The electrophoretic profile of *L. a. marginatus* venom was substantially different than that of its saliva. However, it is evident that obtaining venom completely free of saliva is very difficult, as previously suggested by Hill and Mackessy (2000). Venom of *L. a. marginatus* principally showed proteins in the range of 10.0–30.0 kDa. This protein pattern is in accordance with the very low activity toward azocollagen, casein and hyaluronic acid found in this work, since enzymes of >30 kDa (P-III SVMPs and hyaluronidases) are commonly associated with the degradation of

Table 2
Histological analysis of mouse gastrocnemius muscle injected with 50 μg of *L. a. marginatus* venom.

Lesion	1 h	3 h	6 h	9 h	12 h
Edema	−	−	+	+	++
Interfibrillar hemorrhage	−	−	−	−	−
Inflammatory infiltrate	−	+	++	+	+++
Necrosis (myolytic type)	−	−	+	+	++

−: no lesion detected; +: mild lesion; ++: moderate lesion; +++: severe lesion.

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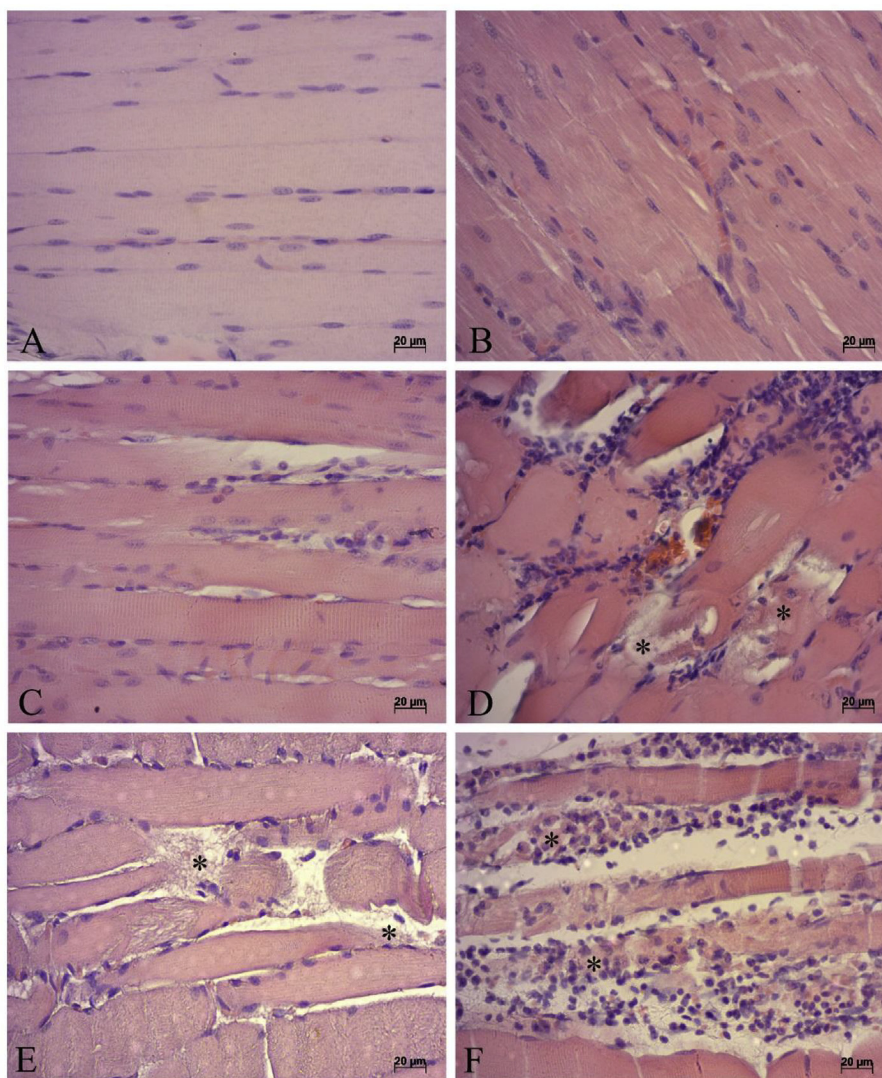

Fig. 6. Light micrographs showing the histopathological changes induced by 50 µg of *L. a. marginatus* venom in mouse gastrocnemius muscle (H&E stain). **A.** Longitudinal section of muscle from mouse in control group. **B.** Longitudinal section of muscle from mouse at 1 h after injection. **C.** Longitudinal section of muscle from mouse at 3 h after injection. **D.** Longitudinal section of muscle from mouse at 6 h after injection. Note inflammatory infiltrate of polymorphonuclear leukocytes and myonecrosis (*), which are more intense at 9 h (**E**) and 12 h (**F**) after injection.

structural proteins and extracellular matrix components (Mackessy, 2010). The most distinctive enzymatic feature was AChE activity, which was at a similar level to that reported for *Boiga irregularis* venom (Mackessy et al., 2006). Although this enzyme is more typically associated with elapid venoms, there are previous studies demonstrating its presence (but often at lower levels) in colubrid venoms (Broaders and Ryan, 1997; Mackessy et al., 2006; Peichoto et al., 2012).

