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Intraspecific venom variation of Mexican West Coast Rattlesnakes (*Crotalus basiliscus*) and its implications for antivenom production

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ABSTRACT

Intraspecific variation in snake venoms has been widely documented worldwide. However, there are few studies on this subject in Mexico. Venom characterization studies provide important data used to predict clinical syndromes, to evaluate the efficacy of antivenoms and, in some cases, to improve immunogenic mixtures in the production of antivenoms. In the present work, we evaluated the intraspecific venom variation of *Crotalus basiliscus*, a rattlesnake of medical importance and whose venom is used in the immunization of horses to produce one of the Mexican antivenoms. Our results demonstrate that there is variation in biological and biochemical activities among adult venoms and that there is an ontogenetic change from juvenile to adult venoms. Juvenile venoms were more lethal and had higher percentages of crotamine and crotoxin, while adult venoms had higher percentages of snake venom metalloproteases (SVMPs). Additionally, we documented crotoxin-like PLA_2 variation in which specimens from Zacatecas, Sinaloa and Michoacán (except 1) lacked the neurotoxin, while the rest of the venoms had it. Finally, we evaluated the efficacy of three lots of Birmex antivenom and all three were able to neutralize the lethality of four representative venoms but were not able to neutralize crotamine. We also observed significant differences in the LD_{50} values neutralized per vial among the different lots. Based on these results, we recommend including venoms containing crotamine in the production of antivenom for a better immunogenic mixture and to improve the homogeneity of lots.

1. Introduction

When Calmette developed his “Sérum antivenimeux” primarily using *Naja kaouthia* venom in 1894, he initially proposed it as a universal snake antivenom [1]. Subsequent experiments demonstrated Calmette's antivenom was not universal and since then, antivenom specificity was not underestimated [2]. The following century of research has documented inter- and intraspecies variation in venoms which has led to recent research into optimal immune mixtures. It is best if the venom pool consists of a large number of specimens including individual across the geographical range representing the variation present in this species [3]. Capturing intraspecific variation is particularly important for

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antivenom producers who rely on captive-bred venomous snakes, often large adults from a single locality, which produce only a fraction of the antigens present in the species as a whole [1].

Intraspecific variation has been observed in individuals from different geographic regions as well as from individuals of different age classes. For example, Schenberg generated a distribution map for the presence of crotamine in populations of *Crotalus durissus* in São Paulo, Brazil, and found regions where crotamine was present, where it was absent, and an area of hybridization between the two types of venom [4]. *Crotalus scutulatus* has been well studied and three venom types were identified: 1) neurotoxic and low proportion of SVMPs, 2) not neurotoxic and high proportion of SVMPs and 3) both neurotoxic and a high proportion of SVMPs [5–8].

Examples of ontogenetic venom variation include *Crotalus polystictus* which exhibits variation in presence and enzymatic activity between adults and neonates [9]. In *C. molossus nigrescens*, venom from juvenile specimens showed greater toxicity, rigid paralysis caused by crotamine and higher procoagulant activity compared to adults [10]. For *C. culminatus* venoms, a reduction in the amount of crotamine as snakes grow has been described [11] whereas *C. v. viridis* [12] and *C. cerastes* [13] are the inverse. Intraspecific variation has an important role in the production of antivenoms because, by accounting for variation, antivenoms can be produced that better neutralize other species [14,15]. However, using more individuals to produce the antivenom may result in lower reproducibility in the neutralization potency among batches [16].

In Mexico, there are two antivenoms used to treat patients envenomated by pitvipers: Antivipmyn®, manufactured by Bioclon, uses the venom of *Crotalus simus* and *Bothrops asper* as immunogens and "Faboterápico polivalente antiviperino", manufactured by Birmex (hereafter, Birmex), uses the venom of *C. basiliscus* and *B. asper* as immunogens. Both antivenoms are of equine origin, have $F(ab')_2$ and are sold lyophilized. The venom *of C. simus* from Mexico has been studied recently [16–18] and the venom of *Bothops asper* from Mexico is poorly studied [19]. The venom of *C. basiliscus* has only been analyzed once and included a pool of venom from 15 captive adults of unknown origin [20]. They reported the proteome was composed of 68% SVMPs, 14% PLA_2s, 11% SVSPs, 4% SVMPs-inhibitor tripeptides (SVMP-ITs), 2% bradykinin-potentiating peptides (BPPs), 0.6% cysteine-rich secretory proteins (CRISPs), and 0.2% L-amino acid oxidases (LAAOs). Due to the unknown origin of the individuals and because they were pooled into one sample, it is necessary to carry out studies on the composition of *C. basiliscus* venom more broadly.

The Mexican West Coast Rattlesnake, *C. basiliscus*, is a large Mexican endemic known to reach a total length of 2 m. *Crotalus basiliscus* inhabit the Mexican Pacific coast west of the Sierra Madre Occidental from southern Sonora to northern Michoacan (Fig. 1) [21,22]. The World Health Organization Guidelines for the production, control, and regulation of snake antivenom immunoglobulins [23] listed this species as medically important. In Mexico, there are no specific statistics on the number of people bitten by each species; however, due to their distribution and large size, *C. basiliscus* likely cause serious health problems where they occur.

Even though *C. basiliscus* is one of only three species used as immunogen to viperid antivenoms in Mexico, very little is known about their venom, and nothing is known about intraspecific variation. To address this, our first objective was to determine if there are differences in the biochemical and biological activities within *C. basiliscus* venom from different localities and between adults and juveniles. Additionally, we wanted to evaluate possible variation in the neutralization of *C. basiliscus* venom by Birmex antivenom. Our study is the first to investigate intraspecific venom variation in *C. basiliscus* and is one of few studies that compares the neutralization capacity of different batches of antivenom. Our results can be used to make recommendations in the design of immunogenic mixtures to treat snakebite in Mexico.

2. Materials and methods

2.1. Animal ethics statement

Animal experiments were approved by the Bioethics Committee of the Institute of Biotechnology of Universidad Nacional Autónoma de México, IBt, UNAM (number 379).

2.2. Venoms and antivenoms

We used venoms from the venom bank of A. Alagón, venom from animals held in captivity (no more than 6 months of captivity after being wild-caught) at the Herpetario Cantil, IBt, UNAM, and from specimens captured in the field and released (Supplementary Table S1). Specimen were collected under collection permit SGPA/DGVS/04788/17 issued by SEMARNAT (Secretariat of Environment and Natural Resources). All specimens were milked manually by the same person. Specimens were milked using glass cups covered with parafilm, the snake was placed over a table or placed inside a plastic tube and the head of the snake was carefully held. The snake's glands were massaged to obtain more venom.

For neutralization tests, we used Birmex lyophilized antivenom. Specifically, we used lots FV044A - October 2020 (B1), FV030A - December 2016 (B2) and FV043A - September 2020 (B3) which are each composed of lyophilized $F(ab')_2$ fragmented antibodies of equine origin. The protein concentrations of the lots were: B1 = 28.1 mg/mL, B2 = 30.1 mg/mL and B3 = 22.2 mg/mL. The technical data sheet of Birmex antivenom indicates that one vial has the capacity to neutralize no less than 780 and 790 LD_{50}s of *Bothrops asper* and *Crotalus basiliscus* venoms, respectively.

2.3. Protein concentration determination

We determined the protein concentration for each of the 27 venom samples using the Pierce® Bicinchoninic Acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions and using bovine serum albumin (BSA) as the standard. We quantified antivenoms using the extinction coefficient for immunoglobulins at 280 nm (molar extinction coefficient: 1.44).

2.4. Electrophoretic profiles

We used 15% polyacrylamide gels made on a Miniprotean III system (Bio-Rad, Hercules, CA, USA) using the discontinuous system. Fifteen micrograms of each of the 27 venom samples were run under reduced and non-reduced conditions. We boiled samples for 5 min and then ran them at 120 V. Gels were stained with 0.2% Coomassie brilliant blue either G-250 or R-250, 10% acetic acid, 10% isopropyl alcohol overnight and then we rinsed off the excess dye with a solution of 10% acetic acid and 10% isopropyl alcohol. Standard molecular markers (AccuRuler RGB PLUS prestained protein ladder, MAESTROGEN) were used as size references.

