Comparative characterization of Viperidae snake venoms from Perú reveals two compositional patterns of phospholipase A$_2$ expression

Bruno Lomonte [a,*], Cecilia Díaz [a,b], Fernando Chaves [a], Julián Fernández [a], Marco Ruiz [a], María Salas [c], Alfonso Zavaleta [c], Juan J. Calvete [d], Mahmood Sasa [a,e]

[a] *Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica*
[b] *Departamento de Bioquímica, Escuela de Medicina, Universidad de Costa Rica, San José, Costa Rica*
[c] *Departamento Académico de Ciencias Celulares y Moleculares, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Peru*
[d] *Laboratorio de Venómica Evolutiva y Traslacional, Instituto de Biomedicina de Valencia, CSIC, Valencia, Spain*
[e] *Escuela de Biología, Universidad de Costa Rica, San José, Costa Rica*

ARTICLE INFO

ABSTRACT

Snake species within the *Bothrops* complex (*sensu lato*) are of medical relevance in Latin America, but knowledge on their venom characteristics is limited, or even unavailable, for some taxa. Perú harbors 17 species of pit vipers, within the genera *Bothrops*, *Bothriechis*, *Bothrocophias*, *Porthidium*, *Crotalus*, and *Lachesis*. This study compared the venoms of twelve species, through chromatographic and electrophoretic profiles, as well as proteolytic and phospholipase A$_2$ (PLA$_2$) activities. Also, proteomic profiles were analyzed for nine of the venoms using a shotgun approach. Results unveiled conspicuous differences in the expression of venom PLA$_2$s among species, six of them presenting scarce levels as judged by RP-HPLC profiles. Since most species within the bothropoid lineage possess venoms with high to intermediate abundances of this protein family, our findings suggest the existence of a phenotypic duality in the expression of venom PLA$_2$s within the *Bothrops* (*sensu lato*) complex. *Bothrops barnetti* and *Bothrocophias andianus* venoms, very scarce in PLA$_2$s, were shown to lack significant myotoxic activity, highlighting that the observed variability in PLA$_2$ expression bears toxicological correlations with effects attributed to these proteins. Finally, an attempt to identify phylogenetic relationships of bothropoid species from Perú presenting low- or high-PLA$_2$ venom phenotypes showed an interspersed pattern, thus precluding a simple phylogenetic interpretation of this venom compositional dichotomy.

1. Introduction

In the Americas, an average of 6.2 snakebites per 100,000 population (about 57,500 accidents) is estimated to occur annually, leading to a fatality rate of 0.04 per 100,000 population (Chippaux, 2017). The genus *Bothrops* (lanceheads), distributed from Mexico to southern Argentina, is responsible for the majority of envenomings in Latin America (Chippaux, 2017). Classified within the Viperidae family, this genus includes 45 species listed in the Reptile Database (http://www.reptile-database.org) and has been the subject of dynamic taxonomic reclassifications within the bothropoid lineages in the last two decades (Wüster et al., 2002; Fenwick et al., 2009; Carrasco et al., 2012, 2016; Alencar et al., 2016). Despite the medical relevance of species within the *Bothrops* complex (*sensu lato*), knowledge on the characteristics of their venoms is still limited, or unavailable, for some species.

The growing body of information on the composition of viperid venoms, as provided by "omics" approaches, has revealed the existence of a limited number of protein families that are generally shared among conspecific species, with both qualitative and quantitative variations (Calvete, 2013, 2017; Lomonte et al., 2014; Göçmen et al., 2015; Tan et al., 2015; Viala et al., 2015; Serrano et al., 2018). Knowledge of this variability is essential for the production of broad-coverage therapeutic antivenoms to confront snakebite envenomings. Also, the study of inter- and intraspecific venom variability may shed light on understanding the evolutionary pathways that have shaped the present-day landscape of the snake venom toxin arsenals.

Perú harbors a rich herpetofauna, which includes 34 species or subspecies of venomous snakes distributed in two families, Elapidae and Viperidae. Sixteen coral snakes represent the former, all included in the genus *Micrurus* of subfamily Elapinae, and a single species of sea snake,

---

Hydrophis platurus within subfamily Hydrophiinae. Viperidae includes 17 pitviper species (Carrillo de Espinoza and Icochea, 1995) within the genera *Bothrops, Bothriechis, Bothrocophias, Porthidium, Crotalus,* and *Lachesis* (taxonomical nomenclature in the present work follows the proposal of Carrasco et al., [2016] for the *Bothrops* complex, *sensu lato*). Viperidae species are responsible for the vast majority of the estimated 1500–2500 snakebites/year recorded in Perú (Ministerio de Salud, 2017), resulting in a mortality rate of not less than 0.043/100,000 population (Chippaux, 2017).

