Toxicon 90 (2014) 15–25

Contents lists available at ScienceDirect

Toxicon

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

Proteomic and toxicological profiling of the venom of *Bothrocophias campbelli*, a pitviper species from Ecuador and Colombia

David Salazar-Valenzuela [a, b], Diana Mora-Obando [c], María Laura Fernández [c], Amaru Loaiza-Lange [b], H. Lisle Gibbs [a], Bruno Lomonte [c, *]

[a] Department of Evolution, Ecology and Organismal Biology, The Ohio State University, 300 Aronoff Laboratory, 318 W. 12th Ave., Columbus, OH 43210-1293, USA
[b] Escuela de Biología, Pontificia Universidad Católica del Ecuador, Avenida 12 de Octubre y Roca, Apartado 17-01-2184, Quito, Ecuador
[c] Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José 11501, Costa Rica

ARTICLE INFO

Article history:
Received 16 June 2014
Accepted 24 July 2014
Available online 1 August 2014

Keywords:
Snake venom
Venomics
Proteomic
Toxicity
Viperidae
Bothrocophias campbelli

ABSTRACT

Detailed snake venom proteomes for nearly a hundred species in different pitviper genera have accumulated using 'venomics' methodologies. However, venom composition for some lineages remains poorly known. *Bothrocophias* (toad-headed pitvipers) is a genus restricted to the northwestern portion of South America for which information on venom composition is lacking. Here, we describe the protein composition, toxicological profiling, and antivenom neutralization of the venom of *Bothrocophias campbelli*, a species distributed in Colombia and Ecuador. Our analyses show that its venom mainly consists of phospholipases A_2 (43.1%), serine proteinases (21.3%), and metalloproteinases (15.8%). The low proportion of metalloproteinases and high amount of a Lys49 phospholipase A_2 homologue correlate well with the low hemorrhagic and high myotoxic effects found. Overall, *B. campbelli* venom showed a simpler composition compared to other crotalines in the region. A polyvalent antivenom prepared with a mixture of *Bothrops asper*, *Crotalus simus*, and *Lachesis stenophrys* venoms cross-recognized *B. campbelli* venom and neutralized its lethal effect in mice, albeit with a lower potency than for *B. asper* venom. Additional work comparing *B. campbelli* venom properties with those of related species could help understand the evolution of different venom protein families during the South American radiation of New World pitvipers.

1. Introduction

The genus *Bothrocophias* (Toadheaded pitvipers) consists of six species of South American pitvipers, whose distribution includes cis-/trans-versants of the Northern and Central Andes, as well as the upper Amazon Basin (Gutberlet and Campbell, 2001; Campbell and Lamar, 2004; Carrasco et al., 2012). They are restricted to primary or slightly disturbed forests; therefore some members (e.g. *Bothrocophias campbelli*) appear to be rare in nature (Campbell and Lamar, 2004; Arteaga, 2013). Nevertheless, some species (e.g. *Bothrocophias hyoprora* and *Bothrocophias microphthalmus*) may be more common, at least in parts of their distribution (Kuch and Freire, 1995; Cisneros-Heredia et al., 2006). Traditionally, these species were included in the genus *Bothrops* with the recognition of the *Bothrocophias* lineage occurring rather recently (see review in Gutberlet and Harvey, 2004). Phylogenetic studies based on morphological and molecular data have found support

* Corresponding author.
E-mail address: bruno.lomonte@ucr.ac.cr (B. Lomonte).

http://dx.doi.org/10.1016/j.toxicon.2014.07.012
0041-0101/

for recognizing the genus *Bothrocophias* as a monophyletic lineage, and the available evidence suggests it constitutes the sister clade of the remaining lineages of South American pitvipers (Fenwick et al., 2009; Jadin et al., 2011; Carrasco et al., 2012). Toadheaded pitvipers are terrestrial snakes of moderate length, a relatively stout body, and a large head (Gutberlet and Campbell, 2001; Campbell and Lamar, 2004).

The Ecuadorian toadheaded pitviper, *B. campbelli* (Freire-Lascano, 1991) is a medium sized snake (mean adult total length: 101.6 cm, mean adult weight: 580.5 g) known from mid-elevation localities (1000–2250 m above sea level) on the western versant of the Cordillera Occidental of the Andes in Ecuador and Colombia (Campbell and Lamar, 2004; Castro et al., 2005; Cisneros-Heredia et al., 2006; Valencia et al., 2008; Arteaga, 2013; personal observations). Its diet appears to be generalist with prey reports based on rodents, caecilians, and snakes (Freire and Kuch, 2000; Zuffi, 2004; Cisneros-Heredia et al., 2006; Rojas-Rivera et al., 2013). Although no snakebite accidents have been reported for this species, envenomation reports for other species in the genus *Bothrocophias* include local signs and symptoms such as severe pain, swelling, and necrosis, whereas systemic effects include coagulopathies, hemorrhages, haematuria, thrombosis, renal failure, and hypovolemic shock (Campbell and Lamar, 2004; Warrell, 2004).

Snake venoms and their constituent toxins have evolved as a result of the actions of different evolutionary and ecological forces (Mebs, 2001; Mackessy, 2010; Calvete, 2013). Comparative studies of snake venom protein evolution (e.g. Gibbs et al., 2013) are hindered without information of venom composition present in different lineages. The genus *Bothrocophias* represents a lineage of early divergence in the South American pitviper radiation, which occurred throughout much of the Caenozoic when this landmass was isolated from North and Central America (Gutberlet and Harvey, 2004; Werman, 2005). To our knowledge, no venom proteomic studies exist for members of the genus. The present work describes the venom composition of *B. campbelli* analysed by the 'snake venomics' methodology (Calvete et al., 2007), and provides an assessment of some of its toxicological properties and its neutralization by antivenom.

2. Materials and methods

2.1. Venom samples

Venom was obtained from three adult *B. campbelli* specimens. Two males were found in primary forest near Mindo river, Mindo, Pichincha Province (0°4′,46″N, 78°45′45″W; datum = WGS84; 1208 m above sea level [asl]). A female individual kept in captivity by local people was provided to us at El Cristal, Esmeraldas Province (0°51′52″N, 78°28′52″W; datum = WGS84; 1116 m asl). Animals were constrained using a set of telescoping clear plastic tubes and then induced to bite the top of a 50 ml glass beaker covered with Parafilm®. The secretion was immediately pipetted into a 1.5 ml tube and dried in a vacuum container containing anhydrous calcium sulphate (Drierite desiccant). Dried venom was stored in a −80 °C freezer. For comparative purposes in functional assays, the recently characterized venom of *Bothrops asper* from the Cauca Department, Colombia (Mora-Obando et al., 2014) was used in some experiments.

2.2. Reverse-phase HPLC and venomic characterization

A venom pool was prepared by mixing equal parts of venom from the three specimens. Approximately 1 mg was dissolved in 200 μL of water containing 0.1% trifluoroacetic acid (TFA), centrifuged at 15,000 × *g* for 5 min to remove debris, and fractionated by RP-HPLC on a C_{18} column (Teknokroma; 4.6 × 250 mm, 5 μm particle) using an Agilent 1200 chromatograph. To assess individual variability, each of the three venom samples was also analysed separately under identical conditions as was the pooled venom. Elution was performed at 1 mL/min by applying a gradient towards solution B (0.1% TFA in acetonitrile), as follows: 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min. Absorbance of the eluent was recorded at 215 nm and the peak areas of the chromatogram were integrated using ChemStation B.04.01 software (Agilent). Venom composition was analysed following the 'snake venomics' strategy (Calvete et al., 2007) with slight modifications as described in Lomonte et al. (2014a). HPLC fractions were manually collected, dried in a vacuum centrifuge, redissolved in water, and further separated by SDS-PAGE under reducing conditions (5% 2-mercaptoethanol at 100 °C for 5 min) in 12% gels. After Coomassie blue R-250 staining, gel images were recorded using ChemiDoc/Image-Lab (Bio-Rad) and analysed by densitometry, which was combined with the integration of HPLC peak areas to estimate protein relative abundances. Protein bands were then excised and subjected to in-gel reduction (10 mM dithiothreitol), alkylation (50 mM iodacetamide), and overnight digestion with trypsin in a ProGest processor (Digilab), following manufacturer's recommendations. The resulting peptides were extracted and characterized by MALDI-TOF-TOF mass spectrometry on an AB4800-Plus Proteomics Analyzer instrument (Applied Biosystems). Peptides were mixed with an equal volume of saturated α-cyano-hydroxycinnamic acid (in 50% acetonitrile, 0.1% TFA), spotted (1 μL) onto an Opti-TOF 384-well plate, dried, and analysed in positive reflector mode. Spectra were acquired using a laser intensity of 3000 and 1500 shots/spectrum, using as external standards CalMix-5 (ABSciex) spotted on the same plate. Up to 10 precursor peaks from each MS spectrum were selected for automated collision-induced dissociation MS/MS spectra acquisition at 2 kV, in positive mode (500 shots/spectrum, laser intensity 3000). For the assignment of proteins to known families, the resulting spectra were analysed against the UniProt/SwissProt database (January 2014) using ProteinPilot v.4 (ABSciex) and Paragon® algorithm at a confidence level of ≥95%. A few peptide sequences with lower confidence scores were manually searched using BLAST (http://blast.ncbi.nlm.nih.gov).