2.5. Reversed-phase high-performance liquid chromatography (RP-HPLC) profiles

We separated each venom using RP-HPLC on an C_{18} analytic column (XChroma™HPLC Column 5 μm, 120 Å, 4.6 × 250 mm). We dissolved 1 mg of each sample in 1 mL of water with 0.1% trifluoroacetic acid (TFA). Elution was performed as described by Ref. [24] using a gradient of solution A (water with 0.1% TFA) and

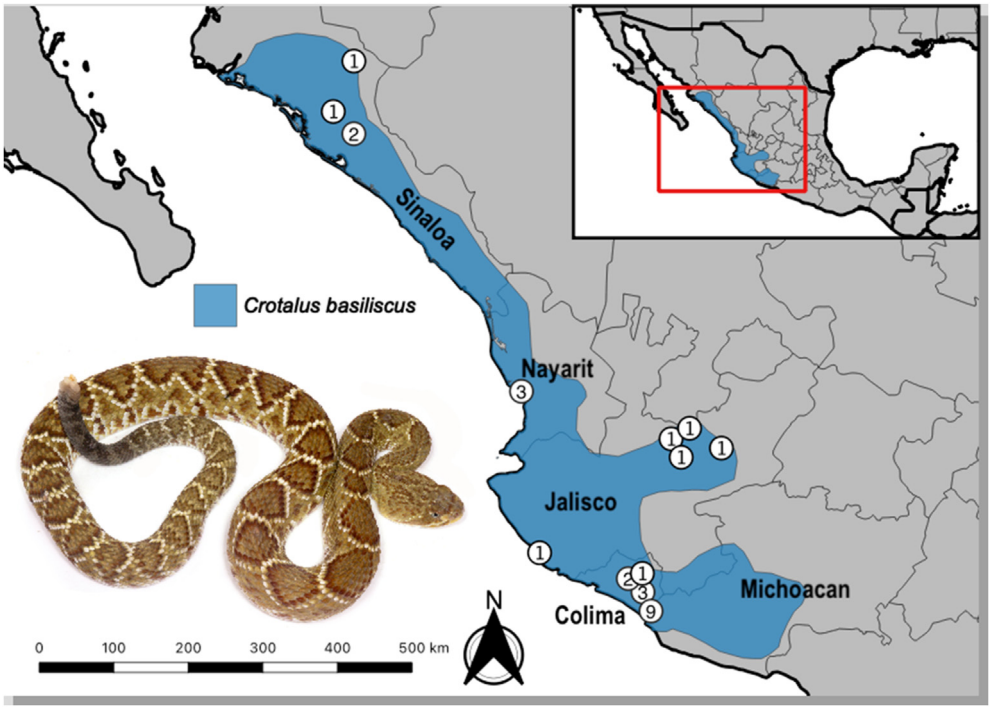

Fig. 1. Geographic distribution of *C. basiliscus* and localities of the twenty-seven *C. basiliscus* specimens analyzed. The circles with numbers indicate the number of specimens collected in that locality.

solution B (acetonitrile with 0.1% TFA) as follows: 5% B for 5 min, 5–25% B over 10 min, 25–45% B over 60 min, 45–75% B over 10 min, and 100% B as of 105 min. Proteins were detected at 214 and 280 nm.

2.6. Proteolytic activity (azocasein hydrolysis)

We evaluated proteolytic activity using azocasein (Sigma-Aldrich) as the substrate as described by (Wang et al., 2004) and modified by (Gutiérrez et al., 2008) for 26 venom samples. We prepared the azocasein solution to a concentration of 10 mg/mL dissolved in 50 mM Tris-HCl, 0.15 M NaCl and 5 mM $CaCl_2$, pH 8.0. We incubated 100 μL of azocasein with 20 μg of each venom individually for 30 min at 37 °C and stopped the reaction with 200 μL of 5% trichloroacetic acid, followed by centrifugation at 10,000 rpm for 5 min. We mixed 150 μL of supernatant with 150 μL of 0.5 M NaOH, ran each venom in triplicate, and recorded absorbances at 492 nm. We defined one unit of proteolytic activity as a change of 0.2 in absorbance per minute. To evaluate the percentage of proteolytic activity caused by SVMPs, ethylenediaminetetraacetic acid (EDTA) was used as a chelator of zinc, an indispensable metal ion for catalytic activity. Venoms were incubated with EDTA for 30 min at 37 °C and then the substrate was added, the rest of the steps are the same as those mentioned above.

2.7. Fibrinogenolytic activity

We estimated fibrinogenolytic activity of each venom using human fibrinogen (Sigma®) [25]. We incubated 10 μg of each venom with 50 μg of human fibrinogen for 40 min in a volume of 50 μL of PBS. We sampled 5 μL of each mixture and then added reducing sample buffer (10% glycerol, 2.5% SDS, 50 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol and 0.02% bromophenol blue) and boiled at 100 °C for 5 min. Samples were separated using SDS-PAGE (12.5%) and compared with human fibrinogen without venom added as control.

2.7.1. Inhibition of fibrinogenolytic activity with EDTA

To evaluate the role of SVMPs and SVSPs on the lysis of fibrinogen, we performed inhibition assays. Inhibition was determined by incubating 10 μg of each venom separately with 5 mM EDTA for 40 min at 37 °C before carrying out the analysis described in 2.7 above. Activity after inhibition with EDTA was subtracted from total activity to determine activity due to SVMPs and SVSPs.

2.8. Phospholipase A_2 activity

We determined phospholipase A_2 activity titrimetrically using 10% w/v egg yolk with a standard solution (0.1 M NaCl, 0.01 M $CaCl_2$, 0.5% Triton X-100) as the substrate. The pH of 0.5 mL of egg yolk was adjusted to 8.03–8.05 with 0.05 M NaOH [26,27]. We performed the assay on a total volume of 500 μL under constant stirring and mild nitrogen bubbling. We added venom samples (1 mg/mL) to the mixture and maintained the pH above eight by constantly adding a solution of 0.05 M NaOH. Given that the consumed NaOH is equivalent to the acidification caused by the fatty acids released after hydrolysis by PLA_2s, specific enzymatic activity units (U/mg) were defined as μmoles of NaOH hydrolysis per minute per milligram of venom.

2.9. Crotoxin homolog detection via Enzyme-linked immunosorbent assay (ELISA)

We sensitized 96-well plates (Nunc MaxiSorp®) with 100 μL of 2 μg/mL "4F6" monoclonal antibody against the basic subunit of crotoxin (CB) [18] using sensitization solution (100 mM $NaHCO_3$, pH 9.5 buffer) for 1 h. We then washed the plates three times with 200 μL of wash solution (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20). Subsequently, the plates were blocked with gelatin (50 mM Tris-HCl, pH 8, 0.5% gelatin, 0.2% Tween 20) and washed again three times with wash solution. The standard control curve started with 5 μg/mL of CB followed by 1:3 serial dilutions. The

venom samples were analyzed at 20 μg/mL followed by 1:3 serial dilutions and left to incubate at 37 °C for 1 h. After dilution, plates were washed, and then we added 100 μL of 2 μg/mL polyclonal rabbit anti-crotoxin antibodies for 1 h. After washing again, we incubated the plate with goat anti-rabbit antibody conjugated with peroxidase HRP diluted to 1:5000 (Millipore) for 1 h. After a final washing step, the colorimetric reaction was developed using ABTS substrate (2,2′-azino-bis(3-ethylbenzothyazolin-6-sulfonic acid)) and absorbance quantified at 405 nm.

2.10. Lethality

We determined the median lethal dose (LD_{50}) by injecting different amounts of venom diluted in PBS to a total volume of 0.2 mL into the caudal vein of CD-1 mice (18–20 g) in groups of three [28]. Deaths were recorded 24 h after inoculation and LD_{50} values were estimated by non-linear regression using the software GraphPad Prism v. 8.

2.11. Hemorrhagic activity

To determine hemorrhagic activity, we injected 25 μg of venom subcutaneously in a volume of 50 μL PBS using groups of five CD-1 mice with weights between 22 and 30 g. Three hours after injection, we sacrificed the mice via CO_2 inhalation, removed their skin at the site of injection, and measured the diameter of the hemorrhagic halo. Level 0 did not generate a hemorrhagic halo, level 1 generated a halo from 0.1 to 10 mm, level 2 generated a halo from 10.1 to 20 mm, and level 3 generated a halo >20 mm [10,29].

2.12. Neutralization test

2.12.1. Neutralization of lethal activity

We used venoms from four *C. basiliscus* with different levels of lethality (V6 = LD_{50} 3.4 μg/mouse, V10 = LD_{50} 2.6 μg/mouse, V9 = LD_{50} 20.1 μg/mouse, V3 = LD_{50} 60.4 μg/mouse) and a venom with a high proportion of crotamine (V6 = 32.5% crotamine) to challenge three lots of Birmex antivenom (B1, B2 and B3). We used venom amounts equivalent to 3 LD_{50} and incubated them with different volumes of each lot of antivenom dissolved in PBS to a final volume of 0.5 mL for 30 min at 37 °C. After incubation, we injected the venom-antivenom solution in the caudal vein of three CD-1 mice (18–20 g) and recorded survival after 24 h post inoculation. Neutralization was expressed as effective dose 50 (ED_{50}) and defined as the amount of antivenom that prevents the death of half the mice.

2.12.2. Neutralization of proteolytic activity

To determine how well Birmex neutralized proteolytic activity, we pre-incubated 20 μg of venom from four venoms (V2, V3, V9, and V12) with different amounts of antivenom. After incubation, we followed the same protocol as 2.6 above. In all experiments, we used the whole venom without antivenom as the positive control at 100% activity. We tested each amount of antivenom in triplicate and the results are expressed as the amount of antivenom needed to inhibit 50% of the proteolytic activity (EC_{50}).

