

## Venomomics of the Central American Lyre Snake *Trimorphodon quadruplex* (Colubridae: Smith, 1941) from Costa Rica



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### ABSTRACT

Rear-fanged colubrid snakes include hundreds of species globally that possess a Duvernoy's venom gland and often one-several enlarged rear maxillary teeth. We investigated the venom proteome of the Central American Lyre Snake (*Trimorphodon quadruplex*), a moderate-sized rear-fanged colubrid snake and the southernmost *Trimorphodon*, using a bottom-up proteomic approach coupled with enzyme and inhibitor assays, cytotoxicity assays and lethal toxicity assays. Several enzymes uncommonly observed in colubrid venoms were purified and characterized further. *Trimorphodon quadruplex* has a rather low complexity venome, typical of many rear-fanged snakes, but its venom contains L-amino acid oxidase, phospholipase A<sub>2</sub>, and a dimeric 3FTx, and 3FTxs dominate the proteome. Its PLA<sub>2</sub> is catalytically quite active, but it lacks myotoxicity or acute toxicity; LAAO exhibits conserved structure and appears to be highly labile. Several P-III metalloproteinases are present and hydrolyze azocasein and the α-subunit of fibrinogen but lack hemorrhagic activity. *Trimorphodon quadruplex* produces venom and retains constriction, utilizing both chemically-mediated and mechanical feeding modes.

**Significance:** We demonstrate that *T. quadruplex* venom proteins similar to those found in front-fanged snake species are present but show different biological activities. Our results underscore the importance of considering the biological roles of venoms from more than a mammal-centric perspective.

### 1. Introduction

Venoms of rear-fanged snakes remain understudied, but the potential for discovery of new compounds or novel activities is high [1–3], because this collection of families within the Colubroidea is diverse and worldwide in distribution [4,5]. Although the origin and evolution of venoms among squamate reptiles is contentious (e.g., 6–8), and the taxonomy of the advanced snakes remains uncertain (cf. 9), it is clear that among the rear-fanged venomous species formerly assigned to the single family Colubridae, venom production is a common trophic adaptation [10–12]. As with front-fanged snakes, venom in rear-fanged snakes is used to facilitate prey capture, but venom toxin diversity is typically lower [2]. Deployment of these chemical weapons typically requires longer contact time with prey, as hollow fangs are absent and venom is delivered under lower pressure than that in front-fanged snakes [13]. The presence of taxon-specific three-finger toxins (3FTxs) has been documented in several species (*Boiga dendrophila*: 14; *Boiga irregularis*: 15; *Oxybelis fulgidus*: 16); these toxins are lethal toward

lizard and/or bird prey but are essentially non-toxic toward mammals. Recently, venom from a single species with two different taxon-specific toxins was described in *Spilotes sulfureus*; a dimeric toxin, sultidotoxin, similar to irditoxin from *B. irregularis* venom, is lethal to lizards but harmless to mammals, and a monomeric 3FTx, sulmotoxin, is lethal to mammals but nontoxic to lizards [17]. Thus, among rear-fanged snakes one sees novel adaptations involving venoms and prey capture, suggesting that other unique venom toxins will be found among the currently unexplored venoms.

The genus *Trimorphodon* (Lyre Snakes) comprises seven species of rear-fanged colubrid snakes, that are distributed from southwestern United States (*T. lambda*, *T. vilkinsonii*) along the Pacific lowlands of Mexico (*T. lambda*, *T. paucimaculatus*, *T. vilkinsonii*, *T. biscutatus*, *T. lyrophanes*, *T. tau*) to Central America (*T. biscutatus*, *T. quadruplex*), with the southernmost limit of distribution in northwestern Costa Rica ([18,19]; [www.reptiledatabase.org](http://www.reptiledatabase.org)). Lyre Snakes are nocturnal predators of a variety of prey, including lizards, birds and mammals, and they possess a Duvernoy's venom gland [20] and one to several grooved

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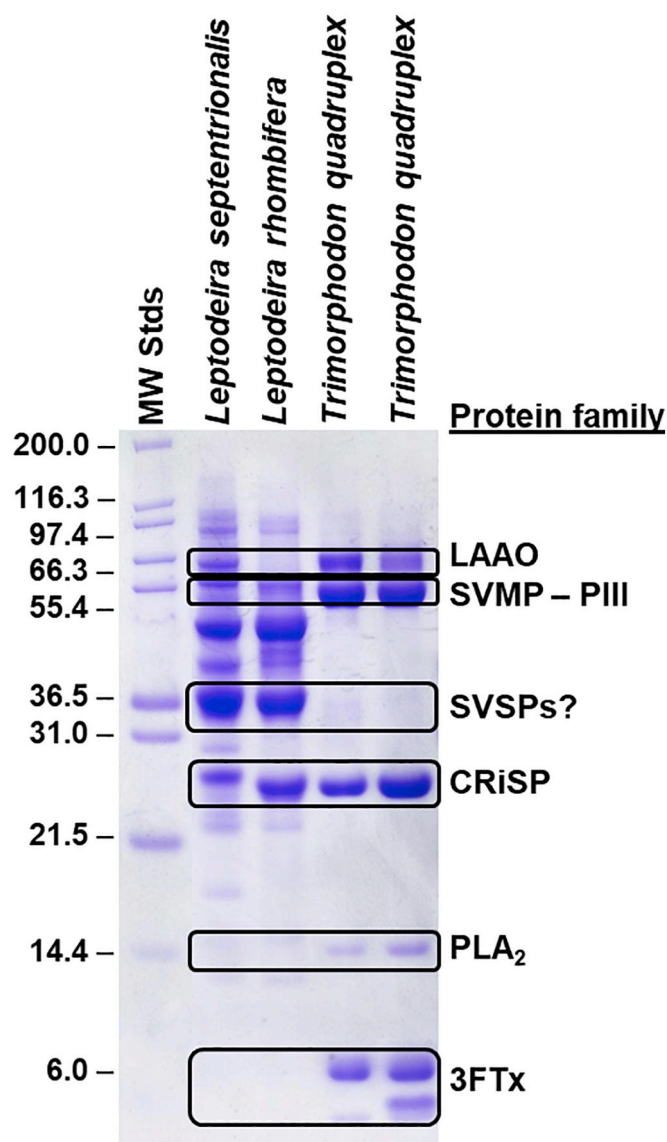


Fig. 1. Reduced venoms from rear-fanged snakes. Two Costa Rica species of Cat-eyed Snakes (*Leptodeira*) were included for comparison with *T. quadruplex*.

and enlarged rear maxillary teeth. Though lay press accounts commonly state that the species possess “weak” venoms, relatively little has been done to evaluate the toxicity of their venom toward native prey or to provide a description of the venom proteome. Several enzyme activities, including phospholipase  $A_2$ , which is uncommon among colubrid venoms, have been described from the venom of *T. biscutatus lambda* (now *T. lambda*) [21], and partial sequence and activity of the venom  $PLA_2$  was reported [22], but venom from the other six species has not been investigated. A cDNA-based study of numerous venom gland transcripts also included sequence data for some of the most abundant Duvernoy’s venom gland components of *T. biscutatus* [23], but secreted venom proteins were not investigated. Herein we report the proteome of the Costa Rican Lyre Snake, *T. quadruplex*, analyze several prominent enzyme components and evaluate the toxicity of the venom and select toxins toward inbred mouse and non-mammalian models.

## 2. Materials and methods

### 2.1. Venom

Venom was obtained from 4 adult *T. quadruplex* specimens,

collected in localities of the Nicoya Peninsula region (Guanacaste province) of Costa Rica, and kept at the Serpentarium of Instituto Clodomiro Picado, University of Costa Rica (Museum of Zoology UCR accession numbers MZUCR 23332–23,335). Venom samples were obtained using pilocarpine stimulation under ketamine anesthesia as described [24], using doses of 25–30  $\mu\text{g/g}$  snake body mass of ketamine-HCl and 6  $\mu\text{g/g}$  pilocarpine-HCl. Venom collected was centrifuged, lyophilized, and stored at  $-20^\circ\text{C}$  until analyzed.

### 2.2. SDS-PAGE fingerprinting

Twenty  $\mu\text{g}$  of venom were run on NuPage 12% acrylamide SDS-PAGE with MES running buffer as described previously [25]. Two samples from adult *T. quadruplex* and one sample each of *Leptodeira septentrionalis* and *L. rhombifera* (formerly *L. annulata*) were treated with 50 mM DTT and run at 100 mA for approximately 45 min. Gels were then stained, destained and imaged on an HP Scanjet.