2.3. Toxicological profiling

2.3.1. Animals

Animal experiments were conducted using CD-1 mice of either sex, following protocols approved by the

Institutional Committee for the Use and Care of Animals (CICUA), University of Costa Rica. Mice were housed in groups of four, and provided food and water *ad libitum*.

2.3.2. Venom lethality

Lethal potency of the venom was tested by intraperitoneal (i.p.) route in mice of 16–18 g body weight. Deaths induced by the injection of varying doses of venom (30–225 μg dissolved in 500 μL of PBS) were recorded after 48 h. The median lethal dose (LD_{50}) was estimated by probits.

2.3.3. Hemorrhagic activity

Various doses of venom (0.6–10 μg) were dissolved in 100 μL of PBS (0.12 M NaCl, 0.04 M sodium phosphate buffer, pH 7.2). These were injected to groups of four mice (18–20 g) by the intradermal (i.d.) route in the abdominal skin. After 2 h, animals were euthanized by CO_2 inhalation, their skin was removed, and the hemorrhagic areas were measured. The minimum hemorrhagic dose (MHD) was defined as the amount of venom causing a hemorrhagic spot of 10 mm of diameter.

2.3.4. Myotoxic activity

Groups of five mice (18–20 g body weight) received an intramuscular (i.m.) venom injection of 50 μg in 50 μL of PBS, into their right gastrocnemius. A control group was treated with PBS alone. After three hr, blood samples were obtained from the tip of the tail and plasma creatine kinase (CK) activity was determined using a UV kinetic assay (CK-Nac, Biocon Diagnostik) (Gutiérrez et al., 1986). CK activity was expressed in units/L. As a comparative control, the venom of *B. asper* from the Cauca Department of Colombia, which has been recently characterized (Mora-Obando et al., 2014), was included in functional assays. Mice were euthanized by CO_2 inhalation 6 h after venom injection, and samples of the injected muscle tissue were obtained, fixed in 10% formalin, embedded in paraffin, and processed for histologic evaluation of hematoxylin/eosin-stained sections.

2.4. In vitro enzymatic activities

2.4.1. Proteolytic activity

Various amounts of venom (1.8–30 μg) were incubated for 90 min at 37 °C with 90 μL of azocasein (10 mg/mL) in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM $CaCl_2$ buffer, pH 8.0, in triplicate wells of a microplate. The reaction was stopped with 200 μL of 5% trichloroacetic acid. After centrifugation, 150 μL of each supernatant were mixed with 150 μL of 0.5 M NaOH, and the final absorbances were recorded at 490 nm. The absorbance of azocasein incubated with PBS

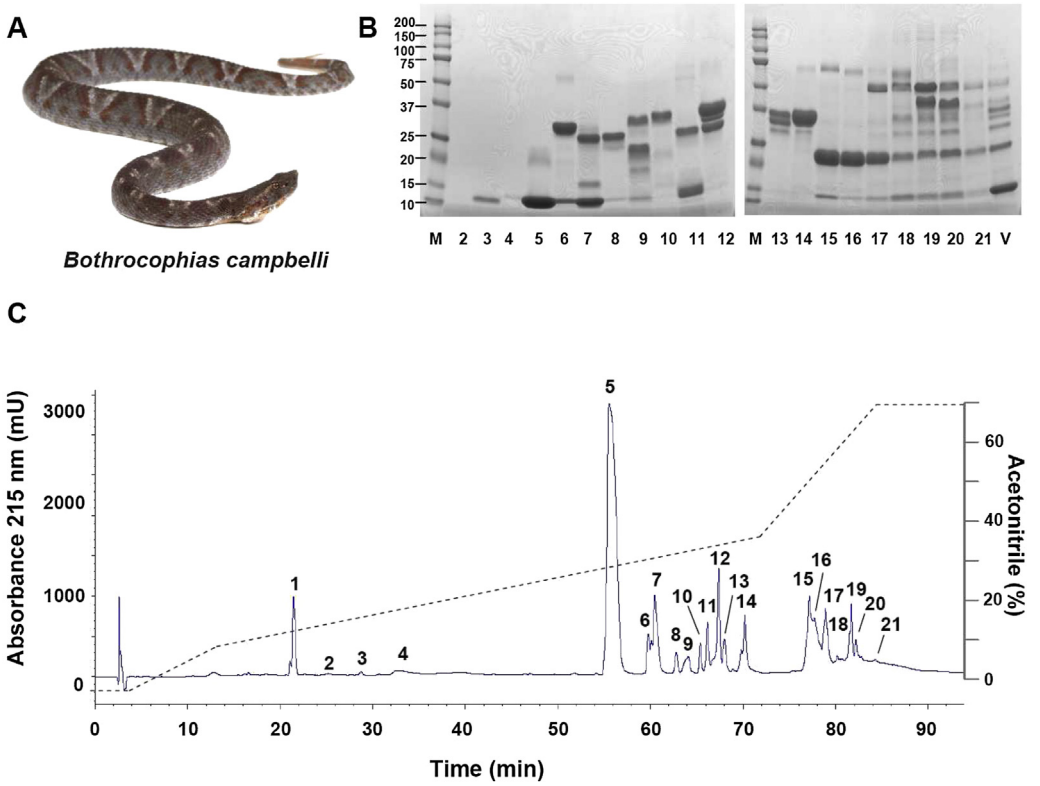

Fig. 1. Elution profile of *Bothrocophias campbelli* (A) venom proteins by RP-HPLC. Venom (~1 mg) was separated on a C_{18} analytical column using an acetonitrile gradient (dotted line) as described in Material and Methods (C). Fractions were further separated by SDS-PAGE under reducing conditions (B). Molecular weight markers (M) are indicated in kDa, at the left, and the migration of crude venom (V) is shown at the right. Tryptic digests of the proteins were characterized by MALDI-TOF/TOF, as summarized in Table 1. *B. campbelli* picture was used with permission from Tropical Herping.

Table 1
Assignment of the RP-HPLC isolated fractions of *Bothrocophias campbelli* venom to protein families by MALDI-TOF/TOF of selected peptide ions from in-gel trypsin-digested protein bands.