Previous studies have examined various biochemical aspects and toxic activities of the venoms of Viperidae snakes inhabiting Perú (Sanz et al., 2008; Núñez et al., 2009; Jimenez et al., 2010; Kohlhoff et al., 2012; Guerra-Duarte et al., 2015; Rodrigues et al., 2018), and their neutralization (Laing et al., 2004; Rojas et al., 2005; García et al., 2008; Estevao-Costa et al., 2016). However, information on most venoms of these species is still partial, and more efforts are needed to gain a broader knowledge of their general properties. In the present study, we have comparatively explored the chromatographic and electrophoretic profiles of the venoms of twelve species of pitvipers, as well as their proteolytic and phospholipase A_2 (PLA$_2$) activities. Besides, proteomic profiles were analyzed for nine of the venoms using a shotgun approach. These comparative analyses revealed important differences in the expression of PLA$_2$s among the species, which relate to variations in toxicological activities attributed to these enzymes and their related PLA$_2$-like homologs.

2. Materials and methods

2.1. Venoms

Venom pools were obtained from adult specimens collected in Perú, from the species and locations indicated in Table 1. In some comparative experiments, venoms of species from other regions of Latin America were included: *Bothrops alternatus* (Coronel Pringles, Argentina), *B. neuwiedii* (Tacuarembó, Uruguay), *B. asper* (Pacific versant, Costa Rica), and *Bothriechis schlegelii* (Costa Rica).

2.2. SDS-PAGE

Venom proteins were separated by SDS-PAGE using pre-cast gradient gels (4–20%; Bio-Rad), either unreduced or after reduction with 2-mercaptoethanol (5 min at 95 °C). Samples (30 μg) were separated at 150 V, along with molecular weight standards (Bio-Rad). Proteins were visualized by Coomassie blue R-250 staining and electrophoretic patterns were recorded with the ImageLab™ software (Bio-Rad).

2.3. Reverse phase-HPLC profiling

Chromatographic profiles of venoms were obtained by RP-HPLC using a C$_{18}$ column (250 × 4.6 mm, 5 μm particle diameter; Phenomenex) in a model 1220 instrument (Agilent) monitored at 215 nm. Venoms (1.5–2.0 mg) were dissolved in purified water (18 MΩ/cm) containing 0.1% trifluoroacetic acid (TFA; solution A), injected, and eluted with a gradient toward acetonitrile with 0.1% TFA (solution B), at a flow rate of 1 mL/min, for a total time of 94 min: 0% B for 5 min, 0–15% B in 10 min, 15–45% B in 60 min, 45–70% B in 10 min, and 70% B for 9 min (Lomonte and Calvete, 2017).

2.4. Phospholipase A$_2$ activity

Phospholipase A$_2$ (PLA$_2$) activity of the venoms was assayed using the synthetic substrate 4-nitro-3-octanoyloxy-benzoic acid (NOBA), as described (Mora-Obando et al., 2014a). Various amounts of venoms (5, 10, or 20 μg), in 25 μL, were added to 25 μL of NOBA (1 mg/mL acetonitrile) and 200 μL of 10 mM Tris, 10 mM CaCl$_2$, 0.1 M NaCl (pH 8.0), in 96-well microplates. Blanks in which venom was replaced with buffer were included. The mixtures were incubated at 37 °C for 60 min, and final absorbances at 450 nm were measured in a microplate reader (Multiskan FC, Thermo), using three replicates for each venom concentration.

2.5. Proteolytic activity

Proteolytic activity of the venoms was determined using azocasein (10 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM CaCl$_2$, pH 8.0) as substrate, as previously described (Jiménez-Charris et al., 2015). Various amounts of venoms (5, 10, or 20 μg) were incubated with azocasein at 37 °C for 90 min, in a total volume of 100 μL, and the reaction was stopped by adding 200 μL of 5% trichloroacetic acid (TCA). Blanks in which venom was omitted were included. After centrifugation of the microplates, 150 μL of the supernatant was transferred to clean wells, and 150 μL of 0.5 M NaOH was added for color development. Absorbances were measured at 450 nm in a microplate reader (Multiskan FC, Thermo), using three replicates for each venom concentration.