2.13. Recognition of venom components by Western blot

To observe any difference in recognition among the three different lots (B1, B2, B3) of Birmex antivenom to the components of 12 representative venoms from *C. basiliscus*, we separated 5 μg of each sample with SDS-PAGE (15%). Samples were selected to capture the variation in lethality and biological activity among adult and juvenile venoms. After electrophoresis, we transferred proteins to a nitrocellulose membrane (*Trans*-blot 0.45 μm Bio Rad®) and loaded the membrane into a semi-dry immunotransference chamber (Thermo Scientific). Subsequently, we blocked the membrane with 5% non-fat dry milk diluted in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20, pH 8.4) for 2 h. Membranes were rinsed three times with TBST and incubated with 10 μg/mL of each lot of antivenom (primary antibody) in 10 mL total for 1 h at room temperature shaking gently. Membranes were rinsed again and then the secondary antibody, goat anti-horse IgG diluted to 1:1000, was added in 10 mL. Following a final rinse step, a third antibody, rabbit anti-goat IgG conjugated with alkaline phosphatase diluted to 1:1000, was added. The reaction was developed with BCIP/NBT substrate for alkaline phosphatase (Sigma®). Membranes were washed, dried, and photographed. In parallel, an SDS-PAGE control gel was carried out under the same conditions and dyed with Coomassie blue R-250.

2.13.1. Transcriptomics

We used transcriptomics to determine if messenger RNA (mRNA) for crotoxin was transcribed for *C. basiliscus* and ultimately did not make it into the venom because it was not detected via ELISA. We used individual V18 (male, 1.3 kg) to obtained total RNA from venom (approximately 1 mL) and a venom gland biopsy (approximately 10 mg of tissue). Five days before the surgical procedure, the individual was milked to increase the rate of transcription. The venom sample was placed directly into a tube of 1 mL TRizol to homogenize it and was used for the venom transcriptome. To obtain the venom gland biopsy, we sedated the individual with a dose of 2 mg/kg Diazepam injected into the dorsolateral musculature and then fully anesthetized the individual with an injection of 30 mg/kg of Ketamine. We made a small incision in the skin from the interior part of the mouth under the supralabial scales using a scalpel (Supplementary Fig. S1). We removed a small piece of venom gland tissue and placed the sample directly into a tube containing 1 mL of TRIzol stored on dry ice in preparation for RNA extraction. We then sutured the gland and the skin back together and returned the individual to its enclosure. The animal was monitored for 12 h until it was fully responsive. The animal has returned to its normal feeding schedule and is able to produce venom from the gland where surgery occurred. The snake was fed 20 days after surgery and the animal ate without a problem. Six months after the surgery, a sample of venom was taken and loaded to get its electrophoretic profile to test for changes (Fig. S2).

2.13.2. Total RNA extraction, cDNA synthesis, and sequencing

We purified total RNA from the venom and venom gland biopsy. For the venom, we divided the sample into two 2 mL tubes and centrifuged at 16,800 g for 10 min at 4 °C to remove impurities, once clean, 200 μL of chloroform/mL of TRIzol was added to each tube and left for 10 min at room temperature. The formation of three phases was perceptible and then we centrifuged tubes for 10 min at 16,800 g and 4 °C, transferred the aqueous upper phase to new RNase-free tubes with the same volume of 100% isopropanol added to each aqueous phase to precipitate RNA. For both the venom and venom gland, tubes were left for 10 min at −60 °C and then centrifuged at 16,800 g for 10 min at 4 °C. Supernatant was removed and the resulting RNA pellet (not visible) was washed with 500 μL of 75% ethanol. We vortexed for 10 s and then centrifuged at 16,800 g for 10 min at 4 °C. This last step was repeated once more, and the pellet was dried for 15 min at 37 °C. Total RNA was resuspended in 30 μL DEPC H_2O and product was visualized on a 1.5% agarose gel. The total RNA yield recovered from ~10 mg of gland tissue was 124.0 μg while total RNA yield recovered from ~1 mL of crude venom was 4.2 μg, both in a total volume of 30 μL of RNAase free water.

2.13.3. Transcriptome assembly, annotation, and analysis

The total RNA was sent to the University Unit of Massive Sequencing and Bioinformatics at the Institute of Biotechnology of the National Autonomous University of Mexico (UUSMB, IBt-UNAM) for sequencing. The venom and venom gland cDNA libraries were sequenced on an Illumina Nextseq 500 using 75 bp paired-end sequencing.

Transcriptome assembly and annotation were conducted as described previously [5] and based on best practices [30]. Briefly, raw paired sequences that passed the Illumina quality control were trimmed with TrimGalore! v. 0.5.0 and then merged using PEAR v 0.9.6 [31]. Merged reads were assembled using Extender [32] and Trinity v 2.6.6 [33]. Assembled reads were annotated based on matches to the UniProt/SwistProt database using BlastX v 2.2.31+ searches and a minimum e-value of 10^{-4}. Duplicate sequences were removed in Geneious Prime v 2020.2.1 (Biomatters Ltd.) and chimeric sequences were removed using ChimeraKiller v 0.7.3 (available at https://github.com/masonaj157/ChimeraKiller). We then combined the transcripts from the venom and venom gland transcriptomes and clustered at 98% (-c 0.98) using cd-hit-est v 4.8.1 [34] to limit allelic variation and generate the final consensus transcriptome for individual V18.

To determine relative expression of each protein family, we mapped merged reads against the consensus transcriptome using Bowtie2 [35] implemented in RSEM v 1.3.2 [36]. We imported the transcripts per million reads (TPM) values into RStudio v March 1, 1093 using R v 4.0.2 (R Core Team, 2020) and graphed the number of reads for each transcript as well as the proportion of each toxin family.

To ensure that crotoxin was not present in the individual, we also mapped merged reads from the venom and venom gland to a known sequence of the basic subunit in *C. basiliscus* (Genbank Accession KU666923; [37]).

3. Results

3.1. Crotalus basiliscus samples

We obtained twenty-seven venom samples of *C. basiliscus* including ten juveniles (<70 cm snout-vent length; SVL) and seventeen adults (>70 cm SVL) from six states in Mexico (Colima, Jalisco, Michoacan, Nayarit, Sinaloa and Zacatecas) (Fig. 1 and Table 1).

3.2. Electrophoretic profiles

The electrophoretic profiles of adult venoms were different in the abundance of some protein bands (~55 kDa) and the presence or absence of bands between 25 and 37 kDa (Fig. 2). The differences were prominent when comparing specimens from different states of Mexico. The protein profiles of the adults presented two abundant bands between 20 and 25 kDa and, in some cases, a band of approximately 55 kDa. The juveniles presented an abundant band at 14 kDa and another near 10 kDa. The 10 kDa band was absent in most adult venoms (Fig. 2).

3.3. RP-HPLC profiles

Twenty-six venoms from *C. basiliscus* were fractionated via RP-HPLC. The chromatographic profiles of the adults (Fig. 3) from Jalisco, Nayarit and Colima were similar in terms of the number of peaks, however, there were differences in the proportion of peaks. The Michoacan and Sinaloa specimens presented differences in the number of peaks between 20 to 40 and 60–80 min. Juvenile samples exhibited differences among samples and were markedly different when compared with adults (Fig. 4; additional figures are available in Supplementary Fig. S3). The primary differences between adults and juveniles were the abundance in the eluting peaks between 24 and 25 min, 42 min (except V11, V12, V24, V25 and V26), 60–75 min and a decrease in the eluting peaks after 80. The peaks that eluted between 24 and 25 min were crotamine isoforms (Myo) as demonstrated by their molecular weight 4197.09, 4395.4, 4506.8, 4065.5 and 4177.7 Da and confirmed by biological activity which included hind limb spastic paralysis observed when injected into the caudal vein of three mice at 10 μg/mouse. The peak eluting at 42 min was detected in venoms of adults and juvenile from all locations but not found in six of seven individuals from Michoacan and individuals from Sinaloa. The peak at 42 min corresponded to the basic subunit of crotoxin (CTX sub B) as demonstrated by its molecular weight 14184.04 and 14295.7 Da via mass spectrometry, indicating the presence of at least two isoforms. We also confirmed the peak's activity by injecting mice in the caudal vein at 20 μg/mouse and identifying signs of neurotoxicity (flaccid paralysis). For the identification of the acidic subunit of crotoxin (CTX sub A), we used data generated in the laboratory (Calvete et al., unpublished data). The venom from six of seven venoms from Michoacan and four from Sinaloa lacked neurotoxic activity confirming the lack of crotoxin.

3.4. Proteolytic activity

Twenty-six venoms were able to hydrolyze azocasein in ranges from 0.081 (V7) to 5.2 U/mg (V2) (Table 1). Adults were significantly ($t = 9.302$; $p < 0.001$) more proteolytic with values ranging from 3.3 to 5.2 U/mg compared to juveniles which ranged from 0.08 to 2.8 U/mg. We observed that Michoacan adult samples that lacked crotoxin exhibited the highest proteolytic activity. Azocaseinolytic activity was completely inhibited with the addition of the chelating agent ethylendiaminetetraacetic acid (EDTA) indicating that the activity over this substrate is completely mediated by SVMPs and not SVSPs.

3.4.1. Neutralization of proteolytic activity (Azocasein hydrolysis)

The venom of four individuals with medium (V3 and V9) and high (V2 and V12) proteolytic activity was used to determine proteolytic neutralization with three lots of Birmex antivenom. All three lots of antivenom neutralized proteolytic activity: B2 and B3 were similar in their EC_{50} to neutralize the three venoms evaluated, while B1 needed more antivenom to neutralize proteolysis (Table 2).