Peak	%	Mass (kDa)▼	Peptide ion		MS/MS-derived sequence[a]	Conf (%)[b]	Sc[b]	Protein family; ~related protein
			m/z	z				
1	3.9		444.3	1	ZBW	*dn*	*dn*	Tripeptide metalloproteinase inhibitor; ~P01021
			466.1	1	ZBW(Na^+)			
2	0.3				–			Unknown
3	0.3	10	2145.9	1	ARGDDM^{ox}DDYCNGXSAGCPR	99.0	8	Disintegrin; ~Q98SP2
			2051.0	1	XRPGABCAEGXCCDBCR	99.0	14	
			1918.8	1	GDDM^{ox}DDYCNGXSAGCPR	98.5	6	
4	1.1	11			–			Unknown
5	41.8	12	2803.4	1	MXXBETGBNPXTSYGAYGCNCGVGGR	99.0	22	Phospholipase A_2, K49; ~Q6JK69
			1902.9	1	NPXTSYGAYGCNCGVGGR	99.0	25	
			1647.8	1	DBTXVCGENNPCXK	99.0	18	
			1578.7	1	TSYGAYGCNCGVGGR	99.0	14	
			1404.7	1	TXVCGENNPCXK	99.0	13	
			1754.8	1	EMCECDBAVAXCXR	99.0	19	
			1104.6	1	DRYSYSWK	99.0	13	
6a	1.9	27	1140.5	1	VSDYTEWXK	99.0	15	Serine proteinase; ~Q072L6
			1342.7	1	XPSSPPSVGSVCR	82.7	6	
6b	0.7	12	2747.3	1	NBPDHYBNBEFCVEXVSYTGYR	99.0	22	C-type lectin/lectin-like; ~Q9PSM4
			1145.6	1	EVWXGXWDK	99.0	15	
			1916.9	1	YGESXEXAEYXSDYHK	99.0	26	
			1330.5	1	AWEDAEM^{ox}FCR	99.0	12	
			1622.8	1	EFCVEXVSYTGYR	99.0	19	
			1472.7	1	YBPGCHXASFHR	99.0	18	
7a	2.6	25	1688.0	1	BBN^{da}VXTDBDXMXXR	99.0	14	Serine proteinase; ~P81661
7b	0.6	16	1140.6	1	VSDYTEWXK	99.0	15	Serine proteinase; ~Q072L6
			2478.3	1	RAAYPWQ^{da}P^{ox}VSSTTXCAGXXBGGK	99.0	11	
			1688.0	1	BBN^{da}VXTDBDXMXXR	99.0	21	
			1063.6	1	EBFXCPNR	97.1	9	
			1314.6	1	BYMWEWTDR	96.6	8	
7c	2.5	12	1622.8	1	EFCVEXVSYTGYR	99.0	18	C-type lectin/lectin-like; ~P86970
			2747.4	1	NBPDHYBNBEFCVEXVSYTGYR	99.0	24	
			2025.9	1	NNCPBDWXPM^{ox}NGXCYK	99.0	15	
			1917.0	1	YGESXEXAEYXSDYHK	99.0	24	
			1472.8	1	YBPGCHXASFHR	99.0	12	
8	1.1	25	2526.4	1	PHCANXNXXDHAVCRAAYSWR	–	9	Serine proteinase; ~Q9DF67
9a	0.7	31	1526.8	1	XXGGDECNXNEHR	99.0	8	Serine proteinase; ~Q8QG86
			942.6	1	XYXGXHAR	99.0	11	
			1978.1	1	GSXCGGTXXNBEWVXTAR	99.0	14	
9b	0.8	23	3024.7	1	SVDFDSESPRBPEXBNEXVDXHNSXR	99.0	10	Cysteine-rich secretory protein; ~F2Q6G1
			1905.1	1	BPEXBNEXVDXHNSXR	99.0	21	
			1537.8	1	MEWYPEAAANAER	99.0	20	
			1798.0	1	BWTEXXHAWHGEYK	99.0	22	
9c	0.1	18	1798.0	1	BWTEXXHAWHGEYK	99.0	17	Cysteine-rich secretory protein; ~F2Q6G1
			1537.7	1	MEWYPEAAANAER	99.0	14	
			2712.3	1	SVNPTASNMXBMEWYPEAAANAER	90.6	8	
9d	0.1	12	1916.9	1	YGESXEXAEYXSDYHK	99.0	18	C-type lectin/lectin-like; ~P86970
			1472.7	1	YBPGCHXASFHR	99.0	9	
			1187.5	1	NAFXCBCBF	99.0	12	
			2747.3	1	NBPDHYBNBEFCVEXVSYTGYR	99.0	12	
			1314.5	1	BYMWEWTDR	98.9	9	
			2052.0	1	DNBDTYDNBYWFFPAK	99.0	13	Phospholipase A_2, D49; ~I2DAL4
			1338.7	1	SXXBFETXXM^{ox}K	95.8	8	
10	1.2	32	1206.7	1	XM^{ox}GWGTXSPTK	99.0	10	Serine proteinase; ~Q072L6
			942.6	1	XYXGXHAR	99.0	10	
			1513.7	1	VXGGDECDXNEHR	99.0	8	
11a	1.3	28	1443.8	1	VXNEDEBTRDPK	99.0	13	Serine proteinase; ~Q072L6
			1190.7	1	XMGWGTXSPTK	99.0	10	
			1571.9	1	BVXNEDEBTRDPK	99.0	16	
			1512.8	1	VXGGDECNXNEHR	99.0	18	
			2477.4	1	VSNSEHXAPXSXPSSPPSVGSVCR	99.0	14	
			2889.6	1	XDSPVSNSEHXAPXSXPSSPPSVGSVCR	99.0	15	
11b	1.3	13	1694.8	1	DTYDNBYWFFPAK	99.0	10	Phospholipase A_2, D49; ~I2DAL4
			1322.8	1	SXXBFETXXMK	99.0	16	
			2221.0	1	SGVWYYGSYGCYCGSGGBGR	99.0	17	
			1338.8	1	SXXBFETXXMK	99.0	15	
			1505.6	1	CCFVHDCCYGK	99.0	16	
			2052.0	1	DNBDTYDNBYWFFPAK	99.0	25	
			958.5	1	YWFFPAK	96.0	6	

Table 1 (*continued*)

