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Protein composition and biochemical characterization of venom from Sonoran Coral Snakes (*Micruroides euryxanthus*)

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ABSTRACT

The elapid genus, *Micruroides*, is considered the sister clade of all New World coral snakes (Genus *Micrurus*), is monotypic, and is represented by Sonoran Coral Snakes, *Micruroides euryxanthus*. Coral snakes of the genus *Micrurus* have been reported to have venoms that are predominantly composed of phospholipases A_2 (PLA_2) or three finger toxins (3FTx), but the venoms of the genus *Micruroides* are almost completely unstudied. Here, we present the first description of the venom of *M. euryxanthus* including identification of some proteins as well as transcriptomic, and biological activity assays. The most abundant components within *M. euryxanthus* venom are 3FTxs (62.3%) and there was relatively low proportion of PLA_2s (14.2%). The venom phenotype supports the hypothesis that the common ancestor of *Micrurus* and *Micruroides* had a 3FTx-dominated venom. Within the venom, there were two nearly identical α-neurotoxins (α-Ntx), one of which was designated Eurytoxin, that account for approximately 60% of the venom's lethality to mice. Eurytoxin was cloned, expressed in a soluble and active form, and used to produce rabbit hyperimmune serum. This allowed the analysis of its immunochemical properties, showing them to be different from the recombinant αNTx D.H., present in the venoms of some species of *Micrurus*. Finally, we observed that the commercial antivenom produced in Mexico for coral snake envenomation is unable to neutralize the lethality from *M. euryxanthus* venom. This work allowed the classification of *Micruroides* venom into the 3FTx-predominant group and identified the main components responsible for toxicity to mice.

1. Introduction

New World coral snakes in the family Elapidae include two genera: *Micrurus,* which includes 89 species and *Micruroides* with only one species, *Micruroides euryxanthus* (hereafter *M. euryxanthus*, Sonoran Coral Snakes [1]). The genus *Micruroides* has been consistently placed as the sister group to all other New World coral snakes (*Micrurus*) in phylogenetic analyses [2,3] with the last common ancestor occurring around 36 million years ago (Mya) [4].

Coral snakes generally cause few bites. In Mexico, between 2003 and 2018, an average of 3850 snake bites occurred per year [5] and we estimate that fewer than 2% of these are caused by coral snakes. Still, patients should be treated quickly because severe envenomations pose a risk of death due to respiratory failure [6]. The majority of coral snake bites are from *Micrurus* sp. Only four cases of bites by *M. euryxanthus* have been reported [7], and were generally mild with no fatalities [8]. Studies on the venoms of *Micrurus* have reported the presence of presynaptic and postsynaptic neurotoxins. The former belong to the phospholipase A_2 (PLA_2) family and the latter belong to the α-neurotoxic subtype of the three-finger toxin (3FTx) family [9]. Presynaptic PLA_2s act on the neuromuscular endplate of skeletal muscle, inhibiting liberation of the neurotransmitter acetylcholine (ACh) and causing destruction of the presynaptic button [10]. α-neurotoxic 3FTxs can also act on neuromuscular junctions by binding with high affinity to nicotinic ACh receptors (nAChRs) and inhibiting muscle contraction.

The relative abundance of PLA_2s and 3FTxs in venom

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composition varies greatly from species to species among *Micrurus*. A dichotomy between PLA_2-rich venoms in North American and 3FTx-rich venoms in South American *Micrurus* has been suggested [9,11], although the mechanisms that generated this pattern and its applicability to all coral snake species need further investigation [12]. It has been suggested that 3FTx-dominated venoms are the ancestral condition, while the appearance of presynaptic PLA_2s is a derived trait [9].

In contrast to the speciose genus *Micrurus*, *Micruroides* is represented by a single species with three subspecies: *M. euryxanthus euryxanthus* distributed from southern Arizona and southwestern New Mexico to northern Chihuahua and Sonora in Mexico, *M. e. australis* distributed in southern Sonora and likely Chihuahua, Mexico, and *M. e. neglectus* distributed near Mazatlán, Sinaloa, Mexico [13–16]. These are small to medium-sized snakes (between 300 and 400 mm [16]) that inhabit deserts and mesquite grasslands, though little is known about their natural history [17]. Like *Micrurus, Micruroides* feeds on small snakes (e.g., *Tantilla, Sonora, Rena,* and *Ficimia*) and some lizards (e.g., *Elgaria, Cnemidophorus* and *Plestiodon*) [13,16,18,19].

Prior to the present study, *M. euryxanthus* venom has been little investigated, although its neuromuscular action and neutralization with antivenom have been reported [20]. Yang and coworkers observed that *Micruroides* venom has postsynaptic blocking activity. Also, they found that the coral snake-specific antivenom, Coralmyn®, produced through immunization with *Micrurus nigrocinctus* venom, is ineffective at neutralizing *M. euryxanthus* venom when tested on isolated neuromuscular tissue preparations. Due to the low venom yields (reported average venom yield per individual is 0.12 mg [16]) and the difficulty of obtaining these animals in the field [17], experiments that require modest to large quantities of purified toxins are generally not possible. Heterologous expression of venom components has been previously achieved [21,22] and is an alternative that can be extremely useful for analyzing the biochemical and immunochemical properties of coral snake toxins.

The aim of this work was to perform a general analysis of the composition of *M. euryxanthus* venom using protein sequencing and transcriptomics and to characterize its biological activities. Also, we specifically tested for heterologous expression of a lethal α-Ntx to use as a tool for further analysis. These data will make it possible to classify *Micruroides* venom and test the hypothesis that it is 3FTx-dominant, given the sister position of *Micruroides* to *Micrurus*.

2. Materials and methods

2.1. Ethics statement

All animals and methods used in this study were approved by the bioethics committee of the Instituto de Biotecnología, Universidad Nacional Autónoma de México (IBt, UNAM) under project # 385 “*Caracterización functional y análisis de especificidad de venenos de coralillos Norteamericanos*”.

2.2. Experimental animals

Mice of both sexes (ICR strain) weighing 18–20 g and New Zealand rabbits weighing approximately 2.5 kg were acquired from the animal facility of the Institute of Biotechnology, UNAM (IBt, UNAM). Mice and rabbits were maintained with food and water *ad libitum*.

Venom was extracted from two *M. euryxanthus* of different subspecies. The first was an adult male *M. e. euryxanthus* from Hermosillo, Sonora with a total body length of 36 cm. The second was a juvenile *M. e. australis* of unknown sex from Los Mochis, Sinaloa with a total body length of 25 cm. Venom from the juvenile was only used to perform reverse phase HPLC and to analyze its protein profile with SDS-PAGE, whereas venom of the adult *M. e. euryxanthus* was used for all other experiments. These specimens were wild-caught under scientific collector license number SGPA/DGVS/8220/19 (SEMARNAT, Mexico).

Venom glands were obtained from two *M. e. euryxanthus* collected near Hermosillo, Sonora and in Gila County, Arizona. The first, used for transcriptomic analysis, was a juvenile of unknown sex with a total body length of 21 cm. This specimen was collected under scientific collector license number SGPA/DGVS/8220/19 (SEMARNAT, Mexico). The second was used for 3FTx cloning (See section 2.11) and was a juvenile of unknown sex kindly donated by Aaron Burk.