### 2.3. Proteomic profiling

The bottom-up “snake venomomics” analytical strategy [26] was followed. In brief, venom (2 mg) was decomplexed by an RP-HPLC step as described [27], followed by one-dimensional SDS-PAGE of each of the obtained fractions. Coomassie-stained protein bands were excised and in-gel digested overnight with sequencing-grade trypsin (Sigma) after reduction and alkylation in an automated workstation (Intavis, Germany). The resulting peptides were subjected to MALDI-TOF/TOF mass spectrometry analysis on a Proteomics Analyzer 4800 Plus (Applied Biosystems) instrument, and peptides were assigned to known protein families contained in the UniProt/SwissProt database (Serpentes, December 2017) based on sequence identity, using ProteinPilot® and the Paragon® algorithm (ABSciex) at a confidence level of  $\geq 95\%$  [27]. In addition, several sequences were obtained by *de novo* manual reconstruction of the spectra. The relative abundance of each protein (% of total venom proteins) was estimated by integration of the RP-HPLC peak signals at 215 nm, using ChemStation® B.04.01 software (Agilent). In the case of HPLC peaks presenting more than one SDS-PAGE band, percent distribution was assigned by densitometry, using a ChemiDoc® recorder and Image Lab® v.2.0 software (Bio-Rad).

### 2.4. In vitro assays

#### 2.4.1. Phospholipase $A_2$ activity on NOBA

Variable amounts of crude venom (in 25  $\mu\text{L}$  of 10 mM Tris, 10 mM  $\text{CaCl}_2$ , 100 mM NaCl, pH 8.0 buffer) were added to 25  $\mu\text{L}$  of the synthetic monodisperse substrate 4-nitro-3-octanoyloxy-benzoic acid (4-NOBA; [28,29]), dissolved at 1 mg/mL in acetonitrile). Reaction wells were filled with 250  $\mu\text{L}$  of the same buffer, and incubated for 60 min at  $37^\circ\text{C}$  for color development. Final absorbances were recorded at 405 nm in a Multiskan reader (Thermo) and activity was expressed as the absorbance change in comparison to the substrate alone. Assays were performed in triplicate. For comparison, the crude venoms of *Naja melanoleuca* and *Micrurus nigrocinctus* were assayed in parallel. In addition, to confirm the enzymatic activity of the major  $PLA_2$  identified in peak 20 of the RP-HPLC separation of the venom, variable amounts of this protein were assayed identically as the venoms.

#### 2.4.2. Proteolytic activity on azocasein

Variable amounts of crude venom (dissolved in 20  $\mu\text{L}$  of 25 mM Tris, 0.15 M NaCl, 5 mM  $\text{CaCl}_2$ , pH 7.4) were added to 100  $\mu\text{L}$  of azocasein (10 mg/mL, dissolved in the same buffer), and incubated for 90 min at  $37^\circ\text{C}$ . The reaction was stopped by adding 200  $\mu\text{L}$  of 5% trichloroacetic acid. After centrifugation, 100  $\mu\text{L}$  of each supernatant were transferred to 96-well microplates, mixed with 100  $\mu\text{L}$  of 0.5 M NaOH, and absorbances were recorded at 450 nm using a Multiskan reader (Thermo) [30]. Activity was expressed as the increase of absorbance at 450 nm in

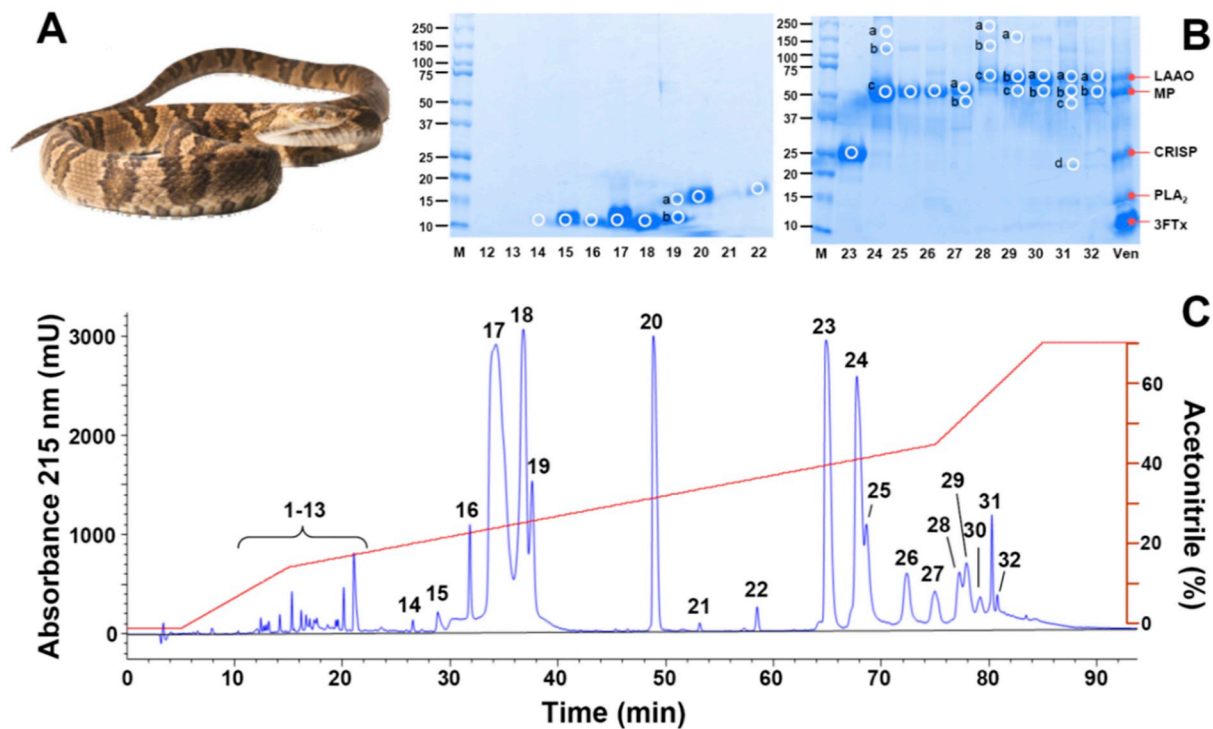


Fig. 2. RP-HPLC fractionation and SDS-PAGE of Costa Rican Lyre Snake (*T. quadruplex*) venom proteins. A. *Trimorphodon quadruplex*. B. SDS-PAGE of venom fractions. C. RP-HPLC fractionation of 2 mg *T. quadruplex* venom.

comparison to the substrate alone. Assays were performed in triplicate. For comparison, the crude venoms of *Naja melanoleuca* and *Bothrops asper* were assayed in parallel. Activity of fractionated venom proteins was also assayed with the same substrate using the method described in Smith and Mackessy [25] and recording absorbance at 342 nm.

#### 2.4.3. L-amino acid oxidase activity

L-amino acid oxidase activity was determined using the method of Kishimoto & Takahashi [31]. Increasing quantities (1–10 µg) of *T. quadruplex* venom in 10 µL 50 mM borax buffer pH 8.5 were added to wells of a microtiter plate, and then 90 µL of a reaction mixture containing 5 mM L-methionine, 2 mM *o*-phenylenediamine, and 0.8 U/mL horseradish peroxidase (Sigma #P8375-1KU) in borax buffer (pH 8.5) was added to each well. After incubation at 37 °C for 30 min, the reaction was stopped with 50 µL of 2 M H<sub>2</sub>SO<sub>4</sub>, and absorbance was recorded at 492 nm. To evaluate relative substrate specificity, different L-amino acids (L-Leu, L-Phe, L-Asn, L-Asp and L-Cys) were substituted for L-Met, and assays utilizing 10 µg venom were conducted as above. For comparison, venoms of *Naja melanoleuca* and *Bothrops asper* were assayed in parallel.

#### 2.4.4. Cytotoxicity assays on MCF-7 breast cancer cells

Toxicity of *T. quadruplex* venom toward MCF-7 cells was evaluated as described previously [32]. In brief, cells in Eagle's minimum essential medium (EMEM; ATCC) were seeded into 96 well plates (100 µL) at a density of 250,000 cells/mL. Venom (in 10 µL medium) was added at 0, 10 or 50 µg/well (total volume 110 µL), incubated for 24 h at 37 °C and 5% CO<sub>2</sub> and then cytotoxicity was measured using the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay [33]. All assays were run in quadruplicate, and results were compared to a standard curve of untreated cells at 10,000–250,000 cells/well.

#### 2.5. Isolation of toxins

Venom (14 mg) was dissolved in 25 mM HEPES buffer pH 6.8 containing 100 mM NaCl and 5 mM CaCl<sub>2</sub>, and size exclusion HPLC fractionation was performed on a Waters HPLC system operating under Empower software with a Yarra 3 µm SEC-2000 300 × 7.80 mm column (Phenomenex, USA) and a flow rate of 0.4 mL per minute of the same buffer for 120 min. Fractions were assayed for metalloproteinase and LAAO activity as above, and proteins in peak I were desalted and concentrated using 10 k cutoff spin columns. This material was then taken up in 20 mM Tris-HCl buffer pH 8.2 and subjected to anion-exchange chromatography using a Tricorn MonoQ column (5 × 50 mm) run on a GE FPLC. Bound proteins were eluted with a linear gradient of 20 mM Tris-HCl pH 8.2 containing 500 mM NaCl. Metalloproteinase and LAAO-containing fractions were identified as above.