2.6. Proteomic profiling

Venoms from nine species were analyzed by a bottom-up shotgun proteomic approach. Samples of 15 μg were reduced with 10 mM dithiothreitol for 30 min at 56 °C, alkylated with 50 mM iodoacetamide for 20 min in the dark, and digested with sequencing grade trypsin at 37 °C overnight, in a total volume of 40 μL. After the addition of 0.5 μL of formic acid, the resulting tryptic peptide mixtures were centrifuged and separated by RP-HPLC on a nano-Easy 1200 chromatograph (Thermo)

Table 1
Venoms from Viperidae species of Perú analyzed in the present study, and their localities.

Species [a]	Common name	n [b]	District	Province	Department
Bothrops atrox	jergón de la selva	60	Llata, Chaglia, Puerto Inca, Codo del Pozuzo	Humalíes, Pachitea, Puerto Inca	Huánuco
Bothrops barnetti	macanche	20	Quebrada Honda, Olmos	Talara, Lambayeque	Piura, Lambayeque
Bothrops bilineatus	loro machaco	10	Pichanaki, Vitoc, Chanchamayo	Chanchamayo	Junin
Bothrops taeniata (castelnaudi)	jergón de árbol	nd	Satipo	Jauja	Junin
Bothrops chloromelas	–	8	Huancabamba	Huancabamba	Piura
Bothrops oligolepis (peruvianus)	jergón negro	8	Satipo	Jauja	Junin
Bothrops pictus	jergón de la costa	5	Pachacamac	Lima	Lima
Bothrocophias andianus	jergona	4	Macchu Picchu	Urubamba	Cusco
Bothrocophias hyoprora	jergón shushupe	10	Puerto Pakuy, Imaza	Bagua	Amazonas
Bothrocophias microphtalmus	jergón pudridora	nd	Pichanaki	Chanchamayo	Junin
Bothriechis schlegelii	loro	4	Reserva de Tumbes	Zarumilla	Tumbes
Lachesis muta	shushupe	10	Alto Marañón, Nazareth, Pijuayal	Bagua, Condorcanqui	Amazonas

[a] Taxonomical nomenclature used in this work follows the proposal of Carrasco et al. (2016), which synonymizes *Rhinocerophis, Bothropoides,* and *Bothriopsis* with *Bothrops,* while maintaining *Bothrocophias* and assigning *Bothrops andianus* to that genus.
[b] n: number of individuals in the venom pool; nd: not determined.

on-line with a Q-Exactive Plus® mass spectrometer (Thermo). Twelve μL of peptide mixture, containing 0.7 μg, were loaded on a C_{18} trap column (75 μm × 2 cm, 3 μm particle; PepMap, Thermo), washed with 0.1% formic acid (solution A), and separated at 200 nL/min on a C_{18} Easy-spray® column (75 μm × 15 cm, 3 μm particle; PepMap, Thermo). A gradient toward solution B (80% acetonitrile, 0.1% formic acid) was developed in a total of 120 min (1–5% B in 1 min, 5–26% B in 84 min, 26–80% B in 30min, 80–99% B in 1 min, and 99% B in 4 min). MS spectra were acquired in positive mode at 2.0 kV, with a capillary temperature of 200 °C, using 1 μscan in the range 400–1600 m/z, maximum injection time of 50 msec, AGC target of 1×10^{6}, and resolution of 70,000. The top 10 ions with 2–5 positive charges were fragmented with AGC target of 3×10^{6}, minimum AGC 2×10^{3}, maximum injection time 110 msec, dynamic exclusion time 5 s, and resolution 17,500. MS/MS spectra were processed against protein sequences contained in the UniProt/SwissProt database (Serpentes, October 2019) using Peaks X® (Bioinformatics Solutions) and matches were assigned to known protein families by similarity. Cysteine carbamidomethylation was set as a fixed modification, while deamidation of asparagine or glutamine and methionine oxidation were set as variable modifications, allowing up to 3 missed cleavages by trypsin. Parameters for match acceptance were set to FDR<0.1%, detection of at least one unique peptide, and −10lgP protein score ≥100.

2.7. Myotoxicity assay

The myotoxic effect of the venoms of *Bothrops barnetti* and *Bothrocophias andianus* (selected as examples of venoms nearly devoid of PLA_2s) was tested in mice. The venom of *B. asper* from the Pacific versant of Costa Rica, known to be abundant in PLA_2s (Alape-Girón et al., 2008), was included in this assay as a positive control. Groups of five CD-1 mice (18–20 g body weight) received an intramuscular venom injection (50 μg/50 μL) in the gastrocnemius. After 3 h, blood was obtained from the tip of the tail into heparinized capillary tubes, centrifuged, and plasma (4 μL) was assayed for creatine kinase (CK) activity using a kinetic commercial kit (CK-Nac, Biocon Diagnostik). A control group of mice received a similar injection of phosphate-buffered saline (PBS; pH 7.2) instead of venom. This experiment followed the ethical guidelines of the Institutional Committee for the Use and Care of Animals (CICUA) of the University of Costa Rica (No. 021–17). Statistical significance of the differences between groups was determined by ANOVA, followed by Tukey-Kramer post-hoc test, using the InStat v.3 (GraphPad) software.