2.3. Venom extraction

Every ~20 days venom was manually extracted as follows. Snakes were held by the head and allowed to bite a small plastic spoon wrapped in parafilm. Venom was obtained by washing the spoon with distilled water, collected in a 1.5 mL tube, and then centrifuged at 17,600 g for 3 min. The soluble fraction was recovered, and the absorbance units (AU) were measured at 280 nm. A quartz cuvette of 1 cm in length (l) was used, assuming an extinction coefficient (E) of 1 to estimate concentration ($[\frac{mg}{ml}] = A_{280}/El$). Venoms were stored at 4 °C and used as soon as possible.

2.4. Electrophoretic analysis (SDS-PAGE)

Electrophoretic separation of whole venoms (15 μg) and fractions (5 μg) was performed on a 15% acrylamide/bisacrylamide gel, using the discontinuous system described by Laemmli [23] under reducing conditions (2-mercaptoethanol). For molecular weight estimation, 3 μL of protein standards from MaestroGEN (AccuRuller RGB PLUS Prestained Protein Ladder; 11–245 kDa) were loaded into the first lane. Gels were fixed and stained with Coomassie Brilliant Blue R-250 (Bio Rad).

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

Five hundred micrograms of adult *M. e. euryxanthus* or 250 μg of juvenile *M. e. australis* venom were separated using RP-HPLC on a C_{18} column (4.6 × 250 mm, 5 μm; XChroma™). Before loading, venom samples were dissolved in a final volume of 1 mL of solution A (0.1% TFA in MilliQ H_2O) and centrifuged at 17,600 g for 5 min. The column was equilibrated for 10 min with solution A and elution was performed at 1 mL/min with a gradient toward solution B (Acetonitrile + 0.1% TFA), as follows: 5% B for 5 min, 5–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min [24]. Absorbance was monitored at 214 nm.

2.6. Mass spectrometry (ESI-MS) and N-terminal amino acid sequencing

In order to classify venom components into protein families and to identify their amino acid sequences, we performed mass spectrometry and N-terminal sequencing of the most abundant RP-HPLC fractions. For mass spectrometry, samples were analyzed on a Finnigan LCQFleet (Thermo Scientific, San Jose, CA) mass spectrometer with an electrospray ionization system (ESI). Samples were injected by direct infusion using 60% acetonitrile and 0.1% acetic acid as the mobile phase with a flow rate of 10 μL/min. The spray voltage was 1.9 kV and pseudo-molecular ions (MH+) were detected in positive ionization mode. Mass spectrometric data were acquired manually using Tune Plus software (Thermo Fischer Scientific) and deconvolution was performed with Xtract software [25]. ESI-MS molecular masses were calculated as isotope-averaged molecular masses.

Amino acid sequencing was performed by Edman degradation in a PPSQ-31 A Protein Sequencer from Shimadzu Scientific Instruments, Inc. (Columbia, Maryland, USA). Each fraction (approximately 250 pmol) was adsorbed on a Glass Fiber TFA-treated Disk (Shimadzu).

2.7. Toxicity

Different amounts of whole venom and selected fractions were injected intravenously (*i.v.*) in a final volume of 0.2 mL of phosphate buffered saline (PBS) to groups of 3 mice [26]. The number of deaths was recorded 24 h after injection. The median lethal dose (LD_{50}) was determined using a non-linear regression as previously described [27] using GraphPad Prism v6.0.

A maximum of 3 μg of fractions were injected, via the intracranial (*i.c.*) route, to ICR mice (18–20 g of body weight) [28] to screen for components that act on the central nervous system. This allowed identification of possible toxin targets, but did not provide information regarding their role in envenomation because, given the molecular weights and proteinaceous nature of coral snake venom components, it is unlikely that they are able to pass through the blood-brain barrier [29]. Each fraction was injected into 1 to 3 mice in a maximum volume of 5 μL of PBS. A negative control mouse was inoculated with 5 μL of PBS.

2.8. Neutralization of venom and αNTx lethality

The commercial antivenom, Coralmyn® (produced in Mexico and manufactured by Bioclon, Batch #B5H-16, Expiration date: Jan 2018), as well as the rabbit hyperimmune serum produced during this study (See section 2.12), were used to analyze neutralization of *M. e. euryxanthus* lethal activity. The commercial antivenom is composed of lyophilized F(ab')$_2$ fragments purified from equine hyperimmune anti-*Micrurus nigrocinctus* sera. A mixture of 3 venom LD_{50}s and 450 μL of antivenom were preincubated for 30 min at 37 °C and injected *i. v.* in a final volume of 0.5 mL to a group of 3 mice. Additionally, rabbit hyperimmune anti-αNTx serum was tested against the main αNTx of *M. e. euryxanthus* venom and a 3FTx designated rD.H. The latter was first described in the venom glands of *Micrurus diastema*. It showed blocking activity on nAChRs and was heterologously expressed in *Escherichia coli* [22].

2.9. Transcriptomic analysis of M. e. euryxanthus venom glands

Venom glands were excised from a single *M. e. euryxanthus* and total RNA was extracted as previously described [22]. The specimen's head had been placed in RNAlater™ (Thermo Scientific). Excision or sampling of the venom gland of live individuals was not attempted because the procedure would have caused suffering.

Construction of RNA-seq libraries and sequencing was done at the University Unit of Massive Sequencing and Bioinformatics at the Institute of Biotechnology of the National Autonomous University of Mexico (UUSMB, IBt-UNAM) as previously described [30]. Briefly, library preparation was performed using a TruSeq Stranded mRNA Library Prep Kit and RNA libraries were sequenced on a Nextseq 500 Illumina sequencer, generating paired-end reads of 75 bp. Read quality was assessed using FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Transcriptome analysis was performed according to the methods of Strickland et al. [31]. Trimming and filtering of raw reads was performed with TrimGalore! v.0.5.0 and paired end reads were merged using PEAR v.0.9.6. *De novo* transcriptome assembly was done with both Trinity v.2.6.6 [32] and Extender [33]. Annotation against the UniProt/SwissProt database was performed using BlastX v.2.2.31+ searches with a minimum e-value of 10^{-4}. Unannotated transcripts were manually checked for toxin matches using BlastN. Identification and removal of duplicate and chimeric transcripts as well as transcript quantification was performed as previously described [30].

2.10. Cloning and expression of an α-NTx from M. e. euryxanthus venom glands

Given the low relative abundance of α-NTxs in the venom and their contribution to overall lethality, the main α-NTx, designated Eurytoxin, was cloned and further expressed in *Escherichia coli*. Total RNA was purified from venom glands of a single specimen of *M. e. euryxanthus* from Sonora, México, as previously described [22]. The cDNAs encoding putative 3FTxs were generated using the 3′RACE technique (Invitrogen). Briefly, total RNA was reverse-transcribed using a poly(T) primer with an adapter following the manufacturer's instructions. The resulting cDNA was amplified by PCR using an adapter primer and a specific antxPS primer encoding the conserved 3FTx signal peptide (5′*ATGAAAACTCTGCTGCTGAC 3′)* [21]. The double-stranded DNA was cloned into a pCR TOPO 2.1 vector (Invitrogen) using standard procedures, as previously described [21]. DNA sequencing was performed using universal primers and the Sanger chain termination method [34] on an ABI 3500 genetic analyzer (Thermo Fisher Scientific, Waltham MA, USA) at the Massive Sequencing Unit of IBt-UNAM. The resulting DNA sequences were analyzed using the software 4-picks and Gene Construction Kit (Textco Biosoftware).