Peak	%	Mass (kDa)▼	Peptide ion		MS/MS-derived sequence[a]	Conf (%)[b]	Sc[b]	Protein family; ~related protein
			m/z	z				
12a	3.5	35	1512.8	1	VXGGDECNXNEHR	99.0	17	Serine proteinase; ~Q5W960
			2575.4	1	FXCGGTXXNBEWVXSAAHCNRK	99.0	14	
			942.6	1	XYXGXHAR	99.0	13	
12b	0.7	32	2352.2	1	VXGGDECNXNEH(1014.3)	Man	Man	Serine proteinase; ~Q5W958
12c	1.2	28	1545.9	1	NDDAXDK^{fo}DXMXVR	99.0	13	Serine proteinase; ~Q5W960
			2477.4	1	VSNSEHXAPXSXPSSPPSVGSVCR	99.0	13	
			1218.7	1	XM^{ox}GWGXXSPTK	99.0	9	
			2492.3	1	DXYPDVPHCANXNXXDHAVCR	99.0	11	
13a	0.8	35	942.6	1	XYXGXHAR	99.0	13	Serine proteinase; ~Q5W958
			1189.7	1	WDBDXMXXR	99.0	11	
			1512.8	1	VXGGDECNXNEHR	99.0	19	
13b	0.8	32	1189.7	1	WDBDXMXXR	99.0	12	Serine proteinase; ~Q8AY81
			1512.8	1	VXGGDECNXNEHR	99.0	12	
			942.6	1	XYXGXHAR	99.0	11	
			1372.8	1	XPSSP^{ox}PSXGSVCR	99.0	16	
			932.5	1	YFCXSSR	98.9	10	
13c	0.3	28	1512.7	1	VXGGDECNXNEHR	99.0	13	Serine proteinase; ~D5KRX9
			1372.8	1	XPSSP^{ox}PSXGSVCR	99.0	11	
14	4.4	34	1561.8	1	SVPNDDEEXRYPK	99.0	13	Serine proteinase; ~Q5W960
			1818.9	1	SVPNDDEEXRYPBEK	99.0	11	
			1746.0	1	BBNDEBDBDXMXXR	99.0	16	
			942.6	1	XYXGXHAR	99.0	12	
15a	1.2	68	2421.2	1	FHECNXGNXXCDAVXYNNVR	99.0	12	5′-Nucleotidase; ~T2HRS9
			1148.6	1	DFPEXXCVR	99.0	11	Transferrin; ~CAK18223
			2507.4	1	ADK^{de}XW^{ky}AXXSYAXEBNBBDBSR	99.0	15	
15b	3.6	23	1313.7	1	YXEXVVVADHR	99.0	16	Metalloproteinase; ~Q5XUW8
			1094.6	1	YNSNXNTXR	99.0	13	
			1581.7	1	VHEMXNTVNGFYR	99.0	11	
15c	1.3	14	2193.0	1	GFCCPXGWSSYDBHCYR	99.0	13	C-type lectin/lectin-like; ~T2HPS7
			1019.6	1	XYVWXGXR	99.0	12	
16	5.4	22	1094.6	1	YNSNXNTXR	99.0	13	Metalloproteinase; ~P0DL29
			1581.8	1	VHEMXNTVNGFYR	99.0	11	
			1313.8	1	YXEXVVVADHR	99.0	14	
			1838.9	1	TRVHEMXNTVNGFYR	98.9	11	
17a	2.0	48	1352.7	1	SAGBXYEESXBK	99.0	11	L-amino acid oxidase; ~Q6TGQ8
			2271.2	1	XYFAGEYTABAHGWXDSTXK	99.0	13	
			1535.7	1	ADNK^{ca}NPXEECFR	99.0	13	
			1915.0	1	ETXSVTADYVXVCTTSR	99.0	20	
17b	2.8	22	2271.2	1	XYFAGEYTABAHGWXDSTXK	99.0	6	L-amino acid oxidase; ~Q6TGQ8
17c	0.8	14	2192.9	1	GFCCPXGWSSYDBHCYR	99.0	10	C-type lectin/lectin-like; ~T2HPS7
18a	0.2	61	1997.8	1	XTPGSBCADGXCCDBCR	99.0	12	Metalloproteinase; ~Q0NZY0
			2210.2	1	XYEXVNTMNEMFXPXNXR	99.0	16	
18b	0.3	50	1535.7	1	ADNK^{ca}NPXEECFR	99.0	13	L-amino acid oxidase; ~Q6TGQ8
			2271.1	1	XYFAGEYTABAHGWXDSTXK	99.0	14	
			3006.5	1	YAM^{ox}GGXTTFTPYBFBHFSEAXTAPVGR	99.0	10	
			1915.0	1	ETXSVTADYVXVCTTSR	99.0	24	
18c	0.1	33	1983.8	1	XTPGSBCADGVCCDBCR	99.0	12	Metalloproteinase; ~E3UJL9
			1561.8	1	SVPNDDEEXRYPK	99.0	13	
			1532.8	1	HRPBCXXNEPXR	99.0	12	
			942.6	1	XYXGXHAR	99.0	12	
			2242.1	1	XYEXVNTM^{ox}NEM^{ox}FXPXNXR	95.1	9	
18d	0.3	23	2363.3	1	XSHD^{cationNa}N^{da}ABXXTAXVFDBBTXGR	99.0	18	Metalloproteinase; ~P0DL29
			1327.8	1	YXEXVVVADHR	99.0	9	
18e	0.2	14	1918.8	1	DCPSDWSSYEGHCYR	99.0	16	C-type lectin/lectin-like; ~P0DJC9
			1887.9	1	Y^{ca}EAWAEESYCVYFK	99.0	18	
			1004.6	1	M^{ox}NVWXGXR	99.0	11	
			1269.8	1	XBADVVWXGXR	99.0	12	
			1019.6	1	XYVWXGXR	98.3	10	
19a	1.3	50	1353.8	1	YVEXXXVADYK^{fo}	99.0	15	Metalloproteinase; ~E3UJL0
19b	1.0	40	1025.5	1	FBGAGTECR	99.0	9	Metalloproteinase; ~F8RTZ7
			1269.6	1	SAECTDRFBR	99.0	10	
			1728.8	1	AABDECDM^{ox}ADXCTGR	99.0	11	
			1183.6	1	ZSNXTPEBBR	99.0	22	
19c	0.2	33	1327.8	1	YXEXVXVADHR	99.0	9	Metalloproteinase; ~J3SDW4
			1983.8	1	XTPGSBCADGVCCDBCR	77.9	6	
19d	0.2	28	1821.8	1	PGE^{dh}BCAEGXCCDBCR	99.0	11	Metalloproteinase; ~O93516
			1183.6	1	ZSNXTPEBBR	99.0	12	
			1327.8	1	YXEXVXVADHR	96.0	8	

(*continued on next page*)

Table 1 (*continued*)

Peak	%	Mass (kDa)▼	Peptide ion		MS/MS-derived sequence[a]	Conf (%)[b]	Sc[b]	Protein family; ~related protein
			m/z	z				
19e	0.9	23	1313.7	1	YXEXVVVADHR	99.0	13	Metalloproteinase; ~ P83512
			1852.9	1	TRVHEMXNTVNGFYR	99.0	13	
			2363.3	1	XSHDNABXXTAXVFDBBTXGR	99.0	22	
19f	0.6	14	1918.8	1	DCPSDWSSYEGHCYR	99.0	15	C-type lectin/lectin-like; ~T1E3Z4
			1887.9	1	Y^{ca}EAWAEESYCVYFK	99.0	20	
			919.4	1	NWEDAER	99.0	10	
			1019.6	1	XYVWXGXR	99.0	11	
			1004.6	1	M^{ox}NVWXGXR	99.0	11	
			1269.7	1	XBADVVWXGXR	99.0	12	
20a	0.6	51	1352.7	1	SAGBXYEESXBK	99.0	13	L-amino acid oxidase; ~B5AR80
			2279.2	1	VXBXQ^{da}BDVBEVTVTYBTSAK	99.0	17	
			1915.0	1	ETXSVTADYVXVCTTSR	99.0	21	
20b	0.6	40	1025.5	1	FBGAGTECR	99.0	13	Metalloproteinase; ~F8RTZ7
			1269.6	1	SAECTDRFBR	99.0	12	
			1821.8	1	P^{dh}GEBCAEGXCCDBCR	99.0	22	
			1183.6	1	ZSNXTPEBBR	99.0	11	
			1728.8	1	AABDECDM^{ox}ADXCTGR	97.2	9	
20c	0.2	35	1983.8	1	XTPGSBCADGVCCDBCR	99.0	9	Metalloproteinase; ~E3UJL9
			1327.7	1	YXEXVXVADHR	99.0	8	
20d	0.2	28	2477.3	1	VSNSEHXAPXSXPSSPPSVGSVCR	99.0	8	Serine proteinase; ~T1E3X0
			2889.5	1	XDSPVSNSEHXAPXSXPSSPPSVGSVCR	99.0	8	
			1512.7	1	VXGGDECNXNEHR	99.0	10	
			1327.8	1	YXEXVXVADHR	99.0	12	Metalloproteinase; ~Q1ZZ79
20e	0.5	23	1313.7	1	YXEXVVVADHR	99.0	14	Metalloproteinase; ~Q072L5
			1852.9	1	TRVHEM^{ox}XNTVNGFYR	99.0	12	
20f	0.3	14	1252.7	1	RPYCTVMVVK	99.0	10	C-type lectin/lectin-like; ~T2HPS7
			1019.6	1	XYVWXGXR	99.0	11	
			1918.8	1	DCPSDWSSYEGHCYR	99.0	17	
			1269.8	1	XBADVVWXGXR	99.0	12	
21	1.2	23	1313.7	1	YXEXVVVADHR	99.0	12	Metalloproteinase; ~Q072L5
			1852.9	1	TRVHEM^{ox}XNTVNGFYR	99.0	12	

X: Leu/Ile; B: Lys/Gln; Z: pyrrolidone carboxylic acid; ^{ox}: oxidized; ^{da}: deamidated; ^{dh}: dehydrated; ^{ca}: carbamylated; ^{fo}: formylated; ^{ky}: kynurenin; de: delta-H_2C_2; *dn*: *de novo* interpreted. ▼: reduced SDS-PAGE mass estimations, in kDa.

[a] Cysteine residues are carbamidomethylated.

[b] Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot®.

alone was used as a blank, being subtracted from all readings (Wang et al., 2004).

2.4.2. Phospholipase A_2 activity

Venom phospholipase A_2 (PLA_2) activity was determined on the monodisperse synthetic substrate 4-nitro-3-octanoyl-benzoic acid (NOBA) (Holzer and Mackessy, 1996). Varying amounts of venom (3.8–30 μg), dissolved in 25 μL of water, were added to 200 μL of 10 mM Tris, 10 mM $CaCl_2$, 0.1 M NaCl, pH 8.0, in triplicate wells of a microplate. After mixing, 25 μL of NOBA (1 mg/mL in acetonitrile) were added, to achieve a final substrate concentration of 0.32 mM. The mixtures were incubated for 60 min at 37 °C, and absorbances at 405 nm were recorded. PLA_2 activity was expressed as the change in absorbance × 1000.