#### 2.6. Mass spectrometry and amino acid sequencing

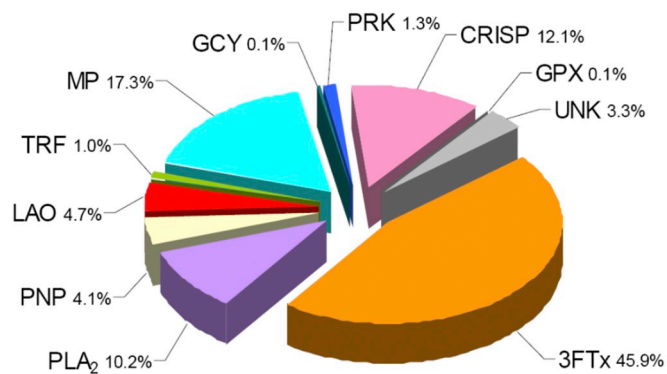
Intact protein masses of selected peaks from the RP-HPLC venom separation were determined by MALDI-TOF, on a model 4800 Proteomics Analyzer (Sciex). Proteins were mixed with an equal volume of a saturated solution of sinapinic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid, spotted (1 µL) onto an OptiTOF target plate and dried. TOF spectra were acquired in positive linear mode using 500 laser shots at an intensity of 3900. External calibration was performed with CalMix-5 standards (Sciex) spotted on the same plate.

The phospholipase A<sub>2</sub> enzyme present in peak 20 of the RP-HPLC venom separation was sequenced by a combination of direct N-terminal Edman degradation (Shimadzu PPSQ-31) and tandem mass spectrometry analysis of peptides obtained from the digestion of the protein with trypsin or chymotrypsin. The protein digest was separated by HPLC and the collected peptide fractions were subjected to MALDI-TOF/TOF in positive reflector mode, to derive *de novo* sequences, as previously described [34].

**Table 1**Summary of the MS/MS protein family assignments of SDS-PAGE protein bands from RP-HPLC peaks of *Trimorphodon quadruplex* venom.

Peak	Mw (kD)	Area (%)	m/z	z	Peptide sequence	Conf	Score	%cov	UniProt best match	Protein family
1–13	–	4.1	–	–	–	–	–	–	–	Peptide/non-protein
14	9	0.5	1891.9	1	Q <sup>py</sup> AIGPPYGLCFQC�NQK <sup>fo</sup>	99.0	17	20.8	$\alpha$ -colubritoxin, P83490, <i>Coelognathus radiatus</i>	Three-finger toxin
15	9	1.3	1891.9	1	Q <sup>py</sup> AIGPPYGLCFQC�NQK <sup>fo</sup>	99.0	17	20.8	$\alpha$ -colubritoxin, P83490, <i>Coelognathus radiatus</i>	Three-finger toxin
16	9	1.7	1891.9	1	Q <sup>py</sup> AIGPPYGLCFQC�NQK <sup>fo</sup>	99.0	17	20.8	$\alpha$ -colubritoxin, P83490, <i>Coelognathus radiatus</i>	Three-finger toxin
17	9	26.9	1891.9	1	Q <sup>py</sup> AIGPPYGLCFQC�NQK <sup>fo</sup>	99.0	17	20.8	$\alpha$ -colubritoxin, P83490, <i>Coelognathus radiatus</i>	Three-finger toxin
18	9	12.9	1160.5	1	SPHCFIEDR	99.0	14	18.0	Three-finger toxin-2, A0A193CHM5, <i>Trimorphodon lambda</i>	Three-finger toxin
19a	13	1.3	969.5	1	Q <sup>py</sup> AIGPPFTR	99.0	14			
			1166.5	1	TYSYTCSEK	99.0	10	24.3	PLA2(II)-Tri1, A7X418, <i>Trimorphodon biscutatus</i>	Phospholipase A2
			1929.9	1	ASKHPACTSFLDSAYFK	99.0	8			
			1643.7	1	HPACTSFLDSAYFK	99.0	18			
19b	9	2.6	1555.6	1	CC <sup>dl</sup> QVHDDCYDR	84.2	10			
			1160.5	1	SPHCFIEDR	99.0	11	18.0	Three-finger toxin-2, A0A193CHM5, <i>Trimorphodon lambda</i>	Three-finger toxin
			1430.6	1	NRSPHCFIEDR	99.0	9			
			969.5	1	Q <sup>py</sup> AIGPPFTR	99.0	12			
20	13	8.2	1174.5	1	SP <sup>py</sup> HCFIEDR	97.9	10			
			1943.9	1	ASKHP <sup>py</sup> ACTSFLDSAYFK	99.0	16	11.2	PLA2(II)-Tri1, tr A7X418, <i>Trimorphodon biscutatus</i>	Phospholipase A2
			1929.9	1	ASKHPACTSFLDSAYFK	99.0	14			
			1643.7	1	HPACTSFLDSAYFK	99.0	16			
			1929.9	1	ASKHPACTSFLDSAYFK	99.0	12			
21	13	0.5	–	–	–	–	–	Unknown	Unknown	
22	13	0.7	1929.9	1	ASKHPACTSFLDSAYFK	99.0	13	17.1	PLA2(II)-Tri1, A7X418, <i>Trimorphodon biscutatus</i>	Phospholipase A2
			1643.8	1	HPACTSFLDSAYFK	99.0	13			
23	26	12.1	1931.0	1	NVDFNSESPRRPEKQK	99.0	10	24.2	Cysteine-rich secretory protein TRI1, Q2XXP4, <i>Trimorphodon biscutatus</i>	CRISP
			1886.9	1	SLVQQNSCQHDWTRK	99.0	8			
			1674.8	1	NVDFNSESPRRPEK	99.0	11			
			1758.8	1	SLVQQNSCQHDWTR	97.8	7			
24a	154	0.2	–	–	–	–	–	Unknown	Unknown	
24b	117	0.9	–	–	–	–	–	Unknown	Unknown	
24c	63	9.3	586.2	2	CAFLFCTPR	<i>de novo</i>			Metalloproteinase, ACS74988, <i>Philodryas ofersii</i>	Metalloproteinase
			571.3	2	CGMLFCTPR	<i>de novo</i>				
			863.9	2	KENGVNIPCEPQDVK	<i>de novo</i>				
25	63	3.1	1726.8	1	KENGVNIPCEPQDVK	96.6	9	2.4	Metalloproteinase, C6JUN2, <i>Philodryas ofersii</i>	Metalloproteinase
26	63	2.9	1726.8	1	KENGVNIPCEPQDVK	96.6	9	2.4	Metalloproteinase, C6JUN2, <i>Philodryas ofersii</i>	Metalloproteinase
27a	63	1.9	1029.5	1	RLSVHN <sup>da</sup> FR	99.0	11	1.1	Retinoblastoma-associated protein, V8PG10, <i>Ophiophagus hannah</i>	Transcription factor
			1834.9	1	V <sup>cm</sup> AVTM <sup>ox</sup> AH <sup>dl</sup> ELGHNLGMR	97.3	9			leucurolysin-A, P84907, <i>Bothrops leucurus</i>
27b	56	1	1029.5	1	RLSVHN <sup>da</sup> FR	99.0	11	1.1	Retinoblastoma-associated protein, V8PG10, <i>Ophiophagus hannah</i>	Transcription factor
			–	–	–	–	–	–	–	–
28a	165	0.1	1369.8	1	YIELLIVVDHK <sup>fo</sup>	99.0	11	9.4	Metalloproteinase, ALA20849, <i>Macropisthodon rudis</i>	Metalloproteinase
28b	122	0.2	–	–	–	–	–	–	Unknown	unknown
28c	78	1.3	1851.0	1	RPAQAYPINYSSNN <sup>da</sup> VR	97.0	8	1.7	Tyrosine-protein kinase receptor Ret, V8P465, <i>Ophiophagus hannah</i>	Protein kinase
			–	–	–	–	–	–	–	–
29a	122	0.2	1492.8	1	FDEIVGGFDQLPR	61.7	5	2.7	L-amino acid oxidase, Q4JHE1, <i>Pseudechis australis</i>	LAAO
29b	75	1.6	1492.8	1	FDEIVGGFDQLPR	99.0	10	7.9	L-amino acid oxidase, Q4JHE1, <i>Pseudechis australis</i>	LAAO
			1648.9	1	RFDEIVGGFDQLPR	99.0	6			
			1216.6	1	FWEADGIHGGK	99.0	11			
			1372.7	1	K <sup>fo</sup> FWEADGIHGGK	99.0	11			
			2259.2	1	IYFAGEHTSK <sup>fo</sup> AHGWLDTGK	99.0	14	11.7	L-amino acid oxidase, A0A098LYF5, <i>Ophedryas aestivus</i>	LAAO
29c	64	0.6	–	–	–	–	–	–	Unknown	Unknown
			–	–	–	–	–	–	–	–
30a	75	1.3	1648.8	1	RFDEIVGGFDQLPR	99.0	21	4.6	L-amino acid oxidase, Q4JHE1, <i>Pseudechis australis</i>	LAAO
			1492.7	1	FDEIVGGFDQLPK <sup>fo</sup>	99.0	19			
			1321.6	1	EGWVYVNLGPMR	99.0	14			
			1125.6	1	A <sup>fo</sup> HGWLDGTIK	99.0	14	7.8	L-amino acid oxidase, A0A098LYF5, <i>Ophedryas aestivus</i>	LAAO
			1947.1	1	SAADIVINDLSLIHQLPK	99.0	16			
30b	64	0.7	1648.8	1	RFDEIVGGFDQLPK <sup>fo</sup>	99.0	8		LAO Pseudechis australis	LAAO
31a	75	0.7	2259.2	1	IYFAGEHTSK <sup>fo</sup> AHGWLDTGK	99.0	11	5.6	L-amino acid oxidase, A0A098LYF5, <i>Ophedryas aestivus</i>	LAAO
			1648.8	1	RFDEIVGGFDQLPR	98.6	6	2.7	L-amino acid oxidase, Q4JHE1, <i>Pseudechis australis</i>	LAAO
31b	64	0.5	–	–	–	–	–	–	Unknown	
31c	42	0.1	1812.9	1	RYPVEDDHIPFLRR	91.9	6	15.7	Glutamyl cyclase, U3TAD9, <i>Protobothrops flavoviridis</i>	Glu-cyclase
31d	23	0.1	1329.7	1	IHDIKWNFEK	99.0	13	27.9	Glutathione peroxidase, J9ZZN7, <i>Thamnophis elegans</i>	Glutathione POX
			1540.8	1	QEPQNSSEILQGIK	99.0	17			
			1492.8	1	LVILGFPCNQFGK	99.0	11			
			1645.7	1	NSCPPVVFETFGDPAK	99.0	15			
			1942.0	1	HVRPGGGFVFNQFLFQK	95.3	11			
32a	75	0.2	1216.6	1	FWEADGIHGGK	99.0	8	4.5	L-amino acid oxidase, A0A098LX00, <i>Pantherophis guttatus</i>	LAAO
			1435.8	1	RINFEPPLPKK	81.4	5			
32b	64	0.2	–	–	–	–	–	Unknown	Unknown	