The sequence encoding the mature peptide of Eurytoxin was determined and amplified by PCR using the respective pCR TOPO 2.1 plasmid as the template. The primers MicrurusFWBam (5′ **GGA TCC** ATG ATA TGT TAC AAC CAA CAG 3) and MicrurusRevHind (5′ **AAG CTT** TCA AGC GTT GCA TTT GCC TGT TG 3') [22], were used because of sequence similarities between Eurytoxin and known 3FTx sequences. These primers included the restriction sites *Bam*HI and *Hind*III (shown respectively in boldface) to allow proper cloning of the product in the expression vector pQE30 (Qiagen, Germany). Competent *E. coli* SHuffle® T7 (Biolabs) were transformed with the pQE30 vector containing the Eurytoxin clone. When the bacterial culture (which contained 80 μg/mL ampicillin) had an A_{600} of 0.6–0.8, protein expression was induced with 0.1 mM IPTG for 24 h at 16 °C.

After cell centrifugation, soluble proteins were isolated using the BugBuster® protein extraction reagent (Novagen, Germany). The soluble protein fraction was then loaded into an agarose-NiNTA histidine-tag purification matrix (Novagen®) and washed with 20 vols. of PBS (pH 7.2). Removal of non-specific proteins was accomplished using PBS with 25 mM imidazole, while the elution of recombinant Eurytoxin (rEury) was achieved with PBS containing 250 mM imidazole. Purified rEury was then dialyzed against PBS (pH 7.2) and stored at −20 °C.

2.11. Generation of rabbit hyperimmune serum

Two New Zealand rabbits (Identification # 22 and 23) were injected subcutaneously (*s.c.*) with increasing doses of the recombinant Eurytoxin (rEury). The first immunization was performed with 50 μg/rabbit and subsequent immunizations were done once a week in a total volume of 500 μL. The dose increased 50 μg each week to 800 μg/rabbit in weeks 13 and 14. Freund's incomplete adjuvant and aluminum hydroxide were alternated each week during injections (250 µL of toxin +250 µL of adjuvant). Blood samples (1–2 mL) were obtained from the central ear vein of each rabbit one week after the 4th, 8th and 13th immunizations. At the end of the three-month immunization period, rabbits were euthanized by exsanguination through cardiac puncture (under ketamine/xylazine anesthesia) and serum was kept at −20 °C until use.

2.12. Determination of anti-rEury antibody titers in hyperimmune rabbit sera

Antibody titers against Eurytoxin were determined using Enzyme-Linked Immunosorbent Assay (ELISA), as previously described [22]. Briefly, 96-well plates (Nunc, Maxisorp) were coated with 5 µg/mL of rEury in 0.1 M carbonate buffer (pH 9.6). Rabbit serum was added starting with a 1:30 dilution and performing serial 1:3 dilutions in reaction buffer (50 mM Tris/HCl pH 8.0, 0.5 M NaCl, 1 mg/mL gelatin, 0.05% Tween 20). Commercial HRP-coupled Goat anti-rabbit IgGs (Millipore) were used as detection antibodies at 1:6000 dilution and plates were developed using 100 µL of substrate 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (50 mg/mL) The colorimetric response was detected using an ELISA plate reader (TECAN) at 405 nm and titers were defined as the dilution at which 50% of the response was obtained. Titer data were fit to a sigmoidal dose-response curve (variable slope) using GraphPad Prism v. 8.0.

2.13. Determination of enzymatic activities

PLA_2 activity of *M. e. euryxanthus* venom and RP-HPLC fractions were analyzed with a titrimetric assay using 10% egg yolk (w/v in standard solution: 100 mM NaCl, 10 mM $CaCl_2$, 0.5% Triton X-100) as substrate, as previously described [30,35]. Initial solution pH was adjusted to 8.05 and 1 µg of venom or fraction was added to the sample. Fifty mM NaOH was used for titration. Enzymatic activity units (U/mg) were defined as the amount of NaOH (µmoles) consumed per minute per milligram of venom or fraction.

Proteolytic activity of *M. e. euryxanthus* venom was determined using a modification of the method of [36] as described elsewhere [37]. Briefly, azocasein at a concentration of 10 mg/mL in standard solution (50 mM Tris-HCl, 150 mM NaCl and 5 mM $CaCl_2$) was used as a substrate and incubated for 30 min at 37 °C with 20 µg of venom. The reaction was stopped with 200 µL of 5% trichloroacetic acid. Samples were then centrifuged at 16,800 g and 150 µL of the supernatant of each sample was added to 150 µL of 500 mM NaOH in a 96-well plate (NUNC) to determine sample absorbance at 450 nm. Units of enzymatic activity (U) were defined as a change of 0.2 in the absorbance of the sample per minute.

3. Results

3.1. Venom yields

Venom was obtained on 45% of extractions from the adult *M. e. euryxanthus*, generating 1840 µg of venom (Table 1). For the juvenile *M. e. australis*, 368 µg of venom was obtained from two extractions.

Table 1
M. e. euryxanthus venom yield.

Extraction date	Venom yield (µg)
04-Dec-18	490
21-Jan-19	300
08-Feb-19	330
06-Mar-19	0
12-Apr-19	0
24-Jun-19	320
04-Sep-19	0
03-Oct-19	0
16-Dec-19	400
11-Feb-20	0
03-Mar-20	0
Total yield	1840

3.2. Venom protein profile

In order to obtain an initial profile of venom composition of both adults and juveniles, whole venoms were separated using RP-HPLC (C_{18}) and 15% SDS-PAGE (Fig. 1). Even though the samples represent two different life stages and different subspecies, the RP-HPLC profiles share most of the abundant components. The *M. e. euryxanthus* profile is slightly more complex, showing approximately 20 peaks in total and more abundant components that elute between 30 and 40% acetonitrile. In SDS-PAGE, most components have apparent molecular weights around 10 and 25 kDa and only 2–3 minor bands were observed >25 kDa. Unfortunately, there was not enough venom from *M. e. australis* to continue characterization and further experiments were performed only for *M. e. euryxanthus*.

A pool of three venom extractions from a single *M. e. euryxanthus* was fractionated using RP-HPLC, yielding 13 major fractions (A-M) that elute between 15 and 40% acetonitrile (Fig. 2A). Each fraction was loaded on a 15% SDS-PAGE gel (Fig. 2B), which showed that they are primarily composed of proteins with apparent molecular weights between 10 and 20 kDa.

Molecular weight determination (ESI-MS) of fractions showed an abundance of components around 7 kDa (nine proteins) (Table 2). Further determination of N-terminal sequences of the most abundant fractions allowed their identification and assignment to specific protein families (Table 2). We observed that the most abundant venom components belong to the 3FTx family, representing 62.3% of the total, with 18.7% belonging to the short-chain αNTx subgroup of 3FTxs. PLA_2s represent 14.2% of the venom protein content (Fig. 2C).