2.4.3. Coagulant activity

Various amounts of venom (8–32 μg) were dissolved in 100 μL 0.15 M NaCl and mixed with 200 μL of citrated human plasma, which was previously warmed at 37 °C for 5 min. Negative plasma controls without venom were included. Clotting times were recorded in triplicate assays and coagulant activity was expressed as the 'minimum coagulant dose in plasma' (MCD-P), defined as the dose inducing clotting in 60 s (Theakston and Reid, 1983).

2.5. Immunochemical and neutralization studies

2.5.1. Immunochemical cross-recognition

The immunochemical cross-recognition of *B. campbelli* venom by the equine polyvalent Viperidae antivenom manufactured at Instituto Clodomiro Picado (ICP, Universidad de Costa Rica; batch 53992014LF, expiry date 2019) was evaluated by gel immunodiffusion (GID) and immunoelectrophoresis (IEP) (Ouchterlony and Nilsson, 1978). GID was performed in 1% agarose dissolved in PBS, pH 7.2, in which wells were punched and filled with 40 μL of either antivenom or venom (5 mg/mL). The venom of *B. asper* was included as a control. After 24 h of diffusion at room temperature, precipitation lines were recorded using the ChemiDoc/ImageLab software. IEP was performed in 1% agarose gel dissolved in 0.1 M Tris, 0.3 M glycine, pH 8.6 buffer. Wells were filled with 25 μL of *B. campbelli* or *B. asper* venoms (20 mg/mL) and the plate was subjected to electrophoresis at 75 V for 90 min (Büchler Instruments). The separated proteins were allowed to diffuse for 24 h against 400 μL of the ICP antivenom placed in a central trough, and precipitation arcs were recorded as described above.

2.5.2. Neutralization by antivenom

Two control groups of mice (16–18 g body weight; $n = 4$ per group) received either four (600 μg) or three (450 μg)

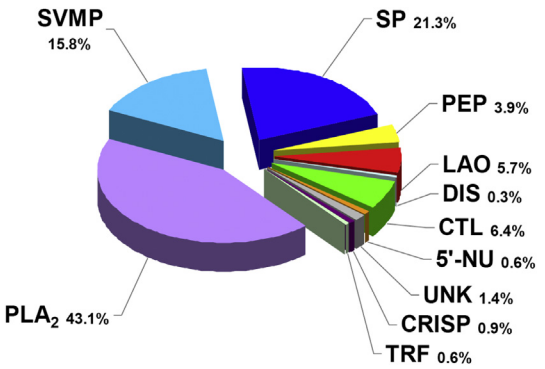

Fig. 2. Composition of the venom of *Bothrocophias campbelli* according to protein families, expressed as percentages of the total protein content: PLA_2: phospholipases A_2; SVPM: metalloproteinases; SP: serine proteinases; PEP: low molecular weight peptides; LAO: L-amino acid oxidases; DIS: disintegrins; CTL: C-type lectin/lectin-like proteins; 5′-NU: 5′-nucleotidases; UNK: unknown/unidentified; CRISP: cysteine-rich secretory proteins; TRF: transferrin.

LD_{50}s of venom in 500 μL of PBS, by the i.p. route. On the other hand, two test groups received the same amounts of venom, previously incubated for 15 min at 37 °C with the ICP antivenom, at a venom/antivenom ratio of 1.36 mg/mL. Deaths were recorded after 48 h.

2.6. Statistical analysis

The statistical significance of differences between two means was determined by Student's *t*-test, at $p < 0.05$.

3. Results and discussion

B. campbelli venom fractionation by RP-HPLC (Fig. 1) showed a chromatographic profile of slightly lower complexity in comparison to venoms from close crotalid species that have been studied by the same methodology (Núñez et al., 2009; Rodrigues et al., 2012; Mora-Obando et al., 2014; Lomonte et al., 2014b). Twenty-one peaks were collected and further resolved by SDS-PAGE into 50 protein bands, which were digested and analysed by MALDI-TOF-TOF. On the basis of the identified amino acid sequences of their tryptic peptides (Table 1), venom components were assigned to ten protein families, whose estimated abundances are summarized in Fig. 2. The three predominant protein types belong to the phospholipase A_2 (PLA_2), serine proteinase (SP), and metalloproteinase (SVMP) families, followed by lower amounts of C-type lectin/lectin-like (CTL) and L-amino acid oxidase (LAO), and minor proportions of cysteine-rich secretory proteins (CRISP), 5′-nucleotidase (5′-NU), disintegrin (DIS) and

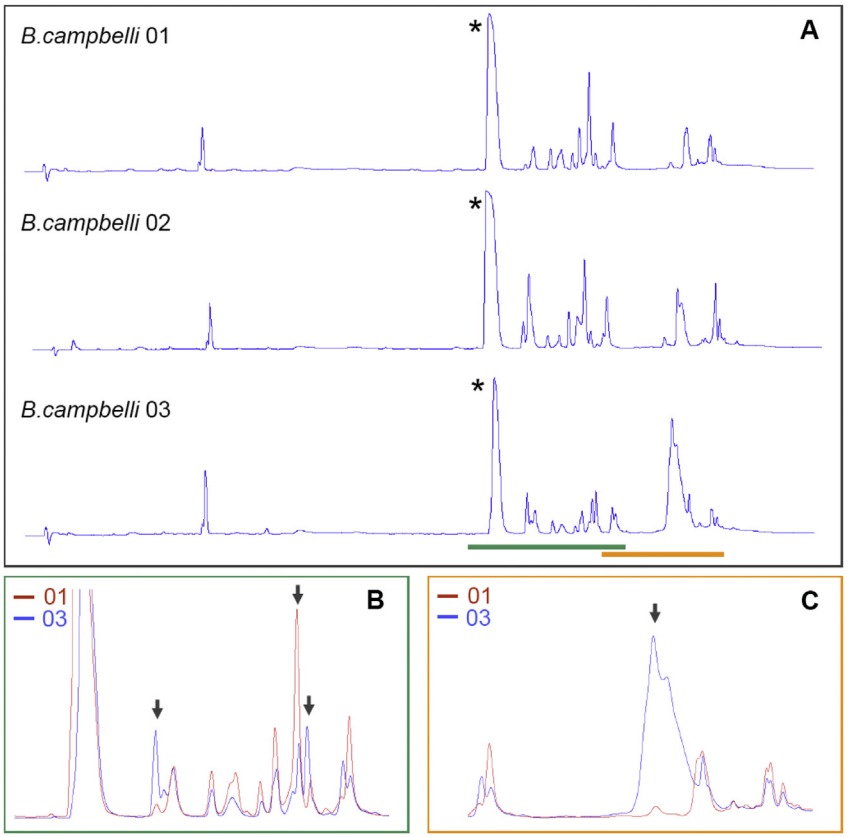

Fig. 3. Comparison of the RP-HPLC elution profiles of the venoms from three individual specimens of *Bothrocophias campbelli* of Ecuador (A). Samples 01 and 02 were males from the same locality (Mindo) and 03 was a female from another locality (El Cristal). The predominant peak identified as a Lys49 phospholipase A2 homologue is indicated by an asterisk. The lower panels (B, C) expand the regions (horizontal green and orange bars in (A), respectively) that showed highest differences between the venom of specimens 01 and 03, indicated by arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transferrin (TRF). Low molecular weight peptides eluting early in the RP-HPLC separation represented a low percentage (3.9%) of the venom, and were directly identified as ZBW-tripeptide metalloproteinase inhibitors (Menin et al., 2008) by tandem mass spectrometry. A low proportion of the venom components (1.4%) remained unidentified by this analytical approach.