Cysteine residues are carbamidomethylated. Confidence (Conf), Score, and coverage (% cov) values were calculated using the Paragon algorithm in ProteinPilot® v.4. Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown with the following superscript. Abbreviations: ox: oxidized; da: deamidated; fo: formyl; dl: delta:H(4)C(2)(H)0.5; cm: carbamidomethyl; py: pyroglutamate (2-oxo-pyrrolidone carboxylic acid).



**Fig. 3.** Relative abundance of protein families detected in the venom of *T. quadruplex*. 3FTx, three-finger toxin; CRISP, cysteine-rich secretory protein; GCY, glutaminyl cyclase; GPX, glutathione peroxidase; LAO, L-amino acid oxidase; MP, metalloproteinase; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PNP, peptide/non-protein; PRK, protein kinase; TRF, transcription factor; UNK, unknown. See also Table 1.

## 2.7. Toxic activities of venom and selected fractions

### 2.7.1. Animal ethics

All procedures involving animal toxicity experiments followed the ethical guidelines of the Institutional Committee for the Use and Care of Animals (CICUA) of the University of Costa Rica (permission No. 021–17). Mice of the CD-1 strain were provided by the animal facility of Instituto Clodomiro Picado. Domestic chicks were purchased from local commercial providers. Toxicity assays toward *Anolis sagrei* lizards were also conducted at UNC under the guidelines of the UNC-IACUC (protocol 1905D-SM-SBirdsLM-22).

### 2.7.2. Lethal toxicity: Mammals

Crude venom was dissolved in 0.12 M NaCl, 0.04 M sodium phosphate buffer (PBS), pH 7.2. Either 50 or 200 µg venom/50 µL buffer was injected by the intraperitoneal route into groups of three mice (18–20 g body weight), and lethality was recorded at 48 h post-injection. A control group received an identical injection of PBS alone. In another experiment, 25 µg/50 µL of fraction 17, identified as a 3FTx, was injected intraperitoneally in two mice.

### 2.7.3. Lethal toxicity: Birds

Crude venom (300 µg/50 µL) was injected by the subcutaneous route in four chicks (40–60 g body weight). In addition, the 3FTx identified in fraction 17 (140 µg/50 µL) was injected in two chicks by the same route. As a control, two chicks received an identical injection of PBS alone.

### 2.7.4. Lethal toxicity: Lizards

The HPLC-purified 3FTx (SE-HPLC peak 2, Fig. 8A; RP-HPLC peak 1, Fig. 13A) was injected IP at several doses (0.5–10 µg/g body weight) in adult *Anolis sagrei* in a volume of 50 µL PBS [35]. Doses were adjusted to individual animal body masses, with lethality expressed as micrograms of venom per gram body mass (mg/g) producing 50% mortality after 24 h [36].

### 2.7.5. Myotoxicity

The purified PLA<sub>2</sub> enzyme obtained in peak 20 of the RP-HPLC separation of the venom (20 µg/50 µL) was injected by the intramuscular route into the gastrocnemius of a group of three mice (18–20 g). After 3 h, a blood sample was collected from the tail of each mouse into heparinized capillary tubes and the plasma obtained after centrifugation was assayed for creatine kinase (CK) activity, using a commercial UV-kinetic kit (CK-Nac, Biocon Diagnostik). A control group of three mice received an identical injection of PBS alone.

### 2.7.6. Hemorrhagic activity

Venom (20 µg/100 µL) was injected by the intradermal route into the abdominal skin of two mice (18–20 g). After 2 h, animals were euthanized by carbon dioxide inhalation, and their skins were dissected to inspect for signs of hemorrhage [37].

## 3. Results and discussion

Owing to their lower significance in terms of human morbidity and mortality, in comparison to highly dangerous snakes of the families Viperidae and Elapidae, many fewer venoms of rear-fanged colubroid snakes have been thoroughly characterized by proteomic and/or transcriptomic analyses (but see [1,2,17,38,39,40]). In the present study, the venom of *T. quadruplex* from Costa Rica was investigated using proteomic, biochemical and biological analyses. SDS-PAGE fingerprinting revealed six prominent bands with approximate masses of 60, 53, 25, 14, 8 and 4 kDa, strongly suggesting toxins in the protein families L-amino acid oxidase, snake venom metalloproteinase (P-III), cysteine-rich secretory protein, PLA<sub>2</sub> and two size classes of three-finger toxins, respectively (Fig. 1). Venom was resolved into 32 fractions by C<sub>18</sub> RP-HPLC (Fig. 2). The early eluting peaks [1–13], together corresponding to 4.1% of the sum of chromatographic areas, were mostly minor, and none presented protein bands when subjected to SDS-PAGE and Coomassie blue staining. Therefore, these peaks were considered to correspond to either very small peptides or non-proteinaceous components (PNP). The remaining chromatographic peaks [14–32] generated protein bands on SDS-PAGE separation (Fig. 2B) that were subjected to tryptic digestion and MALDI-TOF/TOF mass spectrometry. The peptide sequences obtained, and their corresponding assignments to protein families by similarity, are summarized in Table 1.

The ‘snake venomomics’ bottom-up strategy for analysis of venom proteomes allows both identification of protein components and an estimation of abundances of these components [41,42]. The five most prominent peaks of the venom chromatogram were 17, 18, 20, 23, and 24 (Fig. 2C), together representing about 70% of venom proteins. These correspond to proteins of the three-finger toxin (3FTx; peaks 17 and 18), phospholipase A<sub>2</sub> (PLA<sub>2</sub>; peak 20), cysteine-rich secretory protein (CRISP; peak 23), and metalloproteinase (MP; peak 24) families (Table 1). When the whole venom was separated by SDS-PAGE (Figs. 1, 2B), these four protein families were readily identified by tryptic digestion of the major electrophoretic bands, together with another prominent band corresponding to the L-amino acid oxidase (LAO) protein family.

Reconstruction of the venom proteome according to family type assignments and abundances revealed that nearly half of the venom protein content corresponds to 3FTxs (45.9%; Fig. 3). In addition, MP, CRISP, and PLA<sub>2</sub> proteins follow in decreasing order of abundance, representing 17.3%, 12.1%, and 10.2% of the venom, respectively. Altogether, these four protein families add up to 85% of the venom, the remaining 15% distributed among a number of less abundant components, but most notably LAO (4.7%) among them (Fig. 3). A few protein bands remained unidentified in our analyses (unknown; UNK; Table 1), corresponding to 3.3% of the venom. Non-toxin proteins belonging to the protein kinase (PRK), transcription factor (TRF), glutaminyl cyclase (GCY), and glutathione peroxidase (GPX) families were detected in small proportions (0.1–1.3%). Altogether, ten protein families were identified in the venom. Following a general trend observed in the protein composition of many rear-fanged snake venoms compared to most venoms from viperids and elapids, the venom of *T. quadruplex* can be considered of relatively low complexity [26,43–45].