The presence of fibrin(ogen)olytic enzymes, mainly of the α-type which are commonly found in snake venoms (Markland, 1998), suggests that the venom could interfere with the normal blood clot cascade, perhaps by disrupting the balance between fibrin formation and fibrin degradation or by depleting intact fibrinogen, and it may be responsible for the local bleeding reported following human envenomations by *L. ahaetulla* (Beebe, 1946; Minton and Mebs, 1978; Warrell, 2004; Weinstein et al., 2011). However, these results are inconsistent with the minimal local damage observed in mice in this study, as no signs of hemorrhage were seen. This inconsistency may be related to the fact that hemorrhage at the bite site is an event with rapid presentation and is primarily due to the degradation of structural and adhesive components of the connective tissue, and we showed in this study that *L. a. marginatus* venom has no effect toward collagen and hyaluronic acid, two major components of the extracellular matrix. Thus, bleeding reported at the site of the bite by *L. a. marginatus* is likely due primarily to mechanical injury (puncture by teeth), perhaps exacerbated by local depletion of fibrinogen.

By mass spectrometry we identified a cysteine-rich secretory protein (CRiSP) in *L. a. marginatus* venom, and this family of

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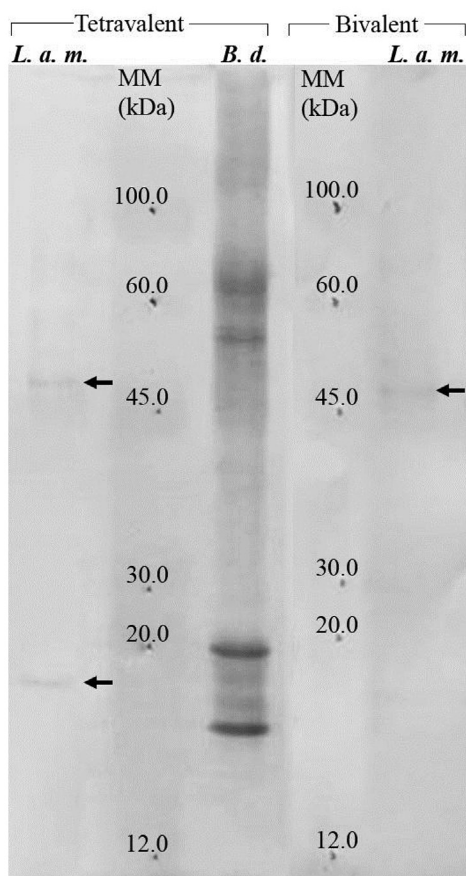

Fig. 7. Western blot analysis of venom from *L. a. marginatus* using bivalent and tetravalent anti-*Bothrops* sera. Proteins were electrophoresed in a SDS-PAGE (12%; non-reducing conditions) and blotted onto a nitrocellulose membrane, which was processed as described in Materials and Methods. *Bothrops diporus* venom (*B. d.*) was used as a positive control. Note that very few *L. a. marginatus* proteins (*L. a. m.*) were recognized by anti-*Bothrops* sera (arrows). MM: molecular mass markers.

proteins is widely distributed among snake venoms. Although to date most CRiSPs have unknown functions (Heyborne and Mackessy, 2010), it is likely that they can exhibit very different pharmacological activities (Saviola et al., 2014). Skeletal myotoxicity is a biological activity previously described for a venom CRiSP purified from the venom of *Philodryas patagoniensis* (Peichoto et al., 2009), and the *Leptophis* CRiSP may be responsible for the slight myonecrosis observed herein; however, this hypothesis needs to be investigated.

Another group of proteins exhibiting a variety of biological roles (Kini and Doley, 2010) are the three-finger toxins (3FTxs), and these were also found in *L. a. marginatus* venom. Although this superfamily of proteins have molecular masses in the range of 6–10 kDa (such as observed in the MS analysis of the venom herein), we found by SDS-PAGE proteins with 15–20 kDa that share partial sequence homology with a 3FTx of the arboreal rear-fanged Brown Treesnake (*Boiga irregularis*) venom. This data indicates the presence of dimeric isoforms of 3FTxs in *L. a. marginatus* venom, perhaps similar to a covalently linked heterodimeric 3FTx, irditoxin, isolated from venom of *B. irregularis* (Pawlak et al., 2009).