3.3. Lethality and enzymatic activity

Venom of an adult *M. e. euryxanthus* had an *i. v.* LD_{50} of 26.2 µg/mouse (CI_{95} = 25.6 to 26.7) equivalent to 1.4 µg/g. Only the A and B RP-HPLC fractions were lethal (Table 3).

When injected *i.c.*, fractions E, F, and L were lethal to mice. Fraction E was initially injected into 3 mice at a dose of 3 µg/mouse and rapidly caused rigid paralysis of hind legs, whole body spasms that looked like convulsions, and death at approximately 15 min. Later, a mouse was injected with 0.5 µg of the same fraction, which caused severe agitation and made it run in circles around the cage for approximately 1 h, dying approximately 75 min after injection. One µg of fraction F also caused rigid paralysis and generalized spasms in the three injected mice. One of these mice died at 5 min and a second at 25 min after injection. The third showed the same symptoms but recovered approximately 1 h after the injection. Finally, fraction L caused tremors in both injected mice that worsened into spasms, dorso-ventral rotations, and death (45 min and approximately 3 h after injection). The remaining fractions (A-D, G-K, and M) caused no evident signs of envenomation at doses of 1 µg/mouse (Table 3).

The whole venom had a PLA_2 activity of 17.9 U/mg and showed no protease activity on azocasein (maximum of 20 µg tested). Only fractions A and B were lethal *i.v.* to mice using less than 20 µg/mouse and only four of the twelve fractions had PLA_2 activity

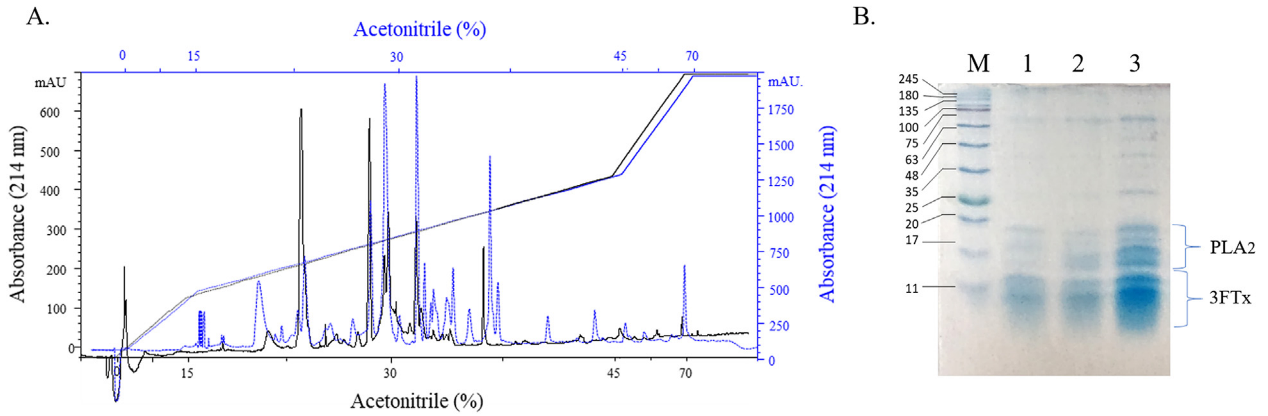

Fig. 1. Protein profiles of two *M. euryxanthus* subspecies are qualitatively similar. A) C_{18} RP-HPLC profile of the venom from 250 μg of juvenile *M. e. australis* (black) and 500 μg of adult *M. e. euryxanthus* (blue). The line on both profiles indicates percentage of solution B: acetonitrile in 0.1% TFA. B) 15% SDS-PAGE of *Micruroides* individual venoms (15 μg/lane) with the molecular weight markers (M) in kDa. First extraction of *M. e. australis* (1), second extraction of *M. e. australis* (2), and a single extraction of *M. e. euryxanthus* (3). Bands with apparent molecular weight corresponding to PLA_2 or 3FTx are indicated to the right of the gel.

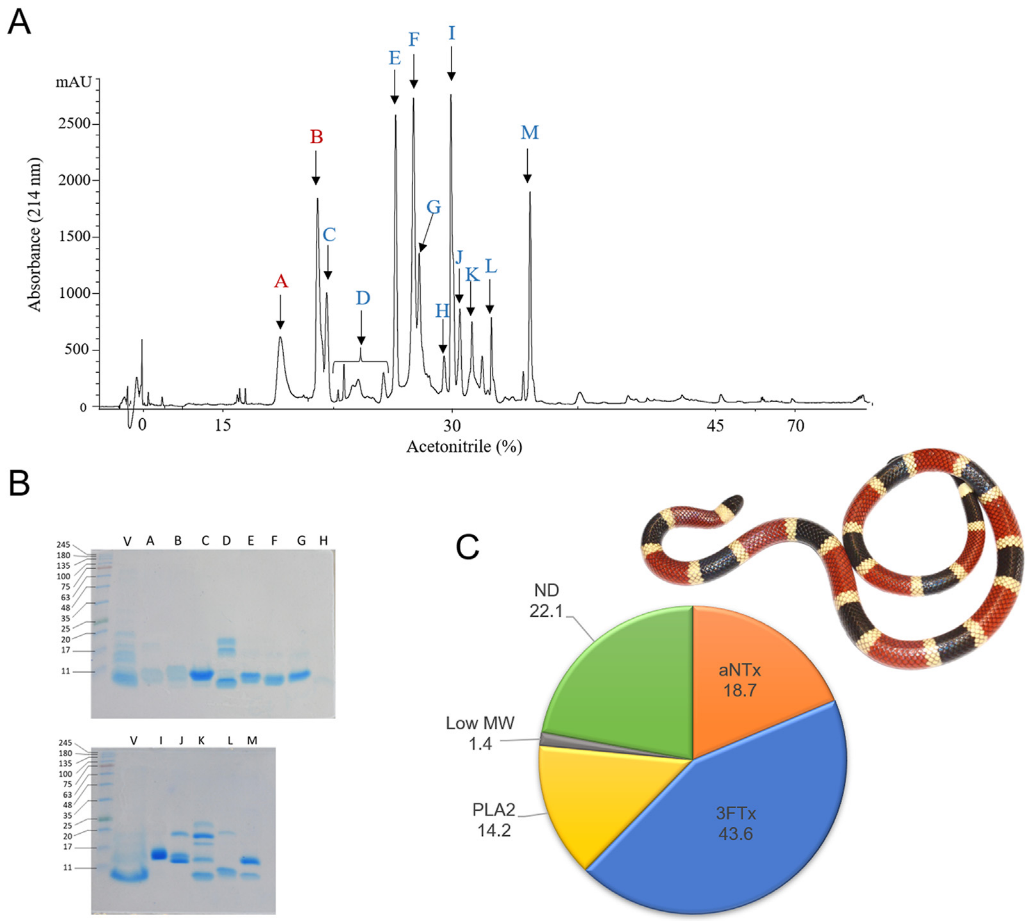

Fig. 2. *M. e. euryxanthus* venom is dominated by 3FTx. A) C_{18} RP-HPLC profile of 0.9 mg of venom from an adult *M. e. euryxanthus*. Pool of four extractions, monitored at 214 nm (mAU). Fractions are labeled as A-M. B) 15% SDS-PAGE of 15 μg of whole venom (V) and each of the RP-HPLC fractions (5 μg/lane) and the molecular marker (MPM). C) Relative abundance of protein families. *ND:* Not determined. *Low MW:* Low Molecular Weight. *PLA_2*: Phospholipases A_2. *3FTx:* Three finger toxins. *aNTx:* α-neurotoxins.