Two enzyme activities that are uncommon in rear-fanged snake venoms but prominent in front-fanged snake venoms were detected in *T. quadruplex* venom: PLA<sub>2</sub> and LAO. Accounting for approximately 10% of the proteomic profile, the identification of a prominent PLA<sub>2</sub> in *T. quadruplex* venom is of particular note, and very few have been identified in rear-fanged snake venoms. A previous study isolated a

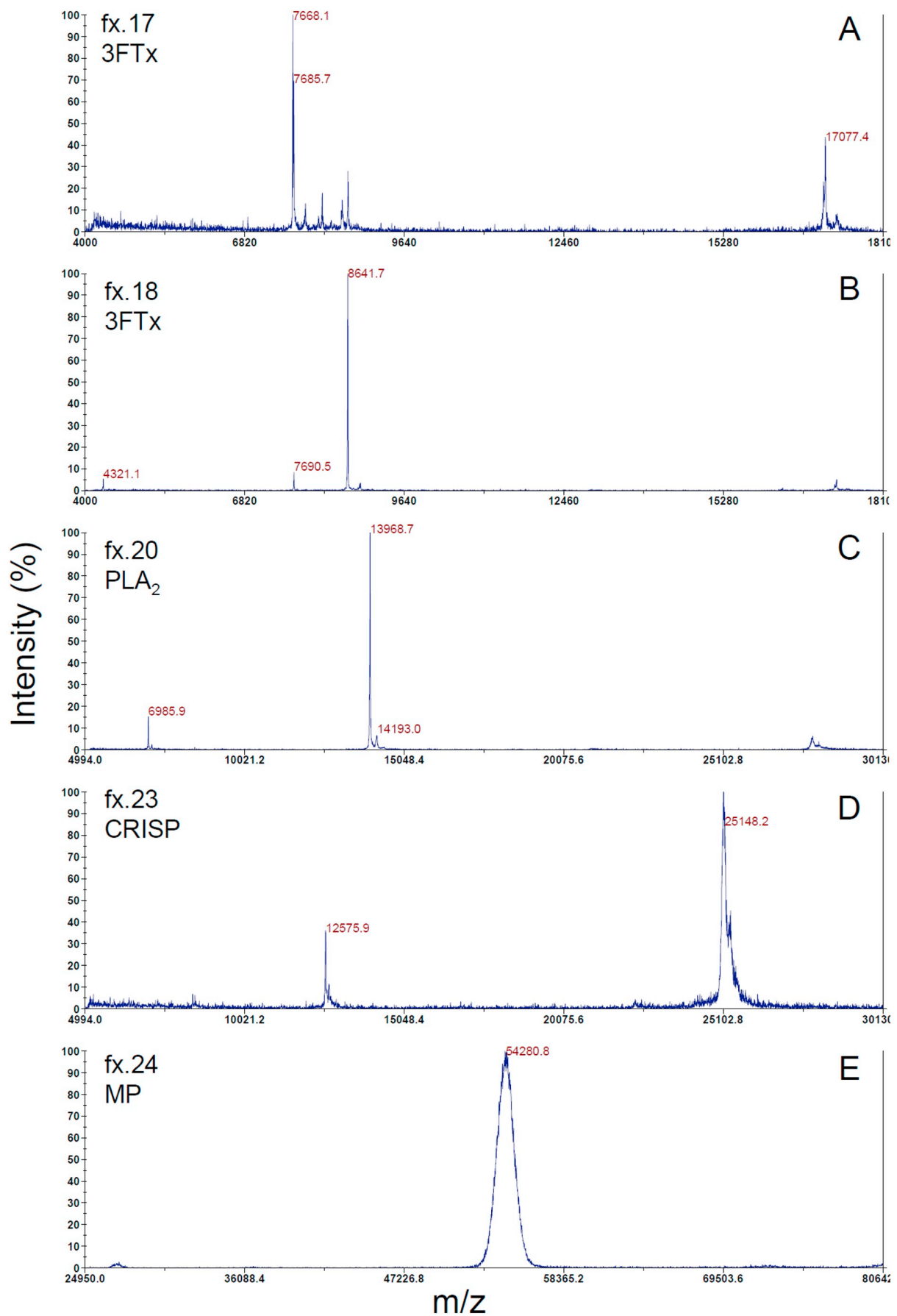


Fig. 4. MALDI-TOF MS spectra of purified toxins from *T. quadruplex* venom. 3FTx, three-finger toxin; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; CRISP, cysteine-rich secretory protein; MP, P-III metalloproteinase.



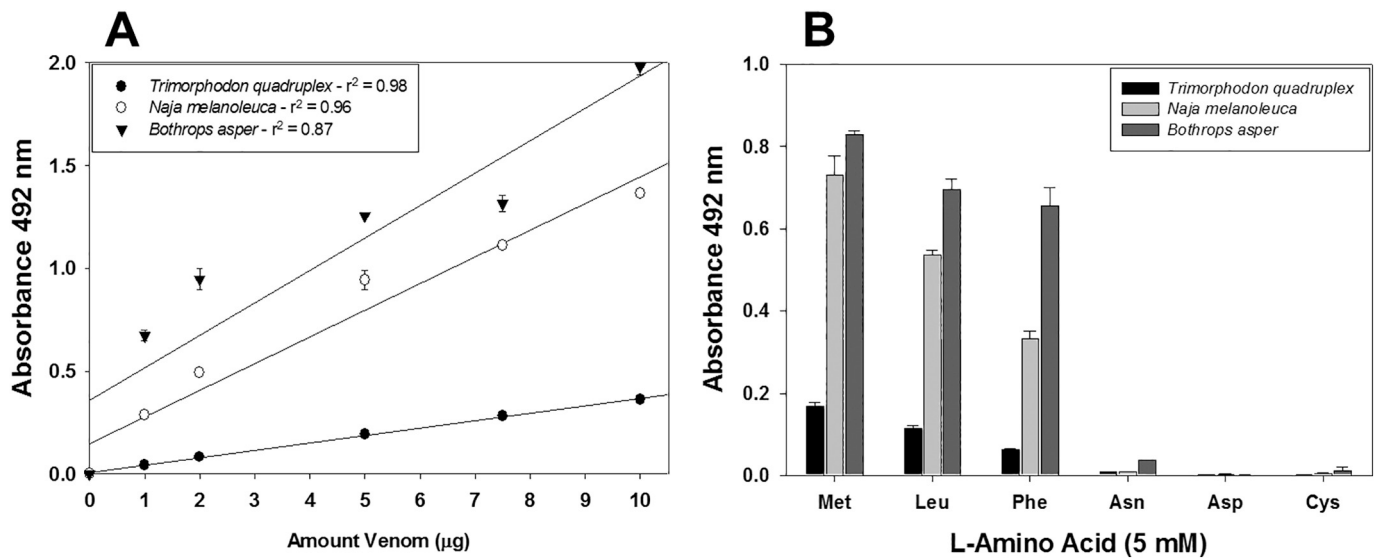


Fig. 7. LAAO activity of *T. quadruplex* and several reference venoms toward L-methionine (A) and *T. quadruplex* venom toward select L-amino acids (B).