Three-finger toxins are commonly associated with lethal prey immobilization (Chang and Lee, 1963) and may display specificity for a particular prey taxon, which appears to follow prey preferences (Mackessy and Saviola, 2016). Several studies have investigated this type of 3FTx in colubrid venoms, e.g. fulgimotoxin, a taxon-specific 3FTx from the venom of *Oxybelis fulgidus*, which exhibits potent toxicity toward lizards, one of its most important prey types, but it has no toxic effects on mice (Heyborne and Mackessy, 2013). The diet of *L. a. marginatus* does not include mice, and instead they feed preferentially on frogs, even toxic species such as *Trachycephalus* sp., which exude an irritating secretion that deters predation by some snakes (Clegg, 2015; Solé et al., 2010). Because of the low availability of venom studied here, toxicity tests could not be conducted on different types of prey animals, and inbred mice were used because they are the standard animal model for testing lethality of snake venoms, according to the World Health Organization (1981). Although showing low toxicity toward mice, venom from *L. a. marginatus* may be highly toxic to their native prey.

McGivern et al. (2014) demonstrated a high level of divergence in the venom types of two rear-fanged snakes, the viperid-like venom of *Hypsiglena* sp. and the elapid-like venom of *Boiga irregularis*. Although we revealed herein a very weak antigenic similarity between *L. a. marginatus* venom and the most important viperid and elapid venoms from Argentina, we found that *L. a. marginatus* (an active forager) produces a venom reminiscent of elapid venoms, containing mainly components such as 3FTxs, CRiSPs and AChE and able to induce some neurotoxic effects in mice. These results support the hypothesis of McGivern et al. (2014) which assumed that selection might favor lethal neurotoxins to subdue prey, rather than abundant tissue-damaging toxins such as SVMPs, in species with active foraging ecologies.

Even though *L. a. marginatus* shows stereotypic aggressive behavior when threatened, it is here considered of relatively low hazard to humans. The weak neurotoxicity toward mammals ($LD_{50} > 20$ µg/g mouse; this study) likely underlies the lack of significant envenomation effects in humans (Beebe, 1946). Further, Weldon and Mackessy (2010) suggested that during a bite by rear-fanged snakes, venom proteins are exocytosed from the serous secretory cells of DVG and flow down the secretory tubules to a central duct leading to the base of (typically 2) enlarged rear maxillary teeth, and venom can be then introduced into the puncture wound(s). Envenomation therefore requires rapid exocytosis of intracellular venom followed by low-pressure delivery into prey tissues, resulting in the requirement of longer contact time for significant envenomation of larger animals such as humans. Based on our observations in the present study (venom yields from the largest adult specimens averaged 60 µL, with ~150 µg protein), which are much lower than those from adult *Boiga irregularis* (Mackessy et al., 2006), we can conclude that serious human envenomation by *L. a. marginatus* will be very unlikely.

In summary, this work provides a biological and toxicological characterization of the venom from *L. a. marginatus*, as well as the main features of its venom-delivery system. Thus, this study contributes to a better understanding about implications of bites with this species, which is very common in forest areas frequented by humans, such as the Iguazu National Park from Argentina. There are hundreds of rear-fanged venomous snake species worldwide whose venoms and venom delivery systems remain unknown, and this study constitutes an initial attempt to understand the biological role of venom in *Leptophis*, and aids in our understanding of the evolution of venom in this complex group of advanced snakes.

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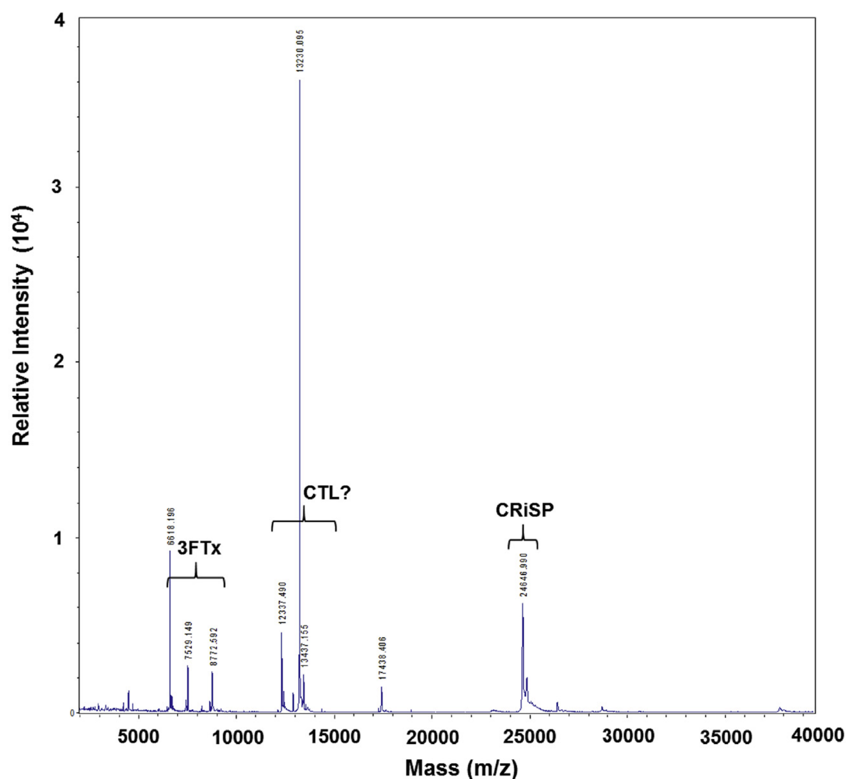