(Table 3).

3.4. Analysis of venom gland transcripts

14,137,216CHECK CHARACTER NOT SUPPORTED reads were obtained from venom glands of *M. e. euryxanthus* and are now available at the SRA database (https://www.ncbi.nlm.nih.gov/sra/PRJNA679946). From *de novo* assembly, we obtained 395 unique annotated transcripts, 38 of which were identified as toxins. Expression in the venom gland was biased towards venom

Table 2
Identification of *M. e. euryxanthus* venom fractions after N-terminal sequencing.

Fraction	RT (min)	% in venom	MW (Da)	N-terminal sequence	BLAST annotation[a] (accession #; species)	Protein family[b]
A	22.1	7.4	6618.0	MIXYNQQSSEPPTTK	MS1 (P86095.1; *M. surinamensis*)	3FTx (αNTx)
B	26.7	11.3	6620.93; 6524.0	MIXH(Y)N(F)QQSSEPPTTKTXPDGQXYKKNWSDH	Frontoxin 1 (P86420.1; *M. frontalis*)	3FTx (αNTx)
C	27.8	4.7	6808.0	nd		3FTx
D	29 to 35	5.5	*nd*	nd		
E	36.2	9.2	6512.3	LMXKKGSXNDYPNSESXPVGQNIXY	Micrurotoxin 2 (C0HJR2.1; *M. mipartitus*)	3FTx
F*	38.4	13.2	7269.3; 7374.9	NLIQFGNMITXSNSRSEGDNLDYGI	Basic PLA_2 (P20256.1; *Pseudechis australis*)	3FTx/PLA_2
G*	39.1	6.0				
H	42.1	1.4	3460.0	nd		Low MW
I	43.0	10.9	13,078.6	SLIHFGNMIHXTTNNSPLVFFNYGG	PLA_2 MALT0035C (F5CPF1.1; *M. altirostris*)	PLA_2
J	44.1	3.3	NI	NLIQFGNMIXDTNNR	PLA_2 MALT0026C (F5CPF0.1; *M. altirostris*)	PLA_2
K	45.6	3.9	7320.4	nd		3FTx
L	47.9	2.4	NI	nd		
M	52.6	6.6	7502.9	LMXKKGSDNRKPNSEAXPVGQNYTY	Micrurotoxin 2 (C0HJR2.1; *M. mipartitus*)	3FTx

*These fractions were analyzed as one for N-terminal sequence and MW determination.
RT. Retention time.
nd. Not determined.
NI. Not identified.
[a] Protein family inferred using MW, N-terminal sequence and presence/absence of PLA_2 activity.
[b] Identity search against UniprotKB/Swissprot database.

Table 3
Lethality and enzymatic activity of whole venom and each RP-HPLC fraction.

	Lethality[a]		PLA_2
	i.v.[b]	*i.c.*[c]	(U/mg ± SD)
M.e.e.	$LD_{50} = 1.39$ µg/g	nd	17.9 ± 2.4
A	$LD_{50} = 0.4–0.5$ µg/g	0/1	0
B	$LD_{50} < 0.5$ µg/g	nd	0
C	nd	0/2	0
D	nd	0/2	0
E	0/1	3/3 (0.025 µg/g)	0
F	0/1	2/3	0
G		0/3	
H	nd	0/2	0
I	0/1	0/3	38.1 ± 1.7
J	nd	0/2	69.2 ± 1.7
K	nd	0/2	9.3
L	nd	2/2	nd
M	0/1	0/1	23.3 ± 2.2

U/mg ± SD. Units of enzymatic activity per milligram ± Standard Deviation.
M.e.e. Micruroides euryxanthus euryxanthus whole venom.
LD_{50}. Median lethal Dose.
0. No enzymatic activity detected with a maximum concentration of 10 µg/mL.
nd. Not determined.
[a] Number of dead over total injected mice.
[b] Intravenous injection 1 µg/g of each fraction (unless other dose is stated).
[c] Intracraneal injection with 0.05 µg/g of each fraction (unless other dose is stated).

transcripts with these representing 47.5% of transcripts per million reads (TPMs) (Fig. 3A).

The most abundant individual transcripts and the most abundant toxin family were 3FTxs, including long chain neurotoxins (3FTx: 64.1%), followed by Kunitz protease inhibitor-like peptides (KUN:15.6%). PLA_2s are significantly less abundant (PLA_2: 12.3%; Fig. 3B and C). Translated sequences for assembled transcripts of 3FTxs, including LCNs, as well as KUN and PLA_2s are presented in Fig. 4.

3.5. Cloning and heterologous expression of eurytoxin

Using a primer specific for the conserved signal peptide of *Micrurus* 3FTxs, 12 transcripts were isolated. Eurytoxin was expressed in soluble and active form with a final yield of 2.6 mg/L of culture (Fig. 5). The recombinant protein, designated rEury, had an LD_{50} of 8.3 µg/mouse ($IC_{95} = 8.3$ to 8.3), equivalent to 0.4 µg/g. rEury caused flaccid paralysis which is characteristic of post-synaptic neurotoxin envenomation.

3.6. Recognition and neutralization of lethal activity by anti-rEury sera

Serum from rabbit 22 showed an antibody titer of 72,335 against rEury and was able to neutralize its lethal activity with an EC_{50} of 224.3 µL/3DL_{50}. Conversely, serum from rabbit 23 showed only partial neutralization with 450 µL/3DL_{50} and had an antibody titer of 37,783. Serum 22 was tested for its ability to neutralize the lethality of whole *M. e. euryxanthus* venom and the recombinant short chain α-neurotoxin rD.H. (DL_{50} = 0.15 µg/g) [22]. No neutralization was observed against either whole venom or rD.H. using the maximum volume of serum possible (450 µL); however, a delay in time of death of 30 min was observed in mice envenomated with rD.H.

Finally, the commercial antivenom, Coralmyn®, was unable to neutralize 3LD_{50}s of *M. e. euryxanthus* venom, even with the maximum amount used (450 µL of AV). No delay in time of death or amelioration of envenomation signs was observed.