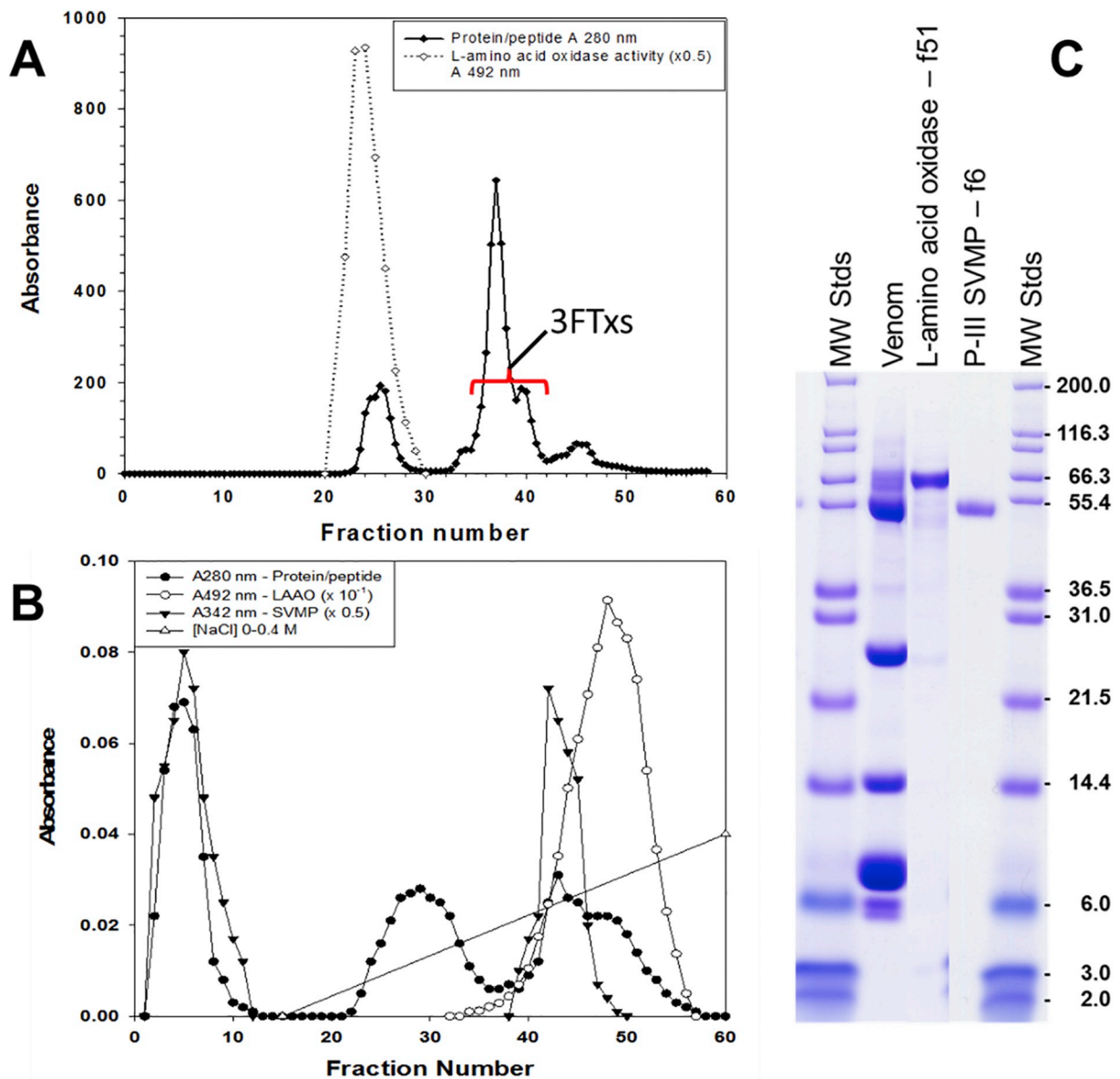


Fig. 8. HPLC size exclusion fractionation of *T. quadruplex* venom (A) followed by FPLC anion exchange of SE peak I (B). Gel (C) shows approximate masses of the purified LAAO and a P-III SVMP. 3FTxs, three-finger toxins.



10	20	30	40	50	60
ADDRRRPLEE	CFREADYEEF	LEIAKNGLQR	TSNPKRVVVV	GAGMAGLSAA	YVLAGAGHQV
AGDRS- <b>PLEK</b>	CF <b>Q</b> EDDYEEF	LEI <b>AR</b> NGLKQ	TSNPKHVVVV	GAGMAGLSAA	YVLAGAGHKV
70	80	90	100	110	120
		<b>EGWYVNLGPM</b> R			<i>T. quadruplex</i>
TLLEASERVG	GRVNTYRNEK	DGWYVNLGPM	RLPERHRI IR	EYIRKFGLEL	NEFIQENDNA Q4JHE1.1 <i>P. australis</i>
<b>TV</b> LLEASERVG	GRV <b>A</b> TYR <b>NDR</b>	<b>EGWYVNLGPM</b>	RLPERHRI IR	EYIRK <b>FG</b> L <b>QL</b>	NE <b>FF</b> Q <b>EN</b> ENA AXL95287.1 <i>S. sulfureus</i>
130	140	150	160	170	180
WYFIKNIKR	VSEVKKDPGV	FKYPVKPSEE	GKSASQLYRE	SLQKVIEELK	RTNCSYILNK
WYFIKNIKR	<b>VW</b> E <b>V</b> KKDPGV	FKYPVKPSEE	GKSASQLY <b>QE</b>	SL <b>KKV</b> REELK	RTNCSYI INK
190	200	210	220	230	240
				<b>RFDEIVGG</b>	
YDTYSTKEYL	IKEGNLSPGA	VDMIGDLLNE	DSSYYLSFIE	SLKSDDIFSY	EKR <b>F</b> DEIVGG
YDTYSTKEYL	IKEGNLSRGA	VDMIGDLLNE	DSSYY <b>I</b> SFIE	SL <b>KN</b> DDIFSY	EKR <b>F</b> DEIVGG
250	260	270	280	290	300
<b>FDQLPR</b>					
FDQLPRSMYQ	AIAEKVHLNA	QVIKIQQNAE	DVRVTYQTPA	KTLSYVIADY	VIVCSTSRAA
FDQL <b>P</b> SMYQ	AIA <b>E</b> MVHLNA	<b>RV</b> IKIQQNAE	<b>KVR</b> VEYQTPA	KTL <b>SN</b> V <b>T</b> ADY	VIVCSTS <b>RAT</b>
310	320	330	340	350	360
<b>RIN</b> FE <b>P</b> PL <b>P</b>	<b>PKK</b>		<b>KF</b> WEAD	<b>GI</b> HGGKS	
RRIHFE <b>P</b> PL <b>P</b>	PKKAHALRSI	HYRSSTKIFL	TCSQKFWEAD	GIHGGKSTTD	LPSRFIYYPN
RR <b>IN</b> FE <b>P</b> PL <b>P</b>	PKKAHALR <b>ST</b>	HY <b>KS</b> GTKIFL	TC <b>IK</b> KFWEAD	GIHGGKSTTD	LPSRFIYYPN
370	380	390	400	410	420
			<b>A</b> ADIVINDLS	<b>LI</b> HQLPK	
HSFTSGIGVI	VAYTLADDT	FFQALDIETS	A-DIVINDLS	LIHQLPKEQI	QALCYPSKI <b>Q</b>
<b>HN</b> FTSG <b>V</b> GV <b>L</b>	VAY <b>V</b> LAD <b>A</b> D	FFQALDF <b>K</b> ST	A-DIVINDLS	LIHQLPK <b>KE</b> I	QALCYPS <b>V</b> V <b>K</b>
430	440	450	460	470	480
			<b>IY</b> FAGE <b>H</b>	<b>TS</b> KAHG <b>W</b> L <b>D</b> G	<b>TI</b> K
KWSLDEYAMG	AITSFTPYQF	QDFFEIVAAP	VGRIYFAGEY	TASVHG <b>W</b> L <b>D</b> S	TIK <b>S</b> GL <b>T</b> AAR
KW <b>S</b> L <b>D</b> KYAMG	<b>S</b> ITSFTPYQF	QDF <b>S</b> ELLA <b>Q</b> P	VGRIYFAGE <b>H</b>	<b>TS</b> K <b>V</b> HG <b>W</b> L <b>D</b> G	TIK <b>S</b> GL <b>T</b> AAR
490	500				
DVNLASQKPS	RIQLSNDNEL				
DV <b>N</b> R <b>A</b> SEKPS	<b>K</b> IR <b>L</b> ISDN <b>Q</b> L				

Fig. 9. Comparison of *Trimorphodon quadruplex* L-amino acid oxidase peptides (green) to sequence from *Pseudechis australis* L-amino acid oxidase (Q4JHE1.1) and sequence from a colubrid snake *Spilotes sulfureus* LAAO-1 (AXL95287.1). Identical amino acid residue matches of peptides from *T. quadruplex* LAAO are shown in green and variants from *P. australis* are shown in red. Note that in spite of these species belonging to separate families, identified peptides share considerable sequence identity, demonstrating the conserved nature of the LAAOs. 87 residues total, ~17.4% coverage. LAAO 1 from *S. sulfureus* has 62 residues that differ from *P. australis* (~12.4%). 3/87 residues differ between *T. quadruplex* and *S. sulfureus* LAAOs (~3.4%).

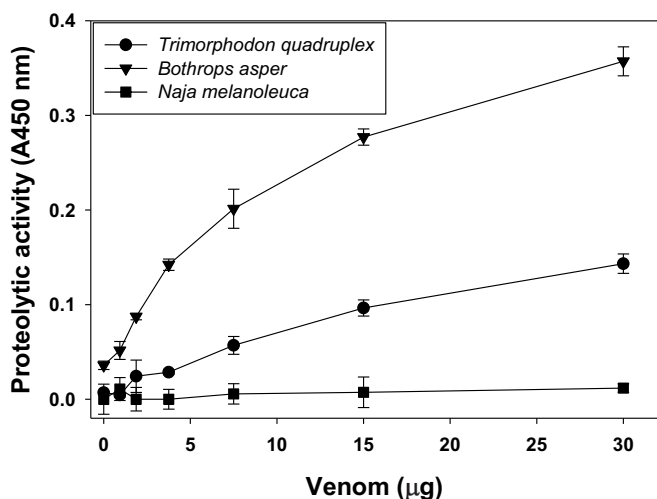


Fig. 10. Metalloproteolytic activity of *T. quadruplex* venom toward azocasein; activities of *Naja* and *Bothrops* are shown for comparison.

corresponding purified enzyme (Fig. 6B). No behavioral alterations or signs of toxicity were observed in the mice that were intramuscularly injected with 20 µg of the isolated PLA<sub>2</sub>. On the basis of sequence homology, it had been speculated that the predicted PLA<sub>2</sub> from *T. biscutatus* would likely be responsible for neurotoxic activity of this venom [23]. Our present findings do not support this assumption, since no signs of neurotoxicity were recorded after the injection of this enzyme in mice.