3.5. Fibrinogenolytic activity

Fibrinogenolytic activity over human fibrinogen of eleven representative *C. basilicus* venoms (one juvenile and one adult form each location) was evaluated. (Fig. 5). The adult and juvenile venoms both degraded the α and β chains of fibrinogen, but the juveniles took longer to degrade the β chain. For juvenile venoms V4 and V11, the β chain was not fully degraded. When the venom was preincubated with EDTA, the degradation of both chains was considerably reduced indicating that SVSPs contribute to the fibrinogenolytic activity.

The numbering of the venoms was given as the venoms were obtained over time.

3.6. Lethality: median lethal dose (LD_{50})

Crotalus basiliscus venoms have a wide range of toxicity from 0.14 μg/g (C.I. 0.13 to 0.15) to 15.5 μg/g (C.I. 15.3 to 15.6) (Table 1). In general, the samples from Michoacan had the lowest lethality.

Table 1
Biological and biochemical activities of juvenile and adult *C. basiliscus*.

		Lethality LD_{50}		Proteolytic activity	PLA_2 activity	Hemorrhage	Fibrinogenolytic activity	Crotoxin homologs		Crotamine
ID	Location	(μg/mice)	(μg/g)	(U/mg)	(U/mg)	Level		ELISA (%)	HPLC (%)	(%)
V1	Tepatitlán de Morelos, Jalisco	8.6 (8.3 a 8.9)	0.45 (0.44 a 0.47)	3.6 ± 0.1	16.4 ± 1.3	1	α , β	+	11.8	1.3
V2	Zapopan, Jalisco	37.5 (36.1 a 38.9)	2 (1.89 a 2.05)	5.2 ± 0.2	60.9 ± 9.5	1	ND	+	4.4	1.7
V3	La Huerta, Jalisco	60.4 (57.8 a 63)	3.2 (3.04 a 3.3)	3.3 ± 0.1	3.4 ± 0.5	1	α , β	+	3.5	0.8
V4*	Ixtlahuacán, Colima	5.9 (5.6 a 6.2)	0.31 (0.3 a 0.33)	0.2 ± 0.04	18.1 ± 0.7	0	α , β**	+	14.7	31.6
V5	Ixtlahuacán, Colima	32.4 (31.9 a 32.8)	1.7 (1.68 a 1.73)	4.8 ± 0.3	4.8 ± 1.0	2	α , β	+	8.3	15.4
V6*	Ixtlahuacán, Colima	3.4 (3.41 a 3.7)	0.2 (0.16 a 0.19)	0.3 ± 0.03	57 ± 2.2	0	ND	+	11	32.5
V7*	Coquimatlán, Colima	4.05 (3.9 a 4.2)	0.21 (0.2 a 0.22)	0.08 ± 0.04	19 ± 1.0	0	ND	+	22	29.9
V8	Coquimatlán, Colima	32.2 (22.8 a 45.5)	1.7 (1.2 a 2.4)	3.6 ± 0.2	16.7 ± 0.7	1	ND	+	15.7	4.5
V9	Colima	20.1 (20.0 a 20.2)	1.06 (1.06 a 1.07)	3.4 ± 0.2	27.8 ± 5.5	1	ND	+	14.3	5.6
V10*	Nayarit	2.6 (2.51 a 2.7)	0.14 (0.13 a 0.15)	0.1 ± 0.03	41.1 ± 3.1	0	ND	+	27	5.8
V11*	Coahuayana, Michoacan	13.1 (10.9 a 15.7)	0.7 (0.6 a 0.8)	1.2 ± 0.1	134 ± 16.5	1	α , β**	−	0	21.3
V12	Coahuayana, Michoacan	104.8 (100.8 a 108.9)	5.5 (5.3 a 5.7)	4.9 ± 0.2	22.7 ± 1.8	1	ND	−	0	3.1
V13	Jalisco	46.5 (45.7 a 47.2)	2.4 (2.4 a 2.5)	3 ± 0.2	6.4 ± 0.7	1	ND	+	5.1	5.1
V14	Zacatecas	234.3 (212.1 a 258.9)	12.3 (11.2 a 13.6)	2.4 ± 0.1	11.1 ± 1.0	1	α , β	−	0	5.9
V15	Coahuayana, Michoacan	73.7 (72.9 a 74.4)	3.9 (3.84 a 3.92)	4 ± 0.2	4.3 ± 1.2	1	ND	+	2.6	0.4
V16	Coahuayana, Michoacan	227.7 (226.6 a 228.9)	12 (11.9 a 12.1)	4.4 ± 0.2	0.8	1	ND	−	0	4.3
V17	Coahuayana, Michoacan	259.8 (255.3 a 264.4)	13.6 (13.4 a 13.9)	4 ± 0.1	0.7	1	ND	−	0	0.3
V18	Coahuayana, Michoacan	162.2 (156.1 a 168.5)	8.5 (8.2 a 8.9)	4.1 ± 0.1	24.6 ± 1.5	1	ND	−	0	2.9
V19	Coahuayana, Michoacan	294.1 (291.4 a 296.7)	15.5 (15.3 a 15.6)	3.9 ± 0.1	0.8	1	α , β	−	0	3.9
V20*	San Blas, Nayarit	7.25	0.5	1.8 ± 0.1	31.8 ± 1.4	1	α , β	+	15.3	13.1
V21	San Blas, Nayarit	24.6 (23.6 a 25.6)	1.3 (1.24 a 1.35)	2.6 ± 0.1	3.4 ± 0.3	1	α , β	+	5.2	2.5
V22	Coahuayana, Colima	11.8 (10.56 a 13.38)	0.6 (0.55 a 0.7)	3.3 ± 0.2	3.6 ± 0.2	1	ND	+	8.6	3.5
V23*	Coahuayana, Colima	ND	ND	ND	ND	ND	ND	+	ND	ND
V24*	Culiacán, Sinaloa	6.1 (3.5 a 7.5)	0.3 (0.2 a 0.42)	0.8 ± 0.03	29.8 ± 1.0	ND	α , β**	−	0	34.1
V25	Culiacán, Sinaloa	81.7 (78.4 a 85.8)	4.5 (4.4 a 4.8)	2.8 ± 0.03	44.8 ± 2.8	ND	ND	−	0	1.8
V26*	Culiacán. Sinaloa	18.3 (16.9 a 20.3)	1.0 (0.9 a 1.13)	1.1 ± 0.04	24.6 ± 2.2	ND	ND	−	0	19.7
V27	Culiacán, Sinaloa	118.1 (116.8 a 119.3)	6.6 (6.5 a 6.6)	2.8 ± 0.07	8.4 ± 0.6	ND	α , β	−	0	15.8

* = Juvenile specimens; ** = Partial degradation.

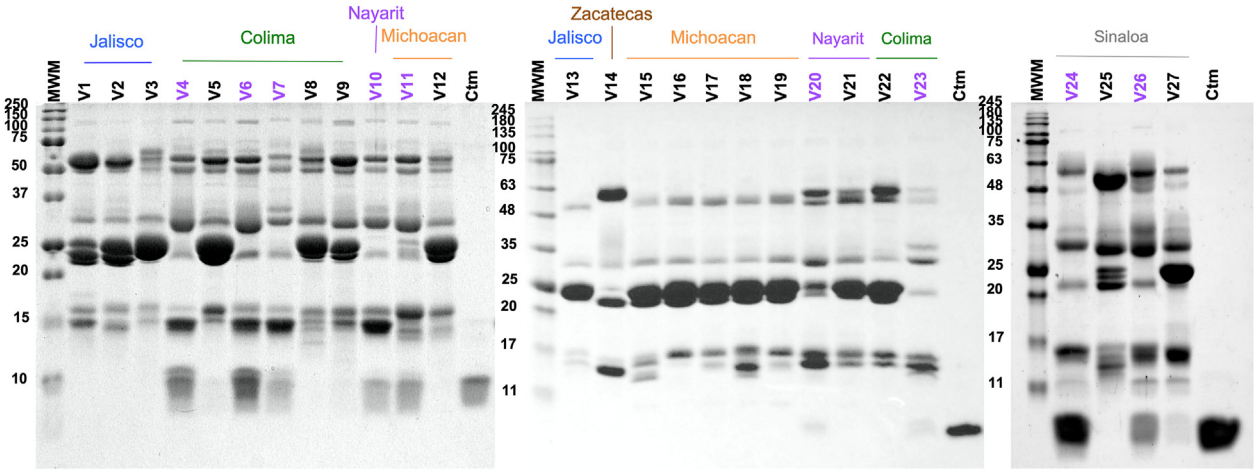

Fig. 2. SDS-PAGE (15%) of *C. basiliscus* venoms. The states of Mexico in which the individual was found is marked with a line of different colors over the IDs of each sample (Jalisco, Colima, Nayarit, Zacatecas, Sinaloa and Michoacan). Juvenile ID numbers are in purple and adults are in black. Molecular weight marker (MWM) with sizes in kDa are in lane one of each gel and two different molecular weight markers were used. Purified Crotamine (Ctm) was added as a reference from *C. durissus terrificus* venom in the last lane.