4. Discussion

This is the first characterization of *M. euryxanthus* venom. We found that it has low lethality (*i.v.* $LD_{50} = 1.4$ µg/g) compared to the venoms of other North American coral snakes, such as *Micrurus fulvius* (0.3 µg/g [38]), *Micrurus tener* (1.2 µg/g [39]), *Micrurus browni* (0.2 µg/g [30]) and *Micrurus laticollaris* (0.5 µg/g [21]). The venom has a predominance of 3FTx, like venoms of many Central and South American *Micrurus* venoms, LD_{50}s of which range between 0.23 and 1.38 µg/g [40]. The relatively low lethality, together with the observed low venom yields (Table 1), likely explain the low severity of the few reported cases of human envenomation [8]. The commercial antivenom, Coralmyn®, had been reported unable to neutralize the neurotoxicity of *M. euryxanthus* venom in *chick biventer cervicis* neuromuscular preparations [20]. Here, we observed that it was also unable to neutralize the lethal activity of *M. e. euryxanthus* venom when 3LD_{50}s of venom and 3.5 mg of antivenom were preincubated and injected into mice. This is not unexpected because *Micrurus nigrocinctus* is the species used to generate the antivenom and it contains a low proportion of α-NTx

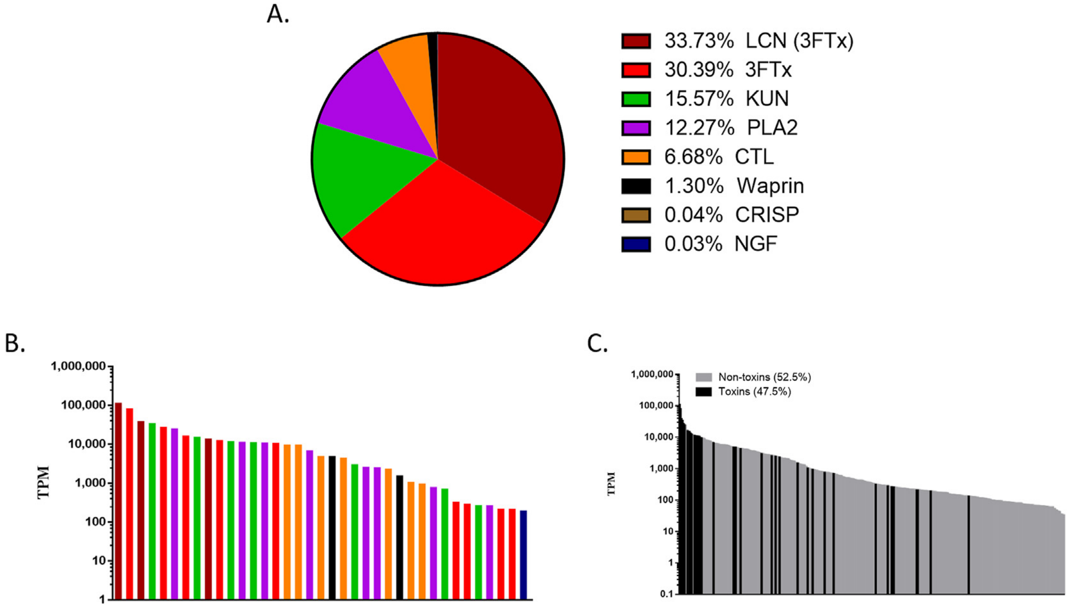

Fig. 3. mRNA expression in venom glands of *M. e. euryxanthus* shows a preponderance of 3FTxs and PLA_2s. A) Proportion of expression per toxin family based on total TPM. *LCN (3FTx)*: Long-chain Neurotoxins, belonging to the 3FTx family. *3FTx:* Three-finger toxins. *KUN:* Kunitz-type serine protease inhibitors. *PLA_2*: Phospholipases A_2. *CTL:* C-type Lectins. *CRISP:* Cysteine-Rich Secretory Peptides. *NGF:* Nerve Growth Factor. B) Expression of each toxin transcript colored by toxin family. C) Expression of toxins and non-toxins in the venom glands. Numbers in parentheses represent relative transcript abundance. *TPM*: Transcripts per million reads.

[41], which are prevalent in *M. e. euryxanthus* venom.

Due to the limited amount of venom, we were unable to perform a complete proteomic analysis of *M. e. euryxanthus* venom, so we focused on identification and characterization of the most abundant components. The general protein profiles of the two individuals from different subspecies are similar, but differences, especially regarding component abundance, are evident in chromatographic profiles (Fig. 1). A deeper analysis, including individuals from all three subspecies and different localities is necessary to assess the extent of intraspecific variation.

The protein profile shows a clear predominance of 3FTxs, specifically short chain α-NTxs (Type I) [42]. A single short-chain α-NTx, designated Eurytoxin, appears to be responsible for most of the *i.v.* lethality in *M. euryxanthus* venom. Two isoforms of this toxin (fractions A and B) which have different retention times, but very similar lethality and MWs, as well as identical N-terminal sequences, were identified in the venom (Table 3). Both in the transcriptome and during cloning of Eurytoxin, we found transcripts encoding this toxin with single amino acid differences. Unfortunately, with the available data, we are unable to be sure which ones are expressed in the venom. Together, fractions A and B represent 18.7% of venom proteins and account for between 50 and 70% of venom *i.v.* lethality. Alpha-NTxs in North American *Micrurus* venoms contribute little to venom lethality [30,39], whereas this is untrue for many South American *Micrurus*, such as *Micrurus mipartitus* [43] and *Micrurus surinamensis* [44], where α-NTxs are the main components responsible for venom lethality. It is interesting that Eurytoxin was not lethal when tested at 1 μg/mouse, *i.c* (Table 2).

In the present work we performed heterologous expression of Eurytoxin in an active, soluble form and used it to produce hyperimmune sera in rabbits. Neutralizing serum was only obtained from one of the immunized rabbits and this neutralization capacity only appeared after 13 weeks of immunization. The absence of cross neutralization with recombinant αNTx rD.H. shows that both proteins are immunochemically different, which could be explained by the relatively low sequence identity between Eurytoxin and other αNTx sequences (Approximately 70%). Fig. 6 shows an alignment with other proteins of the group from North and South American *Micrurus* venoms, as well as two other elapid snake venoms, most of which have been experimentally shown to have αNTx activity [21,22,44–50]. Even though *M. euryxanthus* is not of medical importance, the report of immunochemical variations between lethal 3FTxs in coral snakes could contribute to generation of antivenoms with wider neutralization spectra.

Furthermore, the obtained serum was not able to neutralize whole venom, indicating that there are other proteins, apart from Eurytoxin, that contribute to venom toxicity. These proteins could act synergistically, as has been previously reported for *Micrurus* [30] or additively, with several low lethality proteins accounting for venom lethality.

Our data show that transcripts belonging to the 3FTx family are extremely diverse in *Micruroides* venom glands (Fig. 4A). First, a 3FTx (Fraction E; Table 3) which has an 86% sequence identity with Micrurotoxin 2 (Accession #C0HJR2.1), was identified in the venom. Micrurotoxin 2 was reported in the venom of *Micrurus mipartitus* and modulates activity on $GABA_A$ receptors [51]. Fraction E causes apparent convulsions and death when injected *i.c.* (0.5 μg/mouse) in mice, but has no evident toxicity when injected *i.v.* (20 μg/mouse), providing evidence that this protein targets a receptor in the central nervous system. A similar, though not identical transcript that shares 75% sequence identity with Micrurotoxin 2, was identified in the venom glands. Electrophysiological tests of this fraction on various neuronal receptors are currently being performed in order to identify its possible biological target, though preliminary experiments showed no activity on $GABA_A$ receptors (Data not shown).