L-amino acid oxidase is also commonly found in front-fanged snake venoms, but it is rare in venoms from rear-fanged snakes. Because of this, we decided to characterize the enzyme further. Crude venom showed high activity toward L-methionine (Fig. 7A), and activity was easily detected in as little as 1 µg venom. *Trimorphodon quadruplex* LAAO also showed deamidation activity toward L-leucine and L-phenylalanine, but activity toward L-asparagine, L-aspartate and L-cysteine was very low. This trend in specificity was also noted in the two front-fanged snake venoms assayed (*N. melanoleuca* and *B. asper*) The enzyme appears to be in relatively low abundance, but LAAO was isolated from the crude venom via size exclusion chromatography (Fig. 8A) followed by anion-exchange chromatography on a Tricorn MonoQ column (Fig. 8B). Some venom LAAOs are known to be unstable, and the *T.*

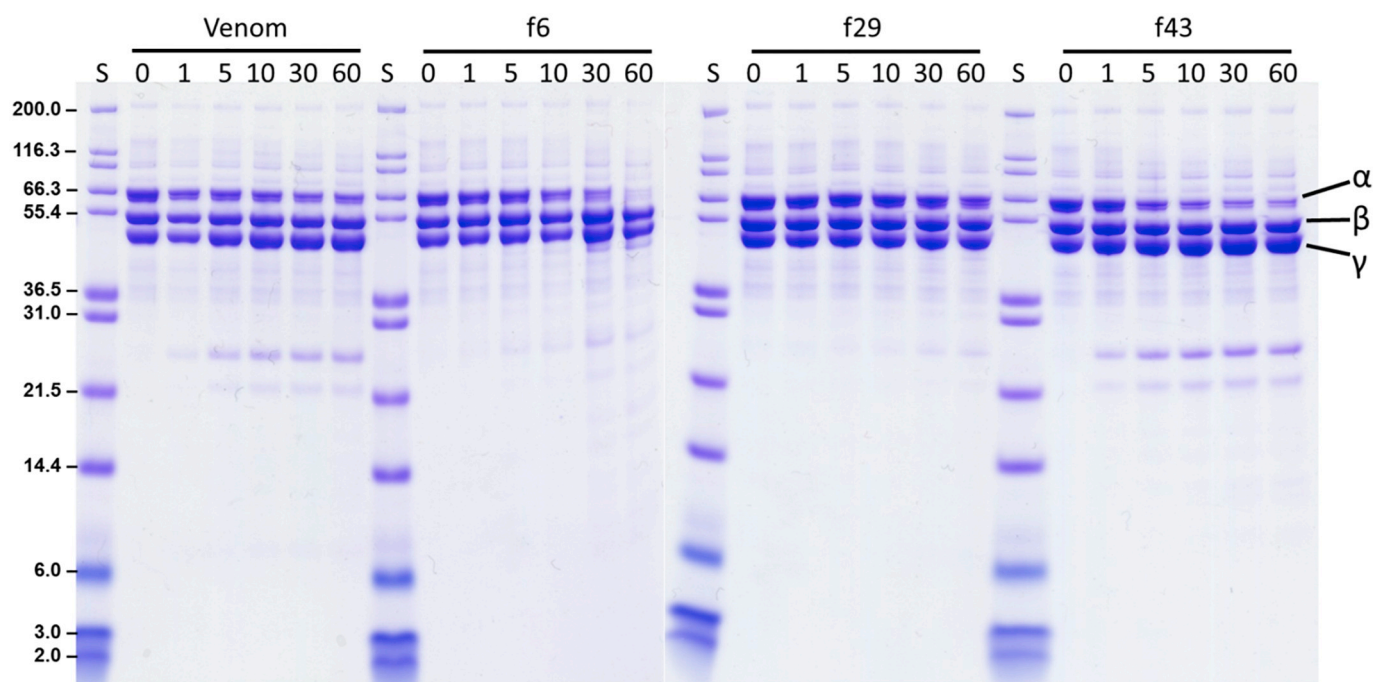


Fig. 11. Activity of crude *Trimorphodon quadruplex* venom and several anion-exchange fractions toward human fibrinogen.

*quadruplex* enzyme appears to be particularly labile, as it was denatured by lyophilization of the pure enzyme and when stored in phosphate buffer for longer than one week at 4°C; activity could not be recovered.

Because of its large size (LAAOs are commonly homodimers: *T. quadruplex* LAAO has a monomer subunit size of approximately 65 kDa; Fig. 8C), LAAOs digested by trypsin typically present an abundance of peptide fragments on MS/MS, even when the parent molecule is a very minor component (cf. 17). Only one LAAO sequence from a rear-fanged snake has been published; LAAOs in general appear to have relatively conserved sequences, and the peptides from *T. quadruplex* LAAO show a high degree of sequence identity (96.6%) with LAAO-1 transcript from the venom gland of *Spilotes sulfureus* (AXL95287.1; 17). These peptides and LAAO-1 also showed high homology with an LAAO from the Australian elapid *Pseudechis australis* (Mulga Snake); an alignment is shown in Fig. 9.

In addition, the presence of a considerable proportion (~17%) of SVMs in the venom prompted us to screen for possible hemorrhagic activity, a toxic effect induced by a number of these enzymes [50]; however, negative results were also obtained for this bioassay (data not shown). The *in vitro* proteolytic activity of *T. quadruplex* on azocasein was moderate, lower than that of the viperid *Bothrops asper*, but higher than that of the elapid *Naja melanoleuca* (Fig. 10). Based on mass (Figs. 4E and 8C) and chromatographic behavior (Fig. 8B), at least two P-III snake venom metalloproteinases (P-III SVMs) are present in the venom of *T. quadruplex*, one acidic and one more basic. Three distinct classes (P-I, P-II, P-III) of these proteinases are abundant in many viper venoms (e.g., [51]) but are generally much less prominent in elapid venoms [52,53]; however, P-III SVMs are often major components of rear-fanged snake venoms [1,10,17,40].

Metalloproteinases often show activity toward basal lamina components and fibrinogen and so are major factors resulting in hemorrhage, tissue damage and necrosis following snakebite [50]. *Trimorphodon quadruplex* venom showed weak activity toward fibrinogen that appeared to be limited to the  $\alpha$ -subunit (Fig. 11). Following anion exchange, this activity is due to both the unbound/basic P-III SVM (Fig. 8B, peak 1; f6) and the acidic P-III SVM (f43); both have a mass on SDS-PAGE of ~53 kDa. The P-III SVMs in rear-fanged snake venoms are likely responsible for the formation of ecchymoses, blebs and

hemorrhagic syndromes occasionally observed in bites by these species [54], particularly those associated with longer contact times.

Cytotoxicity of *T. quadruplex* venom toward MCF-7 breast cancer cells showed a concentration-dependent effect, and at the highest dose (50  $\mu\text{g}/100 \mu\text{L}$ ), cell viability was reduced by 75% (Fig. 12). Interestingly, *T. b. lambda* venom at 19  $\mu\text{g}/100 \mu\text{L}$  had no cytotoxic effects [32], even though these venoms contain most of the same components. *Trimorphodon b. lambda* venom did show cytotoxic activity toward *Leishmania major* promastigotes, and trimorphin (a PLA<sub>2</sub>) from the same venom showed potent cytotoxicity [55]. Lack of sufficient purified material precluded more extensive testing of *T. quadruplex* LAAO toxicity toward this cell line, but at 5  $\mu\text{g}/100 \mu\text{L}$ , no cytotoxic effects were observed (data not shown).