3. Results

3.1. Electrophoresis

Venom SDS-PAGE patterns, under non-reducing and reducing conditions, are shown in Fig. 1. Although none of the inter-species patterns are entirely identical, significant resemblances are evident between several venoms, for example, *Bothrops atrox*, *B. barnetti*, *B. pictus*, and *B. neuwiedii*, especially after disulfide bond reduction. More differences appear in the unreduced electrophoretic patterns than in reduced ones, suggesting inter-species variations in the oligomeric association of
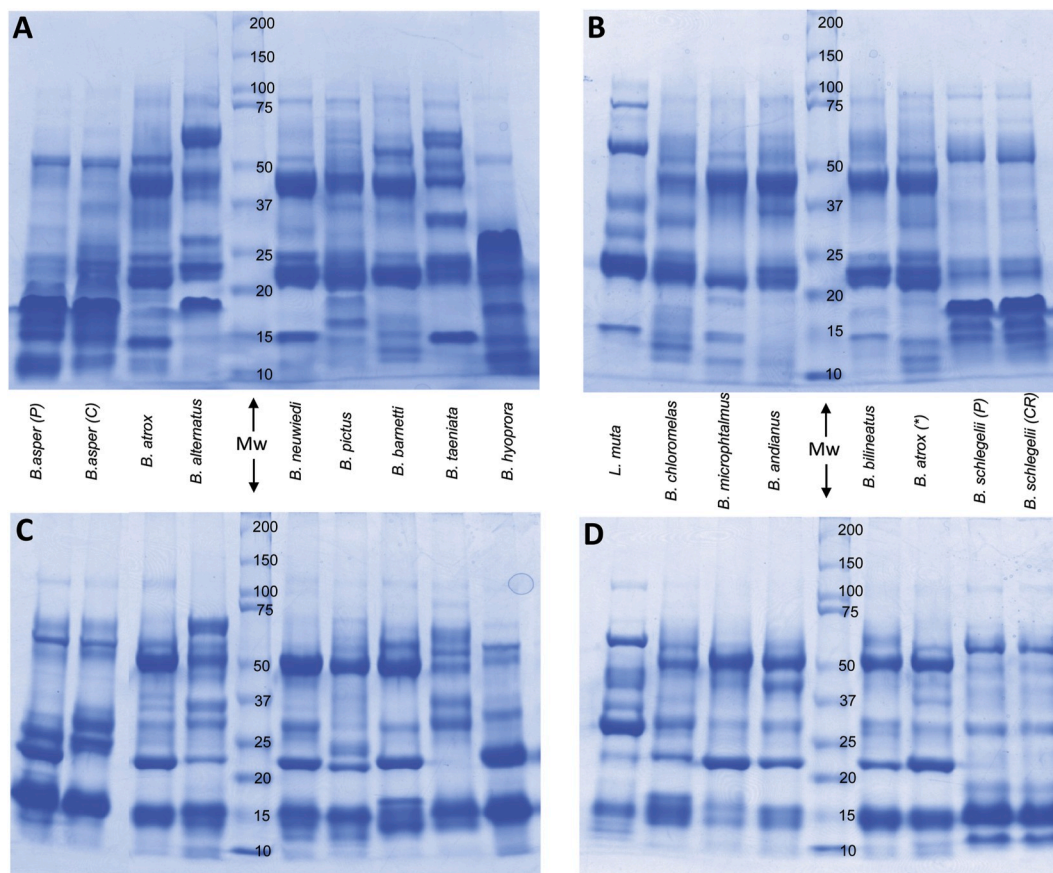

Fig. 1. SDS-PAGE profiles of venoms from twelve viperid species of Perú, unreduced (A, B) or reduced (C, D). Venoms of some species from other locations are included: *Bothrops asper* from the Pacific (P) or Caribbean (C) regions of Costa Rica, *B. alternatus* from Argentina, *B. neuwiedi* from Uruguay, and *B. schlegelii* from Costa Rica (CR). An additional sample of *B. atrox* venom from Perú is indicated with an asterisk. Molecular weight (Mw) markers are indicated in kDa. Proteins were stained with Coomassie brilliant blue G-250. The venom of *B. oligolepis* could not be analyzed in this technique due to insufficient amount. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

subunits of similar molecular mass. Notably, the electrophoretic pattern of *Bothriechis schlegelii* venom from Perú appears indistinguishable from that of the same species originating from Costa Rica, under both conditions (Fig. 1). All major venom proteins distributed below ~100 kDa. The most prominent reduced protein bands migrated at ~50, ~23, and ~15 kDa.