Even though short-chain α-NTxs have been reported as the most important 3FTxs for mammal envenomation by coral snakes [43,45], several α-NTxs that block neuronal nAChRs with varying affinities have been described in elapid venoms [52]. Three transcripts for long chain α-NTxs (Type II) [42] were also assembled, though these proteins could not be clearly identified in the venom. Further purification and N-terminal sequence determination would

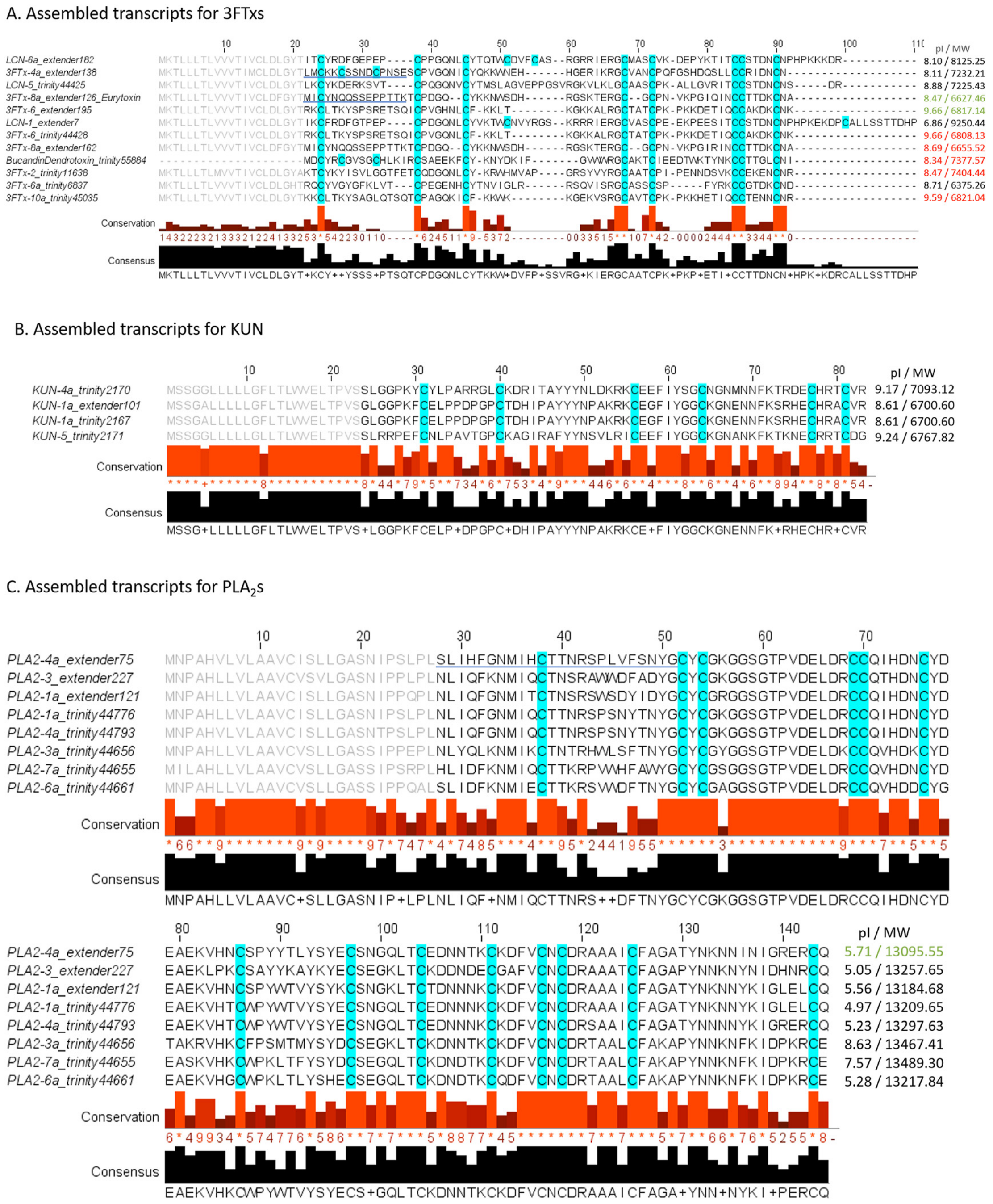

Fig. 4. Alignment of assembled transcripts. A) 3FTxs. B) KUN and C) PLA$_2$s. Signal peptides are shown in grey letters and cysteines are shown in blue. Underlined sequences were identified in the proteome using Edman degradation sequencing. Theoretical MWs of proteins that were identified in the venom are shown in green numbers (less than 2 Da difference) or red numbers (less than 20 Da difference). Conservation and consensus sequences were calculated with the software Jalview 2.10.4.

be necessary to corroborate their presence and testing with higher doses on mice might indicate that they contribute to venom lethality. Another 3FTx, with 74% identity to Bucandin (*Bungarus candidus,* accession #P81782.1), was assembled (Fig. 4) and identified in the venom based on its MW, though no N-terminal sequence was obtained (Fraction F, Table 3). Bucandin belongs to the non-conventional subgroup of the 3FTx family (Orphan group XIX) [42] and reportedly enhances acetylcholine release from presynaptic terminals [53]. This protein could therefore contribute to overall venom lethality.

Some non-conventional 3FTxs have proven lethal to non-mammal prey, such as reptiles or birds [54] and it is possible that some of the venom components hereby described possess reptilian specificity. Studies regarding the effect of coral snake toxins in natural prey are scarce, but higher lethal potency of various *Micrurus* venoms on natural prey models, mostly reptiles, has been previously reported [55]. To date, only one reptile-specific toxin has been described from the venom of *Micrurus tener* [39].

Unlike other North American coral snake venoms [21,30,38,39], PLA$_2$s represent a relatively low portion of *M. e. euryxanthus* venom