In general, the protein composition of *B. campbelli* venom follows a similar qualitative trend of other crotalid venoms, but reveals variations in the proportion of some protein types in comparison to venoms from related species. Specifically, its content of SVMPs (15.8%) is considerably lower than the proportion of these enzymes observed in the venoms of *Bothrops punctatus* (41.5%; Fernández-Culma et al., 2014), *Bothrops ayerbei* (53.7%; Mora-Obando et al., 2014), *Bothrops atrox* from Colombia (48.5%; Núñez et al., 2009) or Perú (58.2%; Kohlhoff et al., 2012), *Bothrops barnetti* (74.1%; Kohlhoff et al., 2012), *Bothrops pictus* (68.0%; Kohlhoff et al., 2012), *Bothrops asper* (41.0–44.0%; Alape-Girón et al., 2008), *Bothropoides pauloensis* (38.1%; Rodrigues et al., 2012), *Cerrophidion sasai* (32.8%; Lomonte et al., 2012), *Porthidium nasutum* (52.1%; Lomonte et al., 2012), *Porthidium ophryomegas* (45.0%; Lomonte et al., 2012) or *Atropoides picadoi* (66.3%; Angulo et al., 2008), although it is closer to the SVMP content in the venom of *Bothrops pirajai* (20.7%; Bernardes et al., 2013) and *Atropoides mexicanus* (18.2%; Angulo et al., 2008). Another noteworthy observation on the venom composition of *B. campbelli* is the presence of a major component, accounting for nearly 42% of its proteins, which was identified as a Lys49 PLA_2 homologue (peak 5, Table 1). The Lys49 proteins are structurally related to PLA_2s, but catalytically-inactive, and are characterized by their ability to induce myonecrosis at the site of injection (Lomonte and Rangel, 2012). This prominent Lys49 myotoxin peak was conserved in the individual venom samples from the three specimens used in this study (Fig. 3), suggesting a relevant trophic role for this species. Due to their myotoxic activity, the Lys49 PLA_2 homologues may contribute to a more efficient digestion of the abundant muscle mass of mammalian prey, among other possible adaptive values (Lomonte et al., 2009). In general, although the venom pool hereby characterized was composed of equal parts of samples obtained from only three specimens, their individual venom variability appears to be minor, with only few changes evidenced in their chromatographic profiles (Fig. 3).

The compositional data gathered for *B. campbelli* venom showed a good correlation with its enzymatic and toxic profiles. The venom presented both proteolytic (Fig. 4A) and PLA_2 (Fig. 4B) activities. In agreement with its low proportion of SVMPs and catalytically-active Asp49 PLA_2,

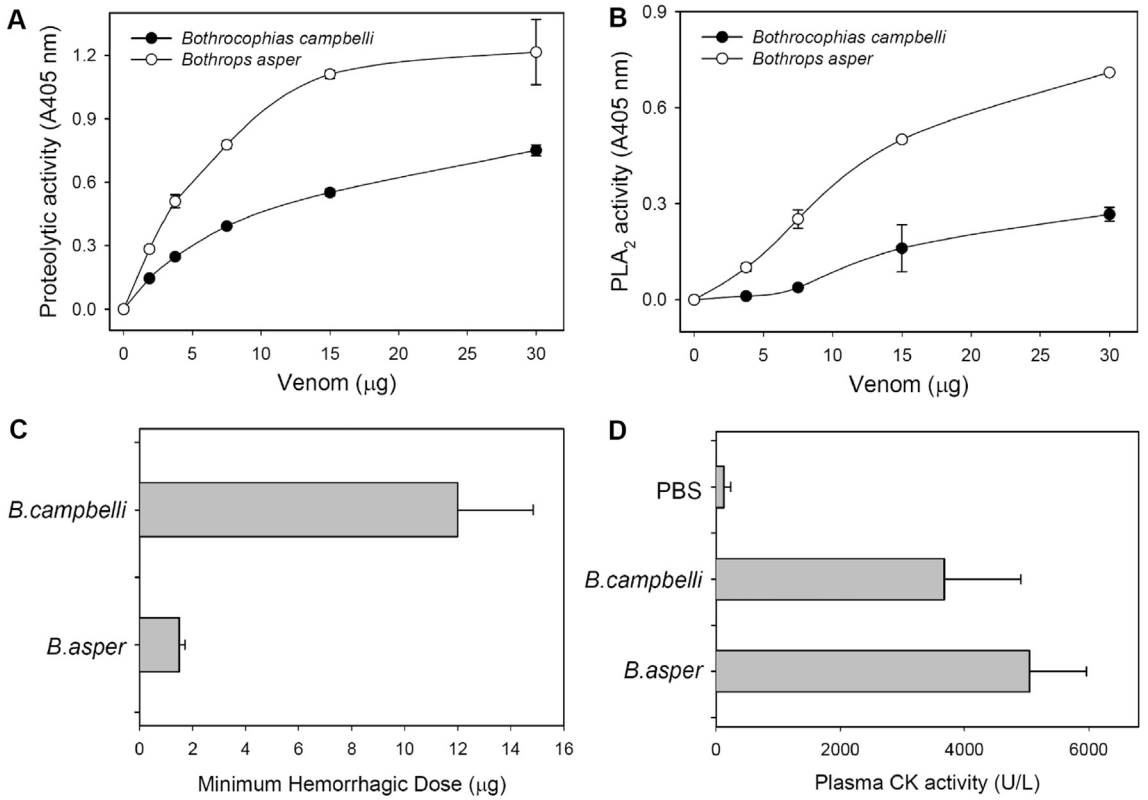

Fig. 4. Enzymatic and toxic activities of the venom of *Bothrocophias campbelli* of Ecuador, compared to the venom of *Bothrops asper* from Cauca, Colombia. (A) proteolytic activity on azocasein, (B) phospholipase A_2 activity on 4-nitro-3-octanoyl-benzoic acid, (C) hemorrhagic activity in the mouse skin assay, and (D) myotoxic activity, as described in Material as Methods. Values represent the mean ± SD of four to five replicates. All differences between *B. campbelli* and *B. asper* venoms in A, B, and C are statistically significant ($p < 0.05$) by Student's *t*-test, whereas the corresponding differences in D are not statistically significant ($p > 0.05$).

respectively, both enzymatic activities were significantly lower ($p < 0.05$ at all concentrations) than those exerted by the venom of *B. asper* from Cauca, Colombia (Fig. 4A and B). On the other hand, in concordance with its high content of Lys49 myotoxin, *B. campbelli* venom induced a strong myotoxic effect in mice (Fig. 4D), which was comparable to that induced by *B. asper* venom ($p > 0.05$). Furthermore, as predicted by its low proportion of SVMPs, the venom of *B. campbelli* showed a weak local hemorrhagic action in the mouse skin assay (Fig. 4C), that was nearly six-fold lower than *B. asper* venom.

Notably, *B. campbelli* venom presented a very low coagulant activity on citrated human plasma, with a 'minimum coagulant dose' (MCD) of 19.4 μg. This activity was approximately 20-fold lower in comparison to the venom of *B. asper*, which presented an MCD value of 0.9 μg in the same assay. Since the coagulant activity of viperid venoms depends mostly on specialized SP enzymes that display thrombin-like activity (Serrano and Maroun, 2005), and considering that SPs represent a considerable proportion in *B. campbelli* venom (21.3%), this finding suggests that most of its SPs would not correspond to the coagulant, thrombin-like type.

The median lethal potency (LD_{50}) of *B. campbelli* venom was estimated at 150 μg/mouse (i.p.), a weaker potency than that reported for *B. asper* venom from Cauca, with an LD_{50} of 100.9 μg/mouse (Mora-Obando et al., 2014). Overall, the compositional and functional profiling of *B. campbelli* venom suggests that envenomations by these snakes would be characterized by significant myotoxicity, but probably only mild or moderate haemostatic alterations and hemorrhagic effects. The validity of these predictions, based on the extrapolation of mouse assays, needs to be confirmed by clinical observations on human envenomations, which are lacking in the literature.