The largest proportion of proteins in *T. quadruplex* venom

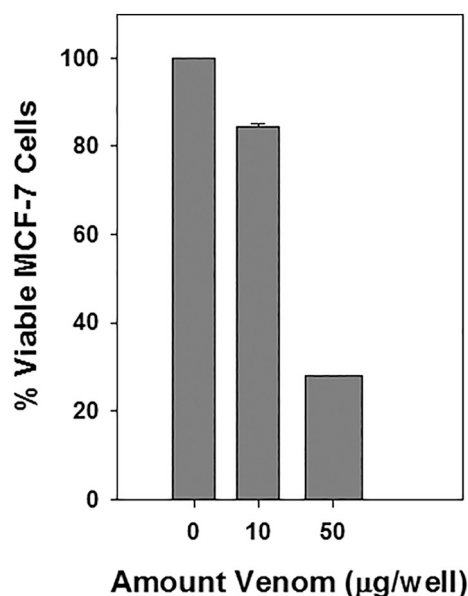
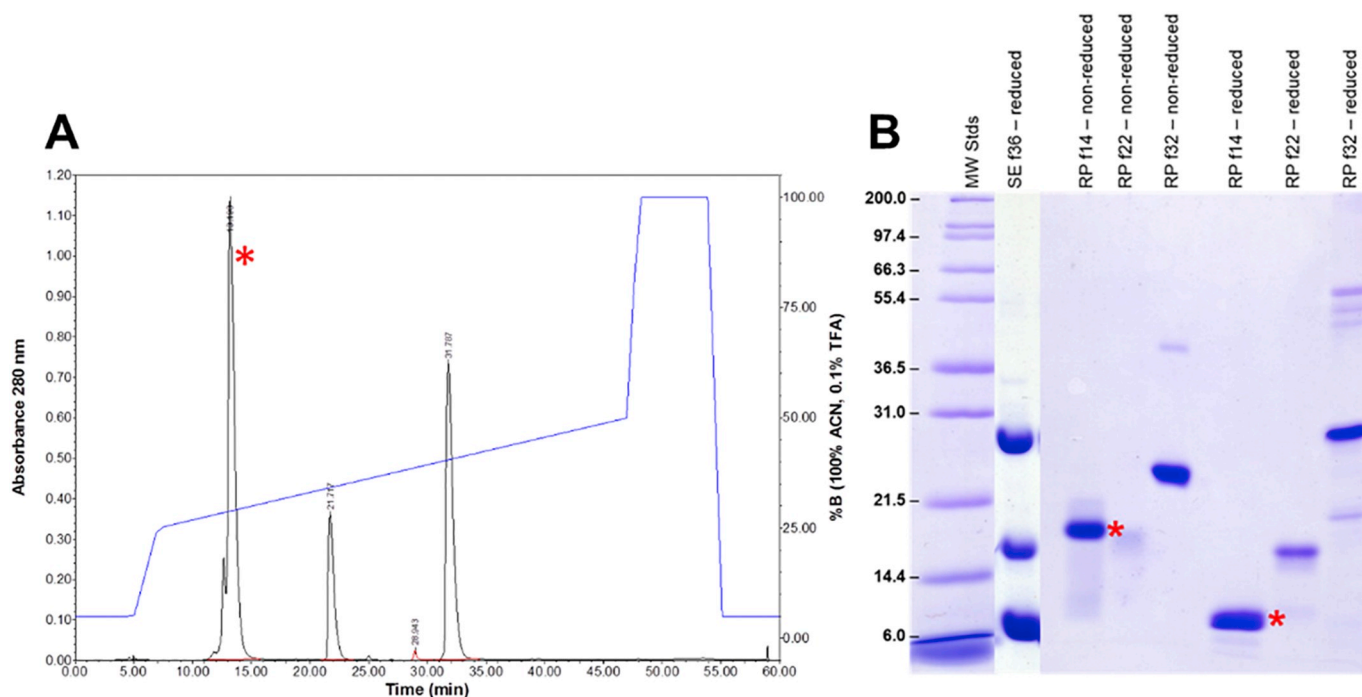


Fig. 12. Cytotoxic effects of *T. quadruplex* venom toward MCF-7 breast cancer cells.



**Fig. 13.** Three-finger toxins in *T. quadruplex* venom. A. RP-HPLC of SE 3FTx peak (fractions 35–39) showed 3 peaks following RP-HPLC. B. SDS-PAGE of peak 1 (fraction 14, \*) contained a dimeric 3FTx, with approximate masses of 17 kDa (non-reduced) and 8 kDa (reduced). Peak 2 (fraction 22) contains PLA<sub>2</sub>, and peak 3 (fraction 32) is a CRiSP.

corresponded to the 3FTx family (Figs. 1, 2), which presented prominent chromatographic peaks eluting within the range of 26–40 min. Peptides identified by MS/MS analysis (Table 1) matched with segments of 3FTxs found in the venoms of *Coelognathus radiatus* and *Trimorphodon lambda*. The former is a well-characterized  $\alpha$ -neurotoxin ( $\alpha$ -colubritoxin; P83490) which displayed post-synaptic blocking effects on the chick neuromuscular junction [56]. Because of this structural similarity, we purified additional material and explored toxicity of this protein. The 3FTx-containing peak obtained from SE-HPLC (Fig. 8A) was subjected to RP-HPLC fractionation, resulting in three protein peaks (Fig. 13A). Reducing and non-reducing SDS-PAGE revealed a dimeric 3FTx in the first RP-HPLC peak, with dimeric (~17 kDa) and monomeric (~8.5 kDa) masses similar to those seen in the taxon-specific toxins irditoxin [15] and sultitoxin [17]; this 3FTx is identical to that of fraction 17 obtained from crude venom RP-HPLC fractionation (Fig. 2). It appears that at least among rear-fanged snake venoms, dimeric 3FTxs are not unusual, and we anticipate that additional examples will be found as venoms from diverse genera are analyzed in the future.

Lethality of the crude venom and of the dimeric 3FTx were screened in both mice and chicks. Three of the four chicks subcutaneously injected with *T. quadruplex* venom (300  $\mu$ g; ~6  $\mu$ g/g body weight) died within the 48 h observation period, while one of two chicks injected s.c. with 140  $\mu$ g of the toxin (~2.8  $\mu$ g/g) died. Both chicks receiving PBS alone survived. Conversely, the crude venom injected by intraperitoneal route caused death in only one out of three mice receiving a dose of 200  $\mu$ g (10  $\mu$ g/g); three mice receiving 50  $\mu$ g (2.5  $\mu$ g/g) and two mice that received 25  $\mu$ g (1.25  $\mu$ g/g) of the purified toxin by intraperitoneal route all survived. *Anolis sagrei* lizards injected IP with > 1  $\mu$ g/g of the purified dimeric 3 FTx showed progressive paralysis, and the LD<sub>50</sub> is ~4  $\mu$ g/g. However, at 3.0  $\mu$ g/g, this toxin was lethal to only 1 of 3 birds. The limited availability of venom (or its fraction) precluded performing complete lethality tests to estimate formal median lethal dose (LD<sub>50</sub>) values on mice or birds. These results present evidence that *T. quadruplex* venom is only weakly lethal to mice, and that chicks and lizards are more susceptible to its lethal

activity, which most likely is related to the neurotoxic effect induced by its prominent 3FTx fractions. Taxon-specific neurotoxins (3FTxs) showing potent toxicity toward lizards and birds, but lacking toxicity toward mice, have been purified, sequenced and characterized from three other rear-fanged snake species (*Boiga irregularis*: 15; *Oxybelis fulgidus*: 16; *Spilotes sulfureus*: 17). Unexpectedly, though this *T. quadruplex* dimeric 3FTx appears to affect avian and saurian prey somewhat more so than mammalian counterparts, it does not show the pronounced taxon-specific effects of either irditoxin or sultitoxin, and further study is needed to define this toxin's actions. This lack of highly potent activity of the dimeric 3FTx toward birds suggests that other venom components, perhaps monomeric 3FTxs, are also contributing to venom lethality, because the crude venom was moderately toxic to birds.

In summary, the present study contributes to our knowledge on the protein composition and toxic properties of rear-fanged snake venoms by showing that the venom of *T. quadruplex* from Costa Rica contains many of the same activities found in other snake venoms, including the dangerously venomous front-fanged species. As with several other recently analyzed rear-fanged snake venoms [17,39], 3FTxs are abundant and account for nearly half of the total protein content. Other protein types present in substantial proportions in this venom belong to the SVMP, CRISP, LAO and PLA<sub>2</sub> families, and the latter two enzymes are uncommon venom components in opisthoglyph species [10]. In spite of this abundance of toxins, *Trimorphodon* venoms/bites are not generally considered dangerous to humans [54]. No myotoxicity or lethality was recorded for the major PLA<sub>2</sub> purified from *T. quadruplex* venom in mice, and therefore its possible toxic functionality remains to be determined. A possibility also exists that this PLA<sub>2</sub> does not play a specific toxic role, but may contribute to prey digestion, as proposed for a number of apparently non-toxic acidic-type PLA<sub>2</sub>s characterized from venoms of viperid snakes [34,57]. On the other hand, a toxic functionality was observed for the major 3FTx component of this venom, which was able to cause lethality in chicks and lizards; this toxin is also a dimeric 3FTx, one of only three presently known from colubrid snake venoms. Although more detailed studies are needed to fully characterize the toxic

activities of *T. quadruplex* venom in different animal models, the present initial observations on the weak lethal effects in mice, and a lack of hemorrhagic activity and myotoxicity in mice, suggest that this venom would be of limited toxicity in human accidents. Indeed, the only documented case of a bite from *Trimorphodon* was an intentionally induced bite *T. lambda*, and no apparent morbidity was recorded [58]. This profound lack of effect illustrates a major bifurcation in the evolutionary trajectory of venoms in advanced snakes: most viperid and elapid venoms show potent toxicities toward mammals (and often other vertebrates), whereas a growing number of rear-fanged snake species are now known to produce venoms with diverse protein families represented, but without significant toxic effects toward mammals. This differential toxicity highlights the observation that biologically-relevant animal toxicity models are critical for determining the biological roles of venoms, as species that do not prey commonly on mammals may produce venoms that are “evolutionarily tailored” with specific effects on particular prey taxa. We predict that as more species of rear-fanged snakes are subjected to functional analyses, as well as proteomic and transcriptomic analyses, this dichotomy will grow, emphasizing the fact that venom evolution in snakes has not followed a single shared compositional strategy.

### Declaration of Competing Interest

The authors have declared no conflict of interest.

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