Fig. 8. MALDI-TOF mass spectrogram of *L. a. marginatus* venom. Tentative protein identifications, based on published masses for rear-fanged snake venoms, are given for three clusters of peaks. CTL, C-type lectin; CRiSP, cysteine-rich secretory protein; 3FTx, three-finger toxin.

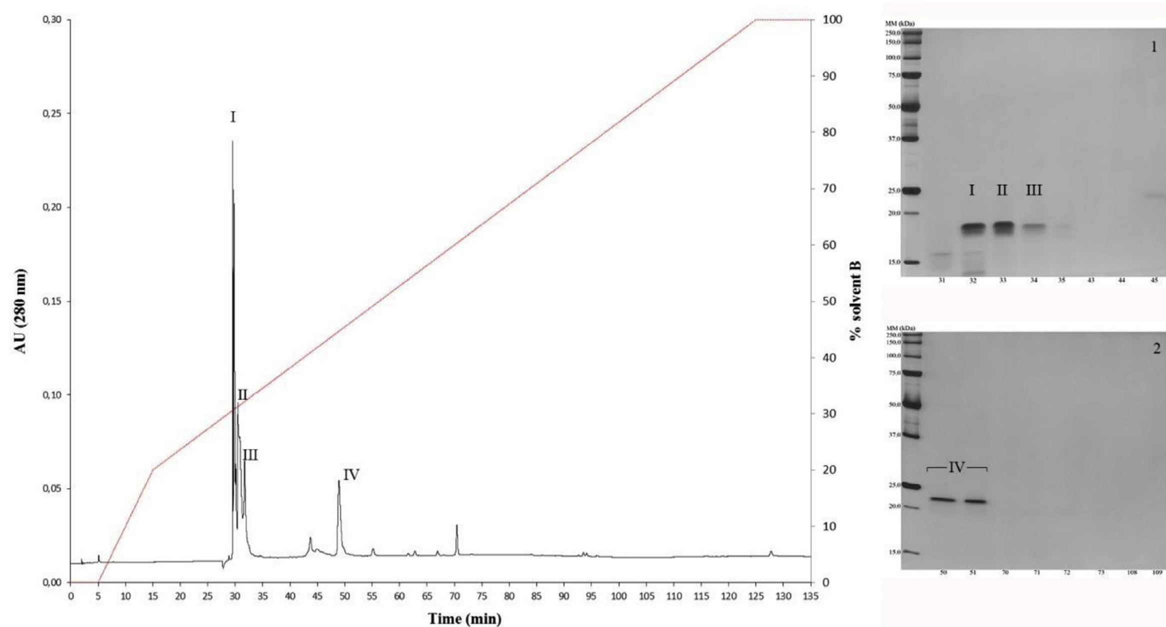

Fig. 9. Reverse-phase HPLC chromatogram of Duvernoy's venom proteins from *L. a. marginatus.* Insert shows SDS-PAGE of the eluted protein peaks on 12% gel run under non-reducing conditions.

Funding

This work was supported by INMeT, CONICET (PIP 112-201301-00126-CO), and Agencia Nacional de Promoción Científica y Tecnológica (PICT-2013-1238) from Argentina, as well as a RDFD travel/research grant from UNC to SPM.

Conflicts of interest

The authors have no conflicts of interest with this work.

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Acknowledgments

M.N. Sánchez is the recipient of a fellowship from Consejo Nacional de Investigaciones Científicas y Tecnológicas, Argentina. This work was carried out in partial fulfillment of the requirements for the PhD degree for M.N. Sánchez at the University of Buenos Aires, Argentina. We would like to thank the National Parks Administration (APN, Argentina) for permission to collect snakes in the Iguazu National Park, and the School of Medicine of the University of Northeastern Argentina (UNNE) for providing experimental animals. We thank Cecilia Galíndez and Cristina Salgado for helping with the SEM sample preparation, and S.L. Maruñak and M.A. Quintana for technical assistance.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.toxicon.2018.04.027.

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