Due to the possibility of eventual accidents by *B. campbelli*, we evaluated its paraspecific recognition by a therapeutic antivenom. Considering the close phylogenetic relationship of this species with those of the genus *Bothrops*, we hypothesized that an antivenom prepared against the latter could cross-recognize and neutralize its venom. The ICP polyvalent Viperidae antivenom, manufactured by using a mixture of *B. asper*, *Crotalus simus*, and *Lachesis stenophrys* venoms for immunization, cross-recognized a number of *B. campbelli* venom components under native conditions by immunoelectrophoresis (Fig. 5A), and generated several identity and partial identity precipitation patterns with *B. asper* venom by GID (Fig. 5B). Moreover, the most abundant protein of *B. campbelli* venom, identified as a Lys49 myotoxin, generated a pattern of complete identity with the Lys49 myotoxin II of *B. asper* venom (Lomonte and Gutiérrez, 1989) when confronted to rabbit antibodies against the latter (Fig. 5C), indicating their high antigenic similarity. The low quantity of *B. campbelli*

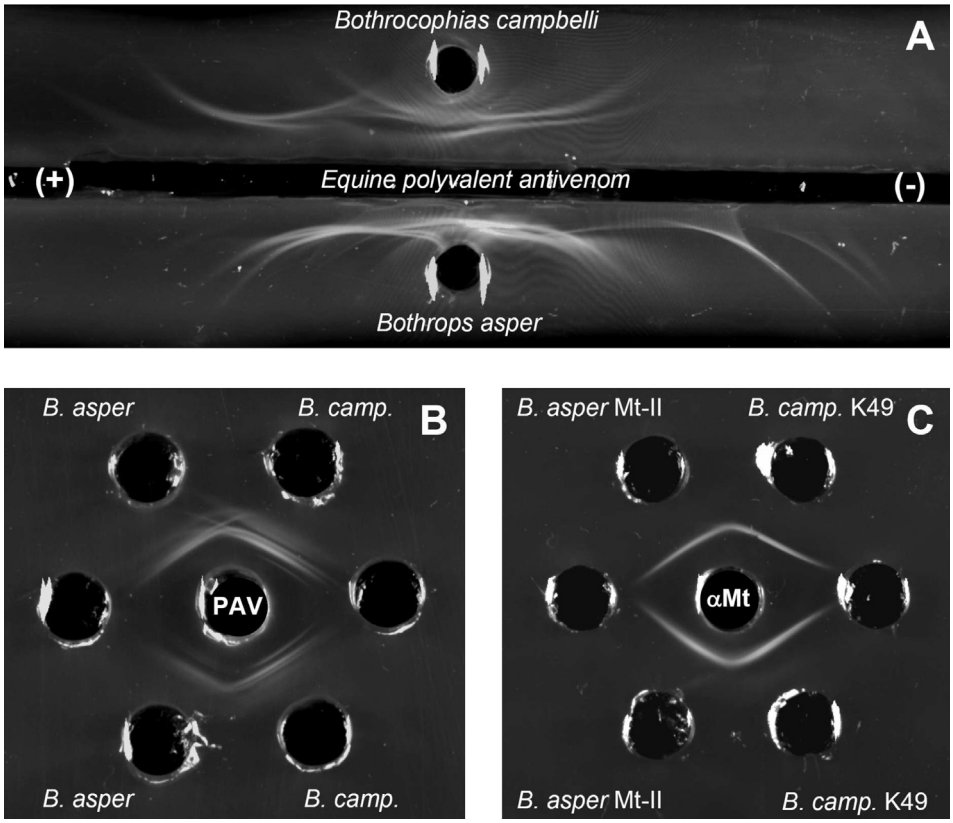

Fig. 5. Immunochemical cross-recognition of *Bothrocophias campbelli* venom of Ecuador by an equine polyvalent antivenom (PAV) to *Bothrops asper*, *Crotalus simus*, and *Lachesis stenophrys*, by immunoelectrophoresis (A) and gel immunodiffusion (B). In (C), the major K49 phospholipase A_2 homologue component of *B. campbelli* venom was tested for cross-recognition by a rabbit antiserum (αMt) to *Bothrops asper* myotoxin II (Mt-II).

venom available precluded further analyses aiming at the detailed identification of the cross-recognized components. Instead, venom was utilized to assess whether the polyvalent antivenom would neutralize its lethal effect in mice, in a preliminary evaluation with a limited number of animals. The antivenom protected two out of four mice from lethality at a ratio of 1.36 mg/mL (venom/antivenom) using a venom challenge of 3 LD_{50} (450 μg), which otherwise caused death in four out of four control animals. Compared to the potency of this antivenom against the homologous *B. asper* venom (3 mg/mL, median effective dose), this preliminary observation evidenced a significant paraspecific neutralization for *B. campbelli* venom. The latter is in agreement with the immunological cross-reactivity results and suggests the potential usefulness of the antivenom for the treatment of eventual envenomings by this species. However, further studies are necessary to properly validate the paraspecific neutralization of *B. campbelli* venom by this polyvalent antivenom.

B. campbelli is the first from the six species within this genus whose detailed venom composition and toxicological profile has been studied. From a biological standpoint, it will be relevant to comparatively analyse the venoms of the other species of this lineage to determine possible similarities and differences that could help to understand the evolution of different venom protein families during the South American radiation of New World pitvipers (e.g. Sousa et al., 2013).

Acknowledgements

This work was funded by grants from Vicerrectoría de Investigación, Universidad de Costa Rica (741-B2-652 and 741-B3-760, "Network for proteomic characterization of snake venoms of medical and biological relevance in Latin America"), and CYTED (project BioTox, P211RT0412). We thank Dr José María Gutiérrez for helpful advice and discussion, as well as Alejandro Arteaga, Lucas Bustamante, Paola Santacruz-Ortega, Omar Torres-Carvajal, Wan-Chih Tsai, Gabriela Solano, and Juan Manuel Ureña-Díaz for their valuable contribution to various aspects of this study.

Conflict of interest

The authors declare that no conflicts of interest are related to this study. M.L. Fernández and B. Lomonte work at the Instituto Clodomiro Picado (Universidad de Costa Rica), where the polyvalent antivenom used in this study is produced.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2014.07.012.

References

Alape-Girón, A., Sanz, L., Escolano, J., Flores-Díaz, M., Madrigal, M., Sasa, M., Calvete, J.J., 2008. Snake venomics of the lancehead pit viper *Bothrops asper*: geographic, individual, and ontogenetic variations. J. Proteome Res. 7, 3556–3571.

Angulo, Y., Escolano, J., Lomonte, B., Gutiérrez, J.M., Sanz, L., Calvete, J.J., 2008. Snake venomics of Central American pitvipers. Clues for rationalizing the distinct envenomation profiles of *Atropoides nummifer* and *Atropoides picadoi*. J. Proteome Res. 7, 708–719.

Arteaga, A., 2013. *Bothrocophias campbelli*. In: Arteaga, A., Bustamante, L., Guayasamín, J.M. (Eds.), The Amphibians and Reptiles of Mindo. Universidad Tecnológica Indoamérica, Quito, pp. 192–193.

Bernardes, C.P., Menaldo, D.L., Camacho, E., Rosa, J.C., Escalante, T., Rucavado, A., Lomonte, B., Gutiérrez, J.M., Sampaio, S.V., 2013. Proteomic analysis of *Bothrops pirajai* snake venom and characterization of BpirMP, a new P-I metalloproteinase. J. Proteomics 80, 250–267.

Calvete, J.J., 2013. Snake venomics: from the inventory of toxins to biology. Toxicon 75, 44–62.

Calvete, J.J., Juárez, P., Sanz, L., 2007. Snake venomics, strategy and applications. J. Mass Spectrom. 42, 1405–1414.

Campbell, J.A., Lamar, W.W., 2004. The Venomous Reptiles of the Western Hemisphere. Cornell University Press, Ithaca, New York.

Carrasco, P.A., Mattoni, C.I., Leynaud, G.C., Scrocchi, G.J., 2012. Morphology, phylogeny and taxonomy of South American bothropoid pitvipers (Serpentes, Viperidae). Zool. Scr. 41, 109–124.

Castro, F., Ayerbe, S., Calderón, J.J., Cepeda, B., 2005. Nuevo registro para Colombia de *Bothrocophias campbelli* y notas sobre *B. colombianus* y *B. myersi*. Noved. Colomb. 8, 57–64.

Cisneros-Heredia, D.F., Borja, M.O., Proaño, D., Touzet, J.M., 2006. Distribution and natural history of the Ecuadorian Toad-headed pitvipers of the genus *Bothrocophias*. Herpetozoa 19, 17–26.

Fenwick, A.M., Gutberlet Jr., R.L., Evans, J.A., Parkinson, C.L., 2009. Morphological and molecular evidence for phylogeny and classification of South American pitvipers, genera *Bothrops*, *Bothriopsis*, and *Bothrocophias* (Serpentes: Viperidae). Zool. J. Linn. Soc. 156, 617–640.

Fernández-Culma, M., Pereáñez, J.A., Núñez-Rangel, V., Lomonte, B., 2014. Snake venomics of *Bothrops punctatus*, a semi-arboreal pitviper species from Antioquia, Colombia. Peer J. 2, e246. http://dx.doi.org/10.7717/peerj.246.

Freire-Lascano, A., 1991. Dos nuevas especies de *Bothrops* en el Ecuador. Universidad Técnica de Machala, Publicaciones de Trabajos Científicos del Ecuador, pp. 1–11.