3.2. RP-HPLC profiles

Venoms were analyzed using a previously standardized C_{18} RP-HPLC separation protocol (Lomonte and Calvete, 2017). Its application in several proteomic studies on snake venoms has shown that monomeric PLA$_2$s and PLA$_2$-like homologs elute within the range indicated with a shadowed dotted box in Fig. 2. Venoms varied largely regarding the presence of chromatographic peaks within this region, and can be separated into two types, namely those presenting conspicuous peaks (blue frame in Fig. 2) and those having barely detectable peaks. The venoms of *Bothriechis schlegelii* and *Bothrocophias hyoprora* showed the most intense peaks in this region, comparable to those of *Bothrops asper* venom from Costa Rica, used as a reference for the elution of PLA$_2$s (Fig. 2).

Since electrophoretic analysis showed a very high similarity of *Bothriechis schlegelii* venoms collected in Perú and Costa Rica (Fig. 1), their RP-HPLC profiles were determined and compared in Fig. 3. As shown, the two profiles are virtually superimposable, confirming their remarkable high similarity.

3.3. Phospholipase A$_2$ and proteolytic activities

PLA$_2$ activity of the venoms on the NOBA substrate was highly variable, as presented in Fig. 4. This activity was strikingly low in the case of *Bothrops oligolepis*, *B. barnetti*, *Bothrocophias andianus*, *Bothrocophias hyoprora*, and *Lachesis muta* venoms. In contrast, *Bothrops atrox*, *B. pictus*, *B. taeniata*, and *Bothriechis schlegelii* venoms were highly active in this assay, while low to intermediate activities were recorded for *Bothrops chloromelas*, *B. bilineatus*, and *Bothrocophias microphtalmus* venoms.

On the other hand, proteolytic activity on the azocasein substrate was more similar among the different venoms (Fig. 5), but *Bothriechis schlegelii*, *Bothrops taeniata*, and *Lachesis muta* venoms showed a slightly lower proteolytic activity than the rest.

3.4. Myotoxicity

The venoms of *Bothrops barnetti* and *Bothrocophias andianus* were selected as examples of venoms virtually devoid of PLA$_2$s in the RP-HPLC profiles, to test whether this phenotypic trait would correlate with toxic activities that are known to depend mainly on these proteins, such as *in vivo* myotoxicity. As shown in Fig. 6, intramuscular injection of these two venoms in mice did not induce a significant increment of plasma CK activity, in contrast to a venom (*Bothrops asper*, used as a reference) known to contain abundant PLA$_2$ (Asp49) and PLA$_2$-like (Lys49) myotoxins.

3.5. Shotgun proteomic profiling

Nine of the twelve venoms here studied were subjected to a bottom-up shotgun proteomic profiling. The complete dataset of matches with known proteins in the Uniprot "Serpentes" database is presented in Supplemental Table S1, and the different protein families identified in each venom are summarized in Table 2. Venom protein families detected by this qualitative approach ranged from 9 (*Bothrops barnetti*) to 13 (*B. atrox*, *B. oligolepis*, *B. chloromelas*). The most variable protein family detected corresponded to hyaluronidase (HYA), found in three out nine venoms, and vascular endothelial growth factor (VEGF), found in five out of nine (Table 2). Nerve growth factor (NGF) was present in seven venoms, while phospholipase B (PLB) phosphodiesterase (PDE) and

cysteine-rich secretory proteins (CRISP) were found in eight. Other major protein families commonly found in viperid venoms were detected in all nine species.

3.6. Relationship of the phospholipase A$_2$ low/high phenotypes to phylogeny

Differences in the expression of PLA$_2$s in viperids of the bothropoid lineage were mapped onto the partial phylogeny described by Carrasco et al. (2016) in Fig. 7, using results of the present study together with data from the literature (evaluated either by proteomic analyses or by reported isolation of PLA$_2$s). The obtained cladogram shows that the majority of bothropoid venoms studied to date typically present PLA$_2$s (indicated with pink dots). One notable exception is *Bothrops cotiara* from Brazil, reported lacking these enzymes (Tashima et al., 2008). However, *B. cotiara* has also been reported to have PLA$_2$s as the most abundant venom components in the case of specimens collected in Argentina (de Roodt et al., 2018), and was therefore indicated with both pink and blue symbols in Fig. 7.