There was a significant positive relationship ($r^2 = 0.55$; $p < 0.05$) between LD_{50} and percentage of crotoxin-homologs. Ontogenetic changes were evident, juvenile venoms were significantly ($U = 5$, $P < 0.001$) more toxic (0.14–1 μg/g) than adult venoms

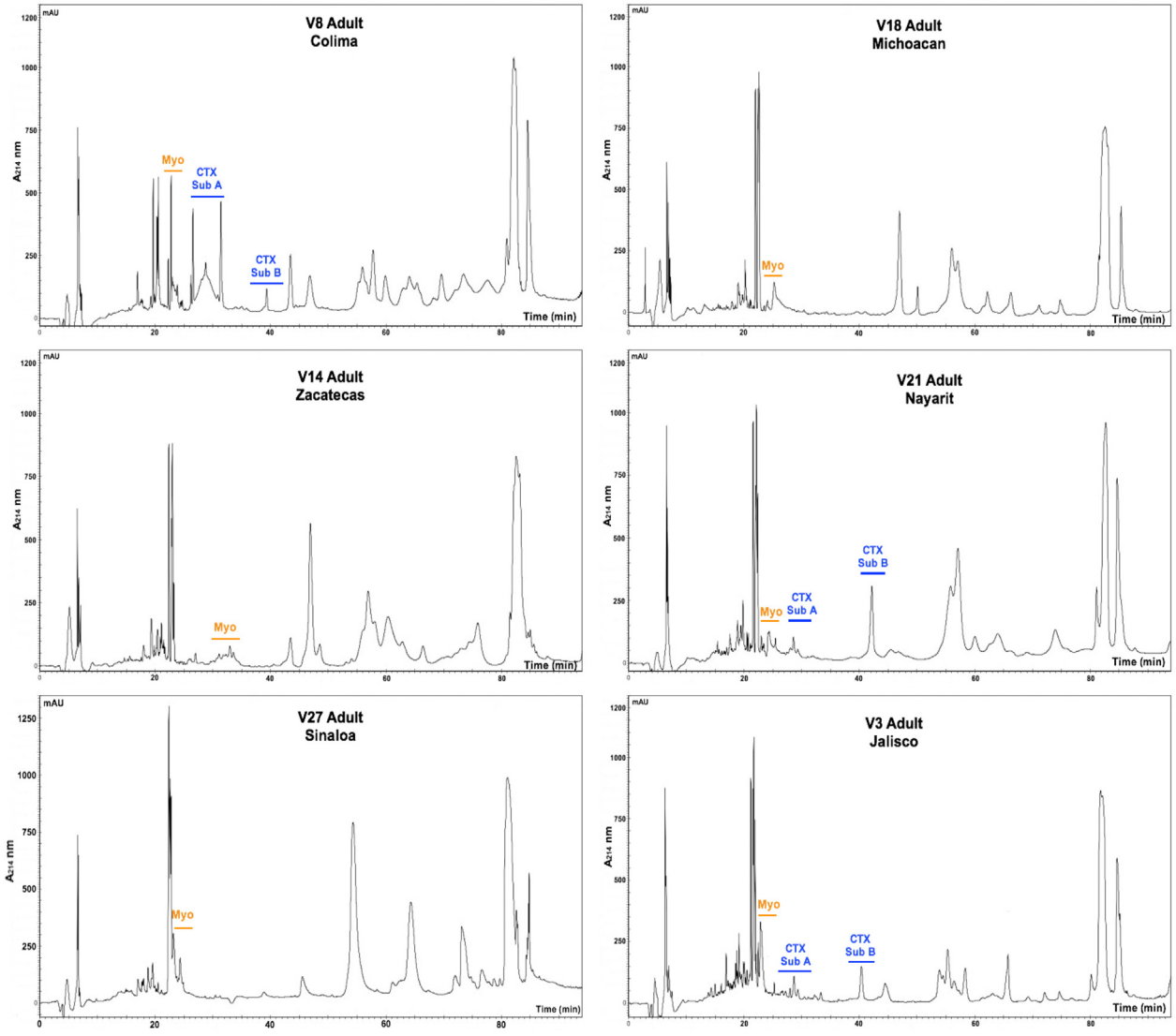

Fig. 3. RP-HPLC chromatograms of 1 mg of *C. basiliscus* venoms of adults showing intraspecific variation among locations. Remaining adult venoms are in Fig. S3. Crotamine (Myo), Crotoxin basic subunit (CTX sub B) and acidic subunit (CTX sub A) are indicated on each HPLC profile.

(0.45–15.5 μg/g). In addition, venoms from juvenile snakes provoked hind limbs spastic paralysis in all mice injected because of their crotamine content. Additionally, of the 27 venoms analyzed, 16 venoms caused flaccid hind limb paralysis caused by crotoxin. The 11 venoms that were not able to induce flaccid paralysis were the ones negative for crotoxin including some from Michoacan (V11, V12, V16–V19), the venom from Zacatecas (V14) and all the venoms from Sinaloa (V24–V27).

3.7. Hemorrhagic activity

Adult venoms presented hemorrhagic levels of 1 and 2 indicating that these venoms have high hemorrhagic activity (Table 1). Most of the juveniles had no hemorrhagic activity, although V11 and V20, juveniles from Michoacan and Sinaloa respectively, had a hemorrhagic activity of 1.

3.8. Phospholipase activity

The twenty-six venoms of *C. basiliscus* exhibited differences in their PLA_2 activity. Venoms showed activities ranging from 0.7 to 134 U/mg (Table 1). Although PLA_2 activity varied among individuals, there was no significant difference between adults and juveniles.

3.9. Crotoxin homologs detection by Enzyme-linked immunosorbent assay (ELISA)

Twenty-seven individuals were tested using ELISA. Sixteen individuals were positive for crotoxin and eleven were negative. The assay permitted us to corroborate the lack of crotoxin for specimens from Zacatecas, Sinaloa and 6 of 7 individuals from Michoacan. One venom from Michoacan (V15) was positive for crotoxin. Overall, crotoxin percentage in HPLC was variable and ranged from 0% to 22% (V7) (Table 1).

3.10. Neutralization of lethal activity by Birmex

The official Mexican regulation establishes that an antivenom must neutralize >780 LD_{50} of *Crotalus* sp. venom to be accepted in the market. Thus, the three lots of antivenom analyzed were able to neutralize more LD_{50}'s than what the official Mexican regulation established. However, there were differences among the three lots (Fig. 6). Looking at the results as milligrams of antivenom needed to

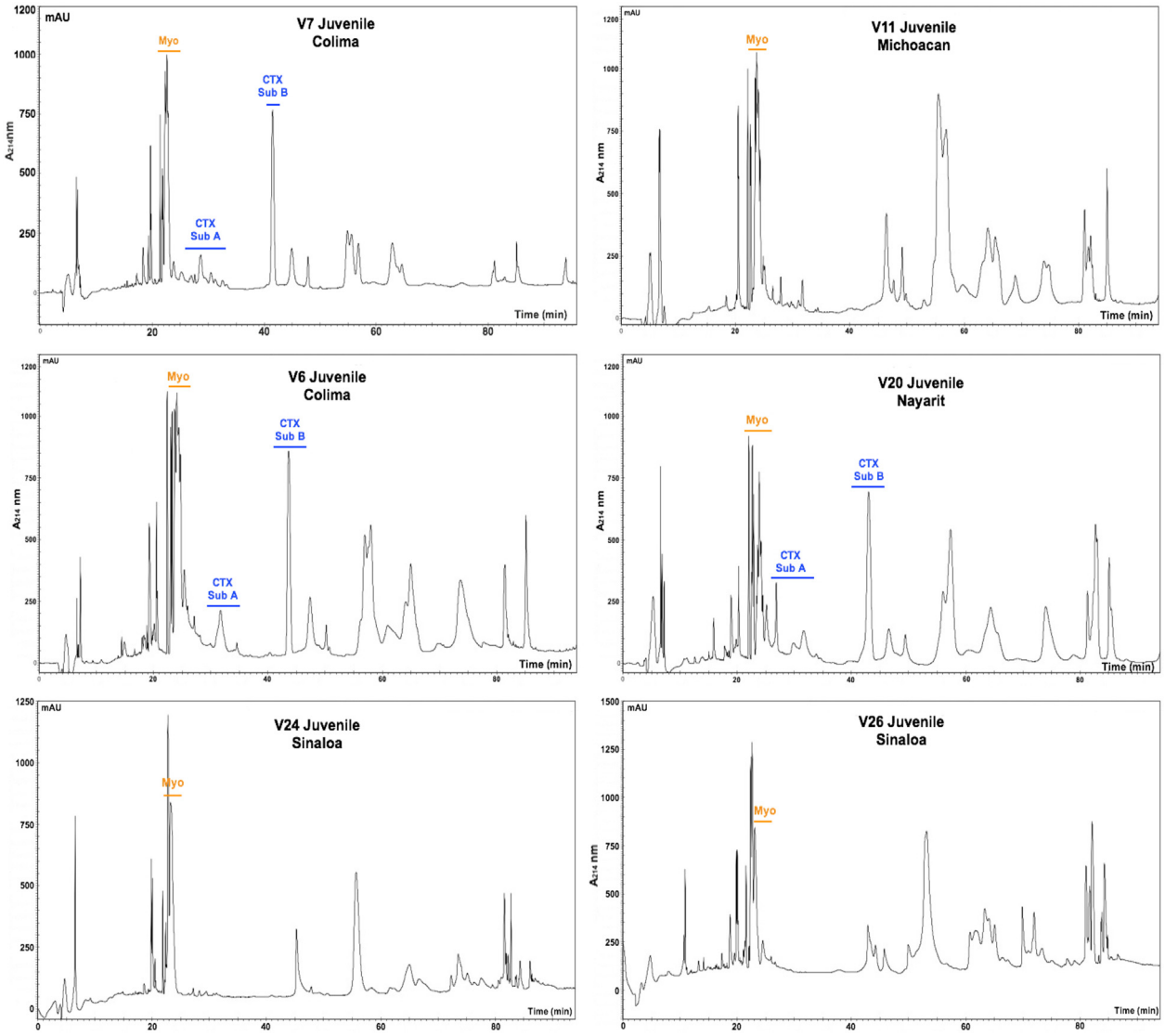

Fig. 4. RP-HPLC chromatograms of 1 mg of *C. basiliscus* venoms of juveniles showing intraspecific variation among locations. Crotamine (Myo), Crotoxin basic subunit (CTX sub B) and acidic subunit (CTX sub A) are indicated on each profile. Remaining juvenile venoms are in Fig. S3.