Freire, A., Kuch, U., 2000. *Bothrops campbelli* (Campbell's lancehead). Diet and reproduction. Herpetol. Rev. 31, 45.

Gibbs, H.L., Sanz, L., Sovic, M.G., Calvete, J.J., 2013. Phylogeny-based comparative analysis of venom proteome variation in a clade of rattlesnakes (*Sistrurus* sp.). PLoS One 8, e67220.

Gutberlet Jr., R.L., Campbell, J.A., 2001. Generic recognition for a neglected lineage of South American pitvipers (Squamata: Viperidae: Crotalinae), with the description of a new species from the Colombian Chocó. Am. Mus. Novit. 3316, 1–15.

Gutberlet Jr., R.L., Harvey, M.B., 2004. The evolution of New World venomous snakes. In: Campbell, J.A., Lamar, W.W. (Eds.), Venomous Reptiles of the Western Hemisphere. Cornell University Press, Ithaca, New York, pp. 634–682.

Gutiérrez, J.M., Lomonte, B., Cerdas, L., 1986. Isolation and partial characterization of a myotoxin from the venom of the snake *Bothrops nummifer*. Toxicon 24, 885–894.

Holzer, M., Mackessy, S.P., 1996. An aqueous endpoint assay of snake venom phospholipase A_2. Toxicon 34, 1149–1155.

Jadin, R.C., Smith, E.N., Campbell, J.A., 2011. Unravelling a tangle of Mexican serpents: a systematic revision of highland pitvipers. Zool. J. Linn. Soc. 163, 943–958.

Kohlhoff, M., Borges, M.H., Yarleque, A., Cabezas, C., Richardson, M., Sanchez, E.F., 2012. Exploring the proteomes of the venoms of the Peruvian pit vipers *Bothrops atrox*, *B. barnetti* and *B. pictus*. J. Proteomics 75, 2181–2195.

Kuch, U., Freire, A., 1995. Notes on morphology, reproduction and medical importance of the poorly known small-eyed lancehead, *Bothrops microphthalmus* Cope, 1876, in Ecuador. Herpetozoa 8, 81–83.

Lomonte, B., Gutiérrez, J.M., 1989. A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). Toxicon 27, 725–733.

Lomonte, B., Rangel, J., 2012. Snake venom Lys49 myotoxins: from phospholipases A_2 to non-enzymatic membrane disruptors. Toxicon 60, 520–530.

Lomonte, B., Angulo, Y., Sasa, M., Gutiérrez, J.M., 2009. The phospholipase A_2 homologues of snake venoms: biological activities and their possible adaptive roles. Prot. Pept. Lett. 16, 860–876.

Lomonte, B., Rey-Suárez, P., Tsai, W.C., Angulo, Y., Sasa, M., Gutiérrez, J.M., Calvete, J.J., 2012. Snake venomics of the pitvipers *Porthidium*

nasutum, *Porthidium ophryomegas*, and *Cerrophidion godmani* from Costa Rica: toxicological and taxonomical insights. J. Proteomics 75, 1675–1689.

Lomonte, B., Tsai, W.C., Ureña-Díaz, J.M., Sanz, L., Mora-Obando, D., Sánchez, E.E., Fry, B.G., Gutiérrez, J.M., Gibbs, H.L., Calvete, J.J., 2014a. Venomics of new world pit vipers: genus-wide comparisons of venom proteomes across *Agkistrodon*. J. Proteomics 96, 103–116.

Lomonte, B., Fernández, J., Sanz, L., Angulo, Y., Sasa, M., Gutiérrez, J.M., Calvete, J.J., 2014b. Venomous snakes of Costa Rica: biological and medical implications of their venom proteomic profiles analyzed through the strategy of "snake venomics". J. Proteomics 105, 323–339.

Mackessy, S.P., 2010. The field of reptile toxinology. In: Mackessy, S.P. (Ed.), Handbook of Venoms and Toxins of Reptiles. CRC Press, Boca Raton, pp. 3–23.

Mebs, D., 2001. Toxicity in animals. Trends in evolution? Toxicon 39, 87–96.

Menin, L., Perchuć, A., Favreau, P., Perret, F., Michalet, S., Schöni, R., Wilmer, M., Stocklin, R., 2008. High throughput screening of bradykinin-potentiating peptides in *Bothrops moojeni* snake venom using precursor ion mass spectrometry. Toxicon 51, 1288–1302.

Mora-Obando, D., Guerrero-Vargas, J., Prieto-Sánchez, R., Beltrán, J., Rucavado, A., Sasa, M., Gutiérrez, J.M., Ayerbe, S., Lomonte, B., 2014. Proteomic and functional profiling of the venom of *Bothrops ayerbei* from Cauca, Colombia, reveals striking interspecific variation with *Bothrops asper* venom. J. Proteomics 96, 159–172.

Núñez, V., Cid, P., Sanz, L., De La Torre, P., Angulo, Y., Lomonte, B., Gutiérrez, J.M., Calvete, J.J., 2009. Snake venomics and antivenomics of *Bothrops atrox* venoms from Colombia and the Amazon regions of Brazil, Perú and Ecuador suggest the occurrence of geographic variation of venom phenotype by a trend towards paedomorphism. J. Proteomics 73, 57–78.

Ouchterlony, Ö., Nilsson, L.Å., 1978. Immunodiffusion and immunoelectrophoresis. In: Weir, D.M. (Ed.), Handbook of Experimental Immunology. Blackwell Scientific Publications, Oxford, pp. 19.1–19.44.

Rodrigues, R.S., Boldrini-França, J., Fonseca, F.P.P., de la Torre, P., Henrique-Silva, F., Sanz, L., Calvete, J.J., Rodrigues, V.R., 2012. Combined snake venomics and venom gland transcriptomic analysis of *Bothropoides pauloensis*. J. Proteomics 75, 2707–2720.

Rojas-Rivera, A., Castillo, K., Gutiérrez-Cárdenas, P.D.A., 2013. *Bothrocophias campbelli* (Campbell's toad-headed pitviper, víbora boca de sapo de Campbell). Diet/ophiophagy. Herpetol. Rev. 44, 518.

Serrano, S.M.T., Maroun, R.C., 2005. Snake venom serine proteinases: sequence homology vs substrate specificity, a paradox to be solved. Toxicon 45, 1115–1132.

Sousa, L.F., Nicolau, C.A., Peixoto, P.S., Bernardoni, J.L., Oliveira, S.S., Portes-Junior, J.A., Mourão, R.A., Helena, V., Lima-dos-Santos, I., Sano-Martins, I.S., Chalkidis, H.M., Valente, R.H., Moura-da-Silva, A.M., 2013. Comparison of phylogeny, venom composition and neutralization by antivenom in diverse species of *Bothrops* complex. PLoS Negl. Trop. Dis., e2442.

Theakston, R.D.G., Reid, H.A., 1983. Development of simple standard assay procedures for the characterization of snake venoms. Bull. World Health Organ. 61, 949–956.

Valencia, J.H., Garzón, K., Betancourt-Yépez, R., 2008. Notes on the reproduction of the Ecuadorian toad-headed pitviper *Bothrocophias campbelli* (Freire-Lascano, 1991). Herpetozoa 21, 95–96.

Wang, W.J., Shih, C.H., Huang, T.F., 2004. A novel P-I class metalloproteinase with broad substrate-cleaving activity, agkislysin, from *Agkistrodon acutus* venom. Biochem. Biophys. Res. Commun. 324, 224–230.

Warrell, D.A., 2004. Snakebites in Central and South America: epidemiology, clinical features, and clinical management. In: Campbell, J.A., Lamar, W.W. (Eds.), The Venomous Reptiles of the Western Hemisphere. Cornell University Press, Ithaca, New York, pp. 709–761.

Werman, S.D., 2005. Hypotheses on the historical biogeography of Bothropoid pitvipers and related genera of the Neotropics. In: Donnelly, M.A., Crother, B.I., Guyer, C., Wake, M.H., White, M.E. (Eds.), Ecology and Evolution in the Tropics: a Herpetological Perspective. University of Chicago Press, Chicago, pp. 306–365.

Zuffi, M.A.L., 2004. *Bothrops campbelli* (Campbell's Lancehead). Diet Herpetol. Rev. 35, 57–58.