In addition to *B. cotiara* from Brazil, another bothropoid species with barely detectable amounts of venom PLA$_2$s (0.7%) is *Bothrops ayerbei* (Supplemental Fig. S1; Mora-Obando et al., 2014b), a lineage of the *B. asper* species complex (Silva-de-França et al., 2019).

In contrast to the widespread presence of PLA$_2$s in characterized venoms of most species included in the genus *Bothrops* (*sensu lato*) (Fig. 7), the chromatographic profiles of six out of the twelve venoms of species from Perú here studied revealed a phenotype in which PLA$_2$s are notably scarce: *Bothrops barnetti*, at the base of the "alternatus" clade (Carrasco et al., 2016), *Bothrocophias andianus* and *B. microphtalmus*, clustered in the "microphtalmus" clade, and *Bothrops chloromelas*, *B. bilineatus*, and *B. oligolepis*, in the "taeniata" clade (Fig. 7). The venom of *Bothrocophias hyoprora* differed sharply in this regard from those of other *Bothrocophias* species (*B. andianus* and *B. microphtalmus*), showing a major protein peak in the PLA$_2$-eluting region, and rather resembling the venom of *Bothrocophias campbelli* (Supplemental Fig. S1; Salazar-Valenzuela et al., 2014), a species not represented in the phylogenetic trees displayed in Fig. 7.

4. Discussion

Compositional, biochemical, and toxicological characteristics of the venoms of several species of the *Bothrops* complex (*sensu lato*) remain unexplored. In this study, the venoms of twelve viperid species that inhabit Perú were analyzed using electrophoretic, chromatographic, and enzymatic profiling for PLA$_2$ and proteolytic activities *in vitro*. Also, their proteomic qualitative profiles were determined using a shotgun approach. Results provide additional information to previous studies on biochemical and toxicological aspects of snake venoms from this South American country (Sanz et al., 2008; Núñez et al., 2009; Jimenez et al., 2010; Kohlhoff et al., 2012; Guerra-Duarte et al., 2015).