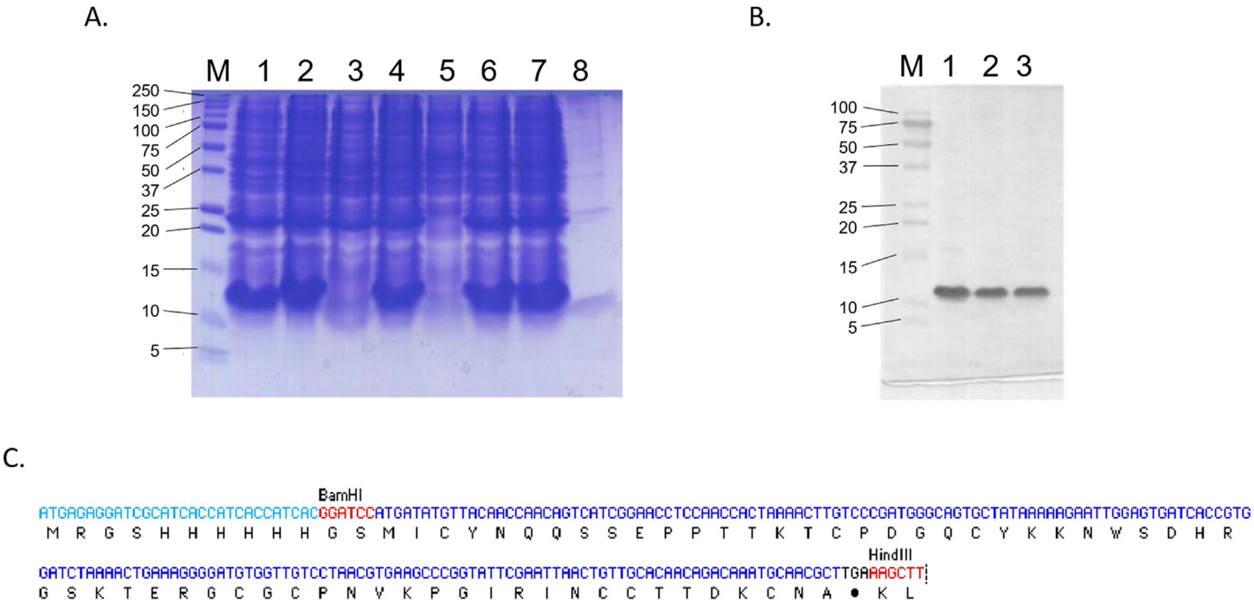

Fig. 5. Heterologous expression of Eurytoxin. A) Expression of Eurytoxin in *E. coli*. *M:* Molecular weight markers. Numbers to the left represent molecular weights of markers in kDa. *Lanes 1–7:* Supernatant from induced culture of different *E. coli* Shuffle clones. *Lane 8:* Recombinant α-neurotoxin rD.H. (for comparison). B) Western Blot with alkaline phosphatase-coupled, anti-Histidine antibody. *M:* Molecular weight markers. *Lanes 1–2:* Supernatant from induced culture of *E. coli* Shuffle clones. *Lane 3:* Positive control (rD.H). C) Nucleotide sequence of recombinant toxin rEury obtained using Sanger chain termination method (colored letters) and amino acid sequence deduced using Gene Construction Kit (black letters). Histidine tag and adapter sequence (light blue), mature peptide sequence (blue) and restriction sites (red) are marked in the nucleotide sequence.

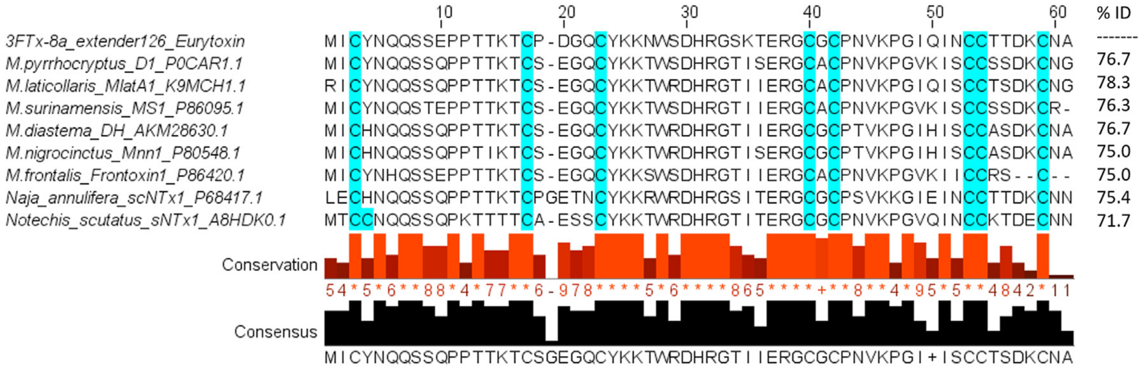

Fig. 6. Alignment of Eurytoxin to its best matching sequences available on GenBank after BlastN searches. Identity percentage (%ID), conservation and consensus were calculated with the software Jalview 2.10.4. Cysteines are highlighted in blue.

proteins and none of them were lethal to mice at 1 μg/g *i.v.* Other North American coral snake venoms, such as those of *Micrurus tener*, *Micrurus nigrocinctus*, and *Micrurus fulvius*, have between two and six components that account for most of venom lethality. In these cases, most of the lethal components were PLA_2s [38,39,56]. Even though some presynaptic PLA_2s could have higher LD_{50}s, most have high lethal potency [57]; therefore, it is likely that those present in *M. e. euryxanthus* venom have no presynaptic action and little role in venom lethality. PLA_2 activity of *M. e. euryxanthus* whole venom is lower than that observed for the previously mentioned North American coral snakes (Table 2). This could be due to variation in catalytic activity of venom PLA_2s, to the presence of non-catalytic PLA_2s (which have been described in *Micrurus* venoms [58]), or simply to the lower abundance of this protein family in the venom (Fig. 2). However, none of the PLA_2s identified that were individually tested had high enzymatic activity, as opposed to some described in the venoms of *Micrurus browni* and *Micrurus tener*. Using the same titrimetric assay performed in the present work, these two venoms have PLA_2s with enzymatic activity over 1000 U/mg [30,39]. PLA_2s with high enzymatic activity have been speculated to have roles in digestion of prey in other snake venoms [59].

Our findings regarding *M. e. euryxanthus* venom composition accord with the hypothesis of a common ancestor of *Micruroides* and *Micrurus* having a 3FTx-dominant venom [9]. After diverging from *Micruroides*, ancestors of *Micrurus* are thought to have colonized South America during the early Tertiary period, with the greatest speciation events occurring there, partly generated by the uplift of the Andean mountain range. Interestingly, a recolonization of North America is thought to have occurred later, around 11 Mya, making other North American coral snakes a consequence of the most recent speciation events of the clade [4,9]. The evolutionary history of *Micruroides* is comparatively simple [4]; thus, it is plausible that *Micruroides* has an ancestral venom phenotype. The absence of neurotoxic PLA_2s in the venom of *M. e. euryxanthus* as well as several South American triadal *Micrurus* venoms, suggests that these proteins appeared after the split of triadal and monadal *Micrurus*. Finally, the presence of abundant type II αNTx transcripts, which are considered plesiomorphic forms within the 3FTx family [60], is further evidence that this venom has ancestral characteristics.

5. Conclusions

The most abundant proteins in *M. euryxanthus* venom belong to the 3FTx family. The phenotype supports the hypothesis that the common ancestor of *Micrurus* and *Micruroides* had a predominately 3FTx venom, and that the PLA_2-predominant venoms of northern *Micrurus* species represent a derived phenotype. *M. euryxanthus* venom has a high diversity of non α-neurotoxic 3FTxs, some of which are lethal when tested *i.c.* Further work is necessary to analyze potential activities of these proteins. Even though it is relatively low in abundance, an α-NTx designated Eurytoxin, with two identified isoforms, accounts for approximately 60% of the venom's lethality. Eurytoxin was cloned and expressed in *E. coli*, in soluble, active form (rEury). Cross neutralization experiments using rabbit serum anti-rEury evidenced the presence of different immunochemical properties among 3FTxs. Finally, the commercial antivenom, Coralmyn®, is unable to neutralize the lethality of *M. e. euryxanthus* venom.

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Author contributions

Conception of work: MBV, ENC, AOR. Collection of data: MBV, ENC, NEM. Data analysis: MBV, ENC, JS, AA, AOR. Writing of manuscript: MBV, ENC. Manuscript revision and editing: AA, GA, JS, AOR. Contributed analysis tools: AA, GA, JS. The manuscript has been approved by all authors.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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