Table 2
Neutralization of proteolytic activity by Birmex antivenom.

	Lots of Birmex		
Venom	B1	B2	B3
	EC50 (μg)		
V2	630.5 (514.6–772.5)	502.1 (424.1–594.5)	438.2 (358.5–535.7)
V3	588.7 (473.8–731.6)	278.9 (225.5–345.0)	266 (185.1–382.2)
V9	184.7 (152.7–223.4)	302.2 (286.3–318.9)	146 (129.7–164.3)
V12	716.4 (599.5–856.1)	454.6 (361.6–571.7)	453.8 (366.7–561.6)

Effective medium concentration (EC_{50}) of three Birmex lots. Results are presented as μg of antivenom capable of neutralizing 20 μg of venom. The 95% confidence interval is indicated in parentheses.

neutralize 1 mg of venom, individual V3 (adult) was better neutralized and individual V10 (juvenile) required more antivenom (Table 3).

3.11. Recognition of venom components by Western blot

Western blot analysis was performed for 12 venoms. The protein bands best recognized by the antivenom were two that run between 50 and 75 kDa, while the lower molecular weight proteins were poorly recognized. The three lots of Birmex were able to recognize venom components ~50 kDa and between 20 and 37 kDa. B3 was better at recognizing components of lower molecular weight at ~15 kDa. In the case of lots B1 and B2, there was poor or no recognition of crotamine in the venoms. However, when lots B1 and B3 were tested on a higher concentration of pure crotamine as a control, there was slight recognition. B2 was not tested at a higher concentration (Fig. 7).

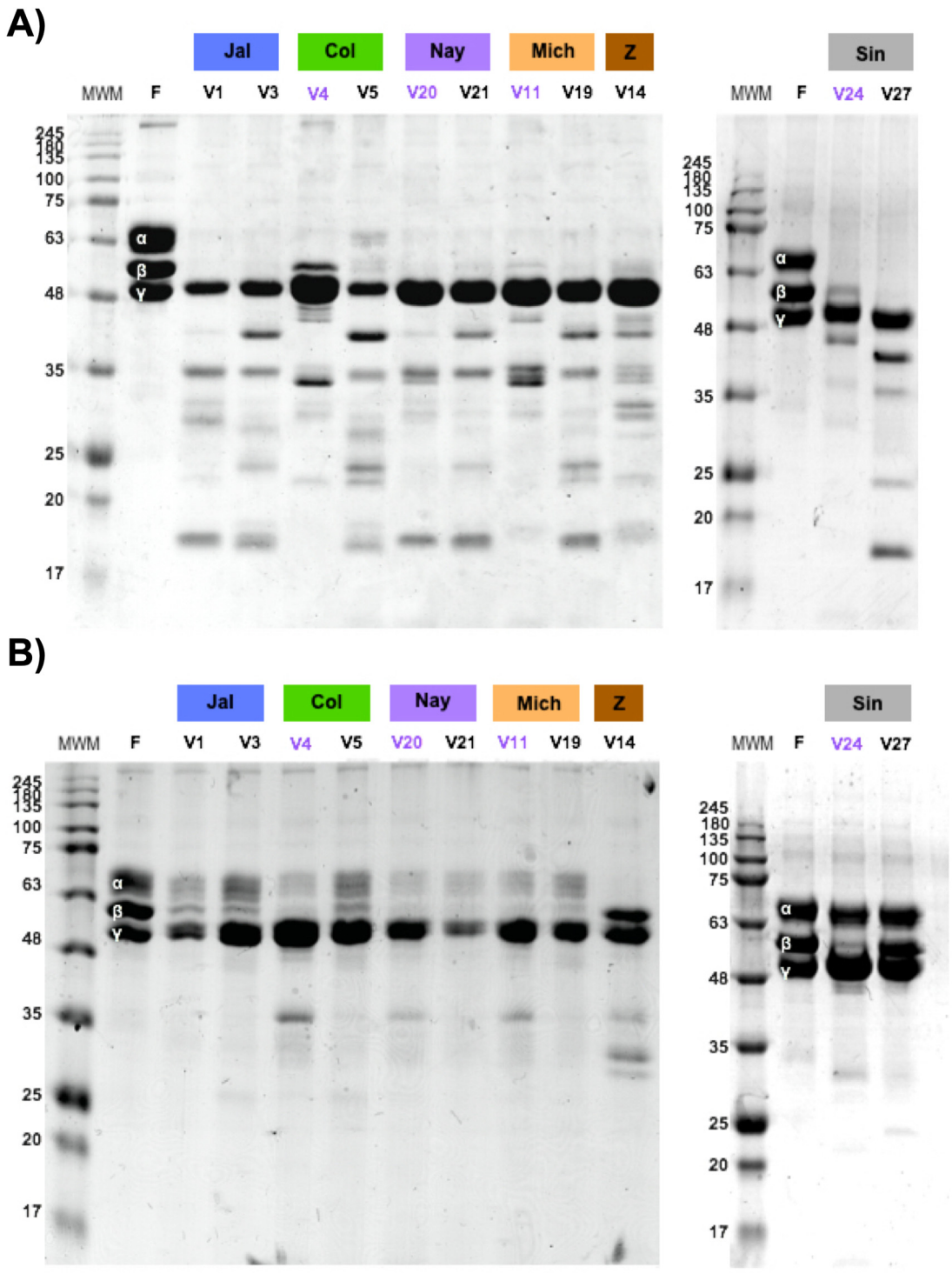

Fig. 5. Fibrinogenolytic activity of crude venoms of *C. basiliscus*. Venom ID numbers in purple and black correspond to juveniles and adults, respectively. Fibrinogenolytic activity was visualized on SDS-PAGE (12.5%). **A)** Fibrinogenolytic activity; **B)** Pre-incubation of the venom with EDTA to identify if SVMPs contribute to fibrinogen degradation. Gels were stained with Coomassie Blue.

3.12. Transcriptomics

We obtained the transcriptome of specimen V18 by isolating mRNA two ways: from the venom directly and using a venom gland biopsy. We annotated 1527 unique transcripts 50 of which were identified as putative toxins. We did not annotate either subunit of crotoxin during annotation nor was the basic subunit present when we mapped reads to a known sequence from Genbank (Accession KU666923; [37]). The proportion of individual toxins and toxin families differed between the venom transcriptome and the venom gland transcriptome (Fig. 8). PLA_2s were the most abundant toxin family when looking only at the venom transcriptome and SVMPs were the most abundant family when looking at the venom gland transcriptome (Fig. 8). Additionally, a larger proportion of reads mapped to nontoxins in the venom compared to the venom gland (Fig. 8).

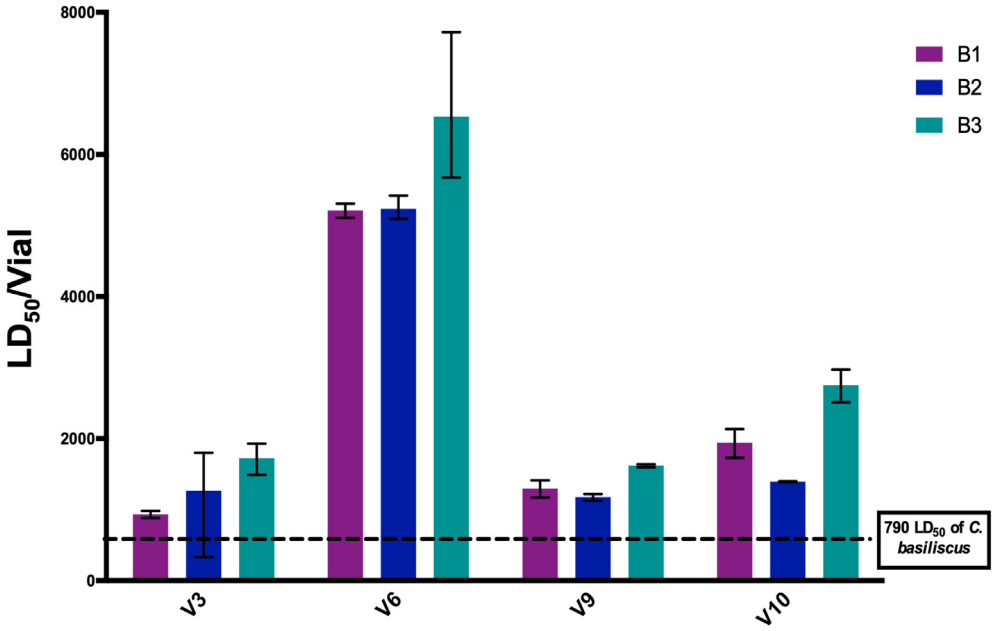

Fig. 6. Four venoms neutralized with three different lots of BIRMEX. The values are expressed as LD_{50} neutralized per vial of antivenom. The three lots of Birmex are shown in different colors (B1, B2 and B3). The dotted line indicates the minimum LD_{50} that must be neutralized per vial as specified by the manufacturer (790 LD_{50} of *C. basiliscus*).