In general, venom SDS-PAGE profiles did not deviate from the typical patterns observed in viperids by this technique but revealed some resemblances and moderate differences among the species. Remarkably, the venom of *B. schlegelii* from Perú showed identical electrophoretic and chromatographic profiles to that of the same species found in Costa Rica, separated by a distance of more than 2000 km. This was unexpected, considering that a previous comparison of *B. schlegelii* venoms from Costa Rica and Colombia reported significant intra-specific differences in electrophoretic and toxicological parameters (Prezotto-Neto et al., 2016). Also, there are marked differences in external characters, mainly scutellation and color patterns, between the Central American populations and those at the southern extreme of the distribution of this species in Ecuador and Perú (Campbell and Lamar, 2014). Moreover, venoms of *Bothriechis* are known for their extremely high inter-species variability (Fernández et al., 2010; Lomonte et al., 2008, 2012; 2014; Calvete, 2017; Pla et al., 2017).
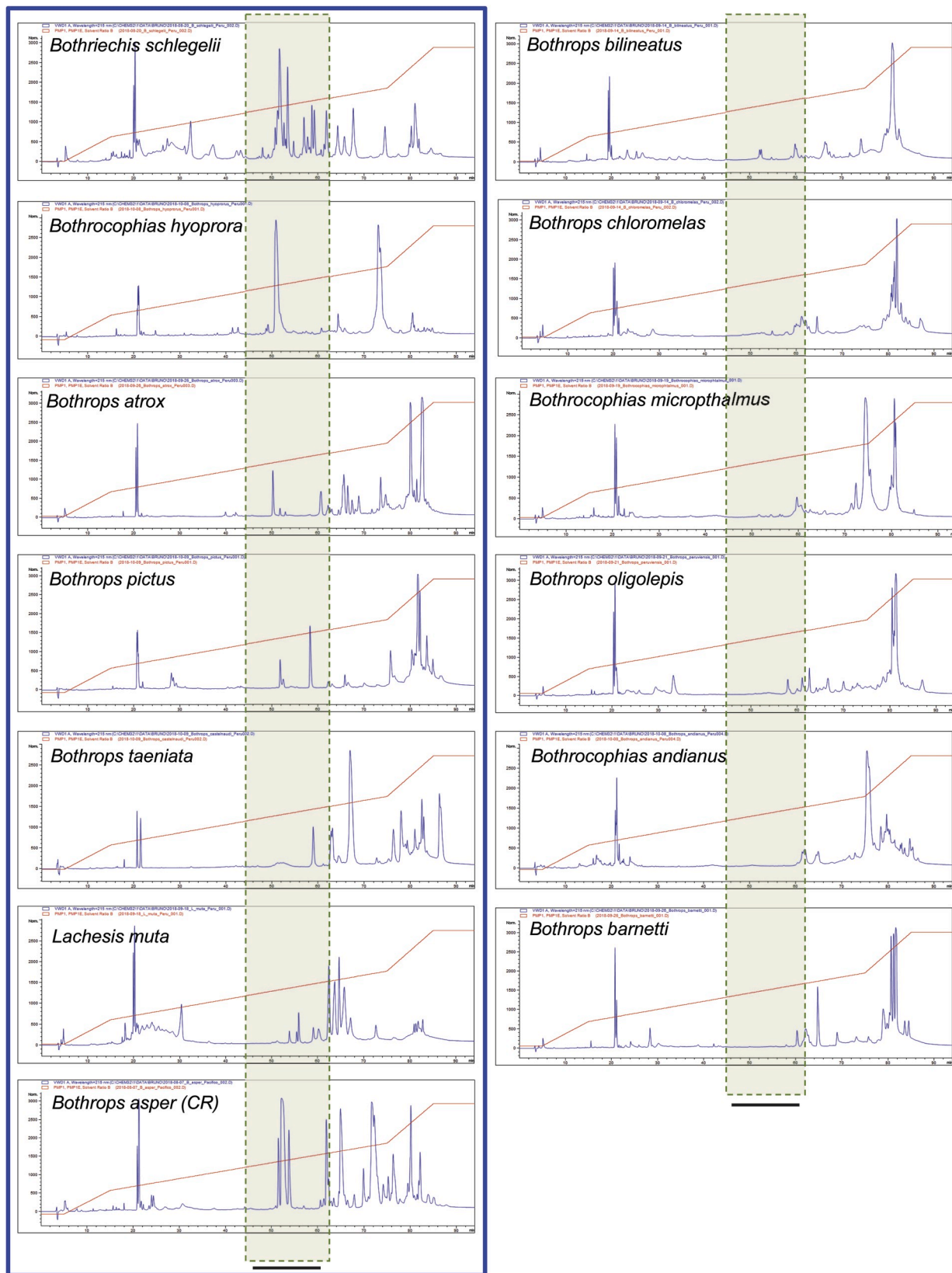
Fig. 2. C$_{18}$ reverse-phase HPLC profiles of venoms from twelve viperid species of Perú. The venom of *Bothrops asper* from Costa Rica was included as a reference. The dotted light green shaded area indicates the known elution region for PLA$_2$/PLA$_2$-like proteins in viperid venoms in this standardized chromatographic gradient. Venoms were divided into two groups, those with evident peaks in such region (enclosed within the blue frame) and those with very minor peaks. For reference to colors, the reader is referred to the web version of this article. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. Superposition of the C_{18} reverse-phase HPLC profiles of the venoms of *Bothriechis schlegelii* from Costa Rica (CR; green trace) and Perú (blue trace). For reference to colors, the reader is referred to the web version of this article. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
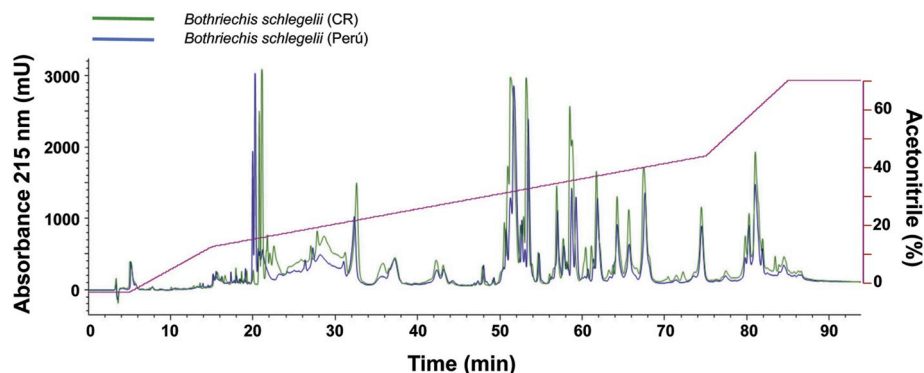
Comparison of venom RP-HPLC profiles revealed an unexpected feature since six out of the twelve species studied presented only minor protein peaks in the elution region known to correspond to PLA_2s as observed in several proteomic studies based on this chromatographic protocol (reviewed by Lomonte et al., 2014; Lomonte and Calvete, 2017). This particular characteristic had been observed in venoms from only a few of the bothropoid species studied until now, such as *B. ayerbei* from Colombia (Mora-Obando et al., 2014b), and *B. cotiara* from Brazil (Tashima et al., 2008). Most commonly, venoms of species within the *Bothrops* complex display prominent to medium-intensity peaks in the PLA_2/PLA_2-like eluting region. In contrast, venoms from six species of Perú here studied presented a low-PLA_2 phenotypic feature. These observations point to an apparent dichotomy among bothropoid venoms, regarding the occurrence of high to scarce levels of PLA_2 expression, and may bear practical relevance in selecting species whose venoms are used for antivenom production.