Table 3
Neutralization of *C. basiliscus* lethality by Birmex. Lots (B1, B2 and B3).

Venom	B1		B2		B3	
	µL AV/3LD_{50}	mgAV/mgV	µL AV/3LD_{50}	mgAV/mgV	µL AV/3LD_{50}	mgAV/mgV
V9	23.2** (21.1–25.5)	10.3	25.5** (24.5–26.5)	13	18.5** (18.2–18.7)	7
V10*	15.5 (13.9–17.2)	53	21.5 (21.4–21.6)	82	10.9 (10.0–11.8)	31
V3	32.2 (30.5–33.9)	5.2	23.7 (13.6–41.2)	3.9	17.4 (15.3–19.7)	2.1
V6	5.8** (5.7 a 5.9)	13.1	5.7** (5.5 a 5.9)	13.8	4.6** (3.9 a 5.3)	9.1

* = Juvenile. ED_{50}: Median Effective Dose, dose of antivenom that induces survival in 50% of intravenous (i.v.) injected mice (18–20 g) with 3X LD_{50} of each venom. mgAV/mgV: milligrams of antivenom that neutralized 1 mg of venom. µLAV/LD_{50}: volume of antivenom that neutralized 3X LD_{50}. ** At all doses of antivenom, the mice presented rigid paralysis.

4. Discussion

We found clear geographic and ontogenetic variation in venom of *C. basiliscus* venoms from throughout its distribution. Our results make *C. basiliscus* the 4th rattlesnake species that is polymorphic for neurotoxic venom in Mexico [8,16–18,38,39] Ontogenetically, the electrophoretic and RP-HPLC chromatographic profiles showed that juvenile venoms contained crotamine as the major component and had a low percentage of SVMPs whereas adult venoms have low percentages of crotamine and had a higher percentage of SVMPs (Fig. 4). Overall, the venom composition we found for adults is similar to the study previously done on *C. basiliscus* pooled venom from 15 captive adults (location not provided) which reported that *C. basiliscus* venom had a high percentage of SVMPs (68%) but crotamine was absent [20].

In the present study, crotamine was present in all venoms but was a clear source of ontogenetic variation as juveniles generally had higher proportions (5.8–34%) of crotamine compared to adults (0.8–15.8%). Ontogenetic variation of crotamine has been widely documented in different rattlesnake species including *C. molossus nigrescens* in which adults present 0–4.3% and juveniles 2–52% [40]. In *C. durissus* it has also been documented that organisms can possess 5–29%, the percentage of crotamine was correlated to the number of copies of the gene which varied from 1 to 7 [41], in another study it was shown that the proteome of juvenile *C. culminatus* presented 11% while adults 6% of crotamine, while for the transcriptomes values where between 10.2% and 24.4%, respectively, the authors suggest that these changes are a consequence of regulation given by microRNA [11,17].

Biological activities also showed ontogenetic differences between juveniles and adults. Azocasein activity, which measures proteolytic activity, was much higher in adults compared to juveniles (Table 1) and was inhibited by EDTA indicating it was caused by SVMPs and not SVSPs. The final fibrinogenolytic activities were similar in juveniles and adults, but the rate of fibrinogen degradation was faster in adults compared to juveniles. We expected fibrinogen degradation because *C. basiliscus* have a known component, Basilase, that is a type II metalloprotease which digests fibrinogen and was purified in the 1990's [42–44]. Although SVMPs have fibrinogenolytic activity *in vitro*, it would be necessary to evaluate whether they have such activity *in vivo*, as pharmacokinetic studies have shown that SVMPs reach the blood in very low proportion [45]. As for the phospholipase activity values, we did not observe differences between juveniles and adults (Table 1).

However, the LD_{50} was lower (more lethal) for juvenile venoms and this is due to the higher amount of crotoxin-like in juvenile

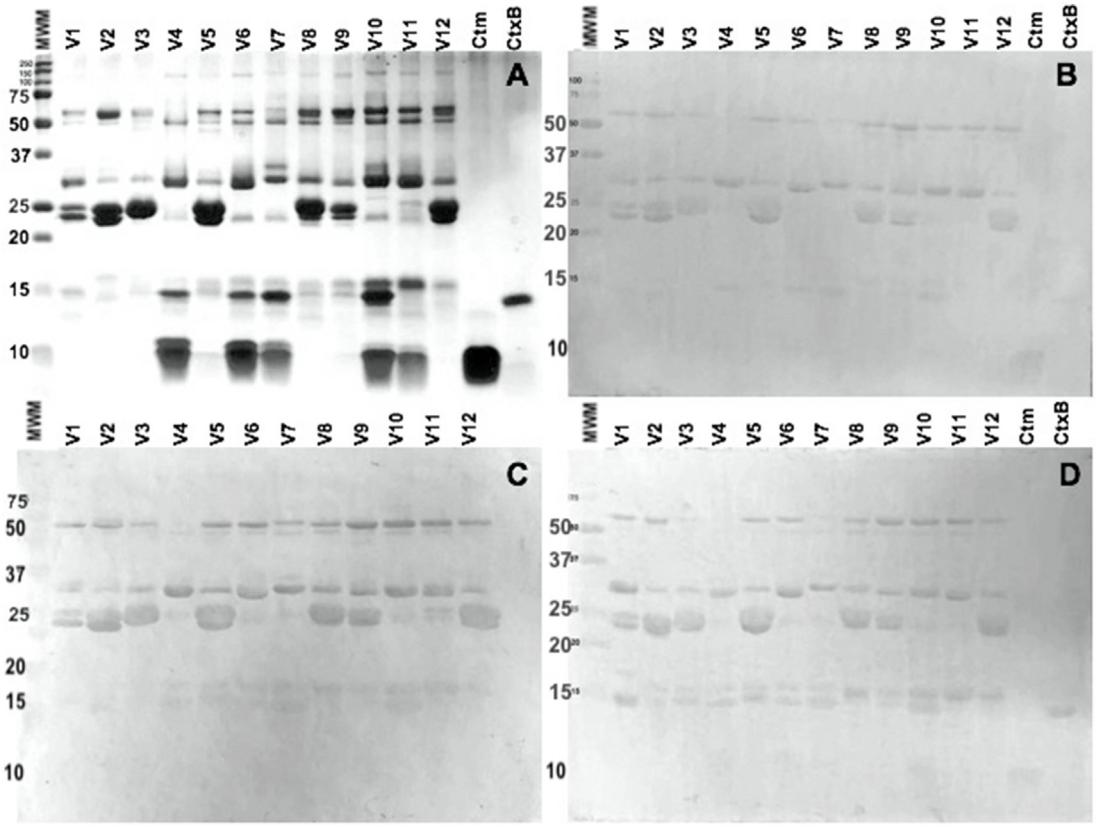

Fig. 7. SDS-PAGE and Western blot analysis of twelve representative *C. basiliscus* venoms. **A)** Venoms (5 μg) were separated by SDS-PAGE (15%). Venoms were transferred on nitrocellulose and detected with Birmex antivenom; lots B1 (**B**), B2 (**C**) and B3 (**D**). **Ctm** = crotamine and **CtxB** = crotoxin basic subunit both isolated from *C. d. terrificus* venom.

venoms and within accordance with what has been reported for other rattlesnake species (Fig. 9) [6,16,40,46], it is interesting to note that the V11 and V24 juvenile venoms do not contain crotoxin and have high lethal potency, more so than adults, suggesting that there is some component with high lethal potency. Based on Mackessy's classification [47] *C. basiliscus* juvenile venoms are type II (LD_{50} < 1 μg/g and low proteolytic activity) while most *C. basiliscus* adult venoms exhibited type I venom (LD_{50} > 1 μg/g and high proteolytic activity), moreover, there are adult samples that presented intermediate characteristics (type I + II; LD_{50} < 1 μg/g and high proteolytic activity) (V1, V9 and V22).

Crotoxin and crotoxin-like molecules (e.g., Mojave toxin, sistruxin, canebrake toxin) have been extensively studied in rattlesnakes and many species exhibit intraspecific variation in the proportion of the toxin in the venom including *C. simus*, *C. mictlantecuhtli*, *C. tzabcan*, and *C. scutulatus* among others and some that are polymorphic for the presence or absence of crotoxin (*C. s. scutulatus, C. lepidus klauberi, C. horridus, and C. tzabcan*) [5,7,11,16,18,40]. *Crotalus basiliscus* exhibited both intraspecific variation in the proportion of crotoxin for the individuals that have it as well as polymorphism as the specimens from Zacatecas and Sinaloa did not have crotoxin. Moreover, all but one of the Michoacan animals lacked crotoxin. The 11 total individuals that lacked crotoxin were all from the same general geographic area suggesting local adaptation could play a role in the polymorphism as hypothesized in *C. scutulatus* [48] which will require further studies to test. From the Michoacan population we only had one negative sample for crotoxin, so it is necessary to have more samples.

To verify that crotoxin was not being transcribed in individuals that lacked it in their venom, we generated a venom and venom gland transcriptome. We were not able to *de novo* assemble and annotate either subunit of crotoxin nor did the reads map to a known sequence of the basic subunit from *C. basiliscus*. Although we cannot confirm the gene is not in the genome, the lack of crotoxin expression in the transcriptome and the lack of the crotoxin in the venom means that functionally, *C. basiliscus* is now listed among other polymorphic rattlesnake species for the presence/absence of crotoxin. However, if *C. basiliscus* follows the pattern found in other rattlesnake species, it is likely the genes for the two subunits of crotoxin are also absent in the genome [49–51]. Overall, after we combined the venom and venom gland biopsy transcriptomes, 50 putative toxin sequences were found which is similar to other rattlesnake species [11,17]. However, there were large differences between the two transcriptome datasets (Fig. 8). The proportion of reads that mapped to the toxins was much lower for the venom transcriptome compared to the glands. The *de novo* assembly from both transcriptome datasets recovered almost all the toxins but the proportions of those toxins was different between datasets (Fig. 8). Particularly, PLA_2s and CTLs were more common in the venom transcriptome compared to the venom gland transcriptome (Fig. 8). Obtaining mRNA sequences from venom sources is feasible as has been shown in other studies [52,53], but in order to examine relative expression, gland extraction is necessary due to differences in the amount of mRNA that can be recovered from the gland.

We evaluated three lots of Birmex antivenom to determine if there was any lot variation in neutralization of *C. basiliscus* venom

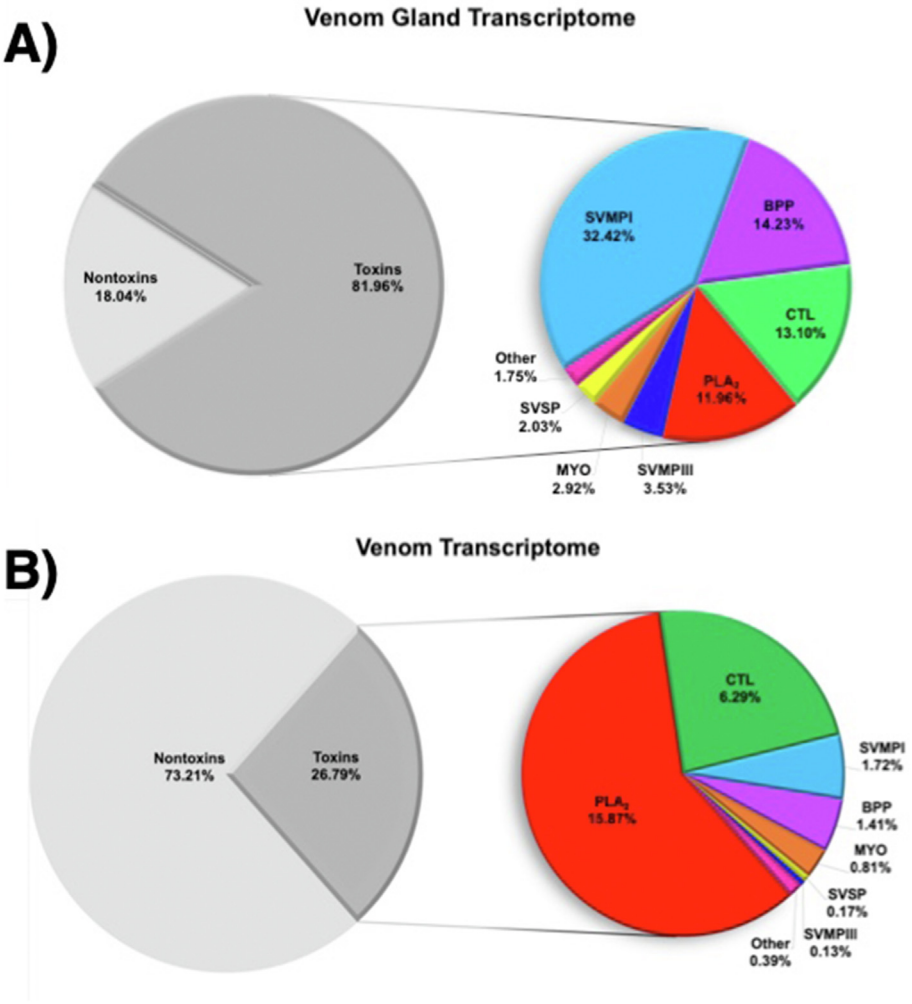

Fig. 8. Each panel shows the proportion of reads that mapped to nontoxins and toxins (left) and then the proportion of reads for each toxin family (right) for *C. basiliscus* specimen V18 (crotoxin negative). The venom transcriptome **(B)** had fewer reads map to toxins overall compared to the venom gland biopsy transcriptome **(A).** Snake Venom Metalloproteinases type I (SVMP-PI), Snake Venom Metalloproteinases type III (SVMPs-PIII), Phospholipases A_2 (PLA_2), Bradykinin-potentiating peptides (BPP), C-type lectins (CTL), Myotoxin/Crotamine (Myo) and Snake Venom Serine proteinases (SVSP).

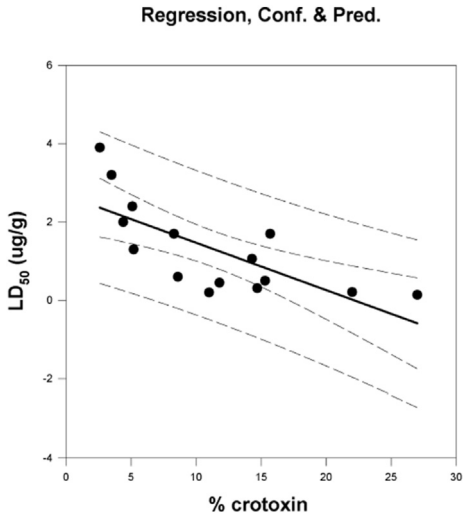

Fig. 9. Correlation of lethality (LD_{50}) and percentage of crotoxin. External dashed lines are the prediction intervals and internal dashed lines are confidence intervals.

and found that all three met the minimum standard of neutralization but varied in efficacy (Fig. 6). In the Western blot assay, it was observed that the recognition of crotamine is minimal, while the rest of the components are well recognized (Fig. 7). In the neutralizations of LD_{50}s it was confirmed that crotamine was not well neutralized because the venom from individual V6 that had 32% crotamine still exhibited rigid paralysis of the posterior extremities (Table 3 and Fig. 6). The inability of commercial antivenom to neutralize crotamine is consistent with other studies and has been shown in *C. molossus nigrescens* [40,54,55]. For snakebite victims, this results in patients suffering from myotoxic damage in addition to pain and severe muscular contractions [56]. Health professionals can use these results when treating envenomations; for instance, doctors must make sure that patients do not develop neurotoxic symptoms. They must also be aware that the effect of crotamine will not be neutralized, so they should expect rigid paralysis in the first few minutes. On the other hand, antivenom producers should pay attention to the neutralization of crotamine. Although, all three lots of antivenom were able to neutralize the crotoxin-like components, we suggest that, due to the batch variation in antivenom, manufacturers work to better homogenize lots to produce consistent results when using them to treat snakebite victims.

5. Conclusions

Our study is the first to demonstrate intraspecific variation in the venom of one of the most important rattlesnake species in Mexico, *Crotalus basiliscus*, due to the number of envenomations and because it is used as an immunogen in the production of one of Mexico's antivenoms. We documented both ontogenetic and geographic venom variation in venom components and highlighted variation in crotamine and crotoxin. The results of our work contribute with useful information in the clinical field and in the production of antivenoms as follows: 1) an envenomation from a *C. basiliscus* specimen containing crotoxin-like will have a shorter time for antivenom administration. If the antivenom is not applied quickly a neurotoxic profile may develop, leading to acute muscular weakness with respiratory involvement. On this context, these patients may also require special medical equipment such as mechanical ventilation, intensive care and prolonged hospital stays, and 2) we recommend that antivenom producers take into consideration the intraspecific variation of the species used for the production of antivenom. We advise using an immunogenic mixture with the venom of juveniles and adults covering the broadest possible distribution. Further consideration must be taken to minimize lot variation and maintain the greatest homogeneity of the product. Based on the composition and biological activities of the juvenile and adult venoms we hypothesize that the optimal ratio is 40% of venom from juvenile specimens and 60% of the mixture from adult venom. In the near future, we will verify these proportions experimentally to determine if they are the optimal ratio.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2021.10.006.

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