Venom compositional dichotomies are not infrequent in other taxonomic groups of venomous snakes. For example, rattlesnakes (*Crotalus* species) display either type I or type II venoms, defined as containing inverse proportions of metalloproteinases and PLA_2s, respectively (Mackessy, 2008, 2010). Another example relates to venoms of New World coral snakes (*Micrurus* species), which express either 'PLA2-rich' or '3FTx (three-finger toxin)-rich' phenotypes (Fernández et al., 2015; Lomonte et al., 2016; Sanz et al., 2019). In contrast to the streamlined 3FTx neurotoxic venoms of non-spitting, particularly African, cobras (*Naja haje, N. nivea, N. annulifera*) (Malih et al., 2014; Silva de Roodt et al., 2018; Tan et al., 2019a; unpublished results), variable expression of PLA_2s has also been observed in cytotoxic venom proteomes among species of African (*N. nubiae, N. pallida, N. nigricollis, N. mossambica*) (Petras et al., 2011) and Asian (*N. sumatrana, N. siamensis, N. philippinensis*) (Chong et al., 2019; Tan et al., 2019a, 2019b) spitting cobras. The emergence of venom spitting in cobras is tightly linked with convergent upregulation of PLA_2 toxins, which potentiate the nociceptive action of their venoms compared to those of their non-spitting counterparts (Kazandjian et al., 2019). Enhanced pain caused by spitting cobras is explicitly associated with the defensive use of venom, rather than for prey subjugation (Kazandjian et al., 2019).

The variable expression of PLA_2/PLA_2-like proteins in the Peruvian *Bothrops* venoms here studied showed only partial correlation with a functional assay for enzymatic hydrolysis of the NOBA substrate. This result is expected, since protein peaks in the known PLA_2-eluting RP-HPLC region may correspond to either Asp49 PLA_2 enzymes (catalytically active) or enzymatically inactive (but highly myotoxic) Lys49 PLA_2-like homologs (Lomonte and Rangel, 2012). Moreover, catalytic efficiency (specific activity) may vary among active PLA_2s. On the other hand, and in contrast to the highly variable expression of PLA_2s, proteolytic activity did not vary widely among the studied venoms.

Regardless of their relative abundances, both PLA_2s and

metalloproteinases were detected by shotgun proteomics in all the studied venoms, together with serine proteinases, C-type lectins/lectin-like, and L-amino acid oxidases, as typical of viperids. Other protein families that appeared common to all venoms were nucleotidases and glutaminyl cyclotransferases. In contrast, some differences were observed among them regarding the detection of cysteine-rich secretory proteins, phosphodiesterases, nerve growth factors, phospholipases B, vascular endothelial growth factors, and hyaluronidases.

An inference of present results revealing the low expression of PLA_2s in several bothropoid venoms from Perú is that they should display low toxicity for effects that depend mainly on this protein family. One of these known effects is myotoxicity (Gutiérrez and Lomonte, 2013). To test this prediction, the venoms of *Bothrops barnetti* and *Bothrocophias andianus* were selected as representatives of the 'low-PLA_2' phenotype and assayed *in vivo* for induction of muscle necrosis. Results demonstrated their negligible myotoxic effect. Whether this lack of myotoxicity confers any restriction on the trophic function of snake venoms that express it is still unknown, since information on diet and basic ecology is still very fragmented. Also, it is reasonable to expect that these differences in myotoxicity among bothropoid venoms, shown experimentally in mice, should translate to clinical envenomings. Envenomings by *Bothrops* (*sensu lato*) species are sometimes described in the Latin American medical literature as a single entity having shared general features. However, present results predict that important differences exist depending on the species, e.g., myonecrosis would not be expected in envenomings by species that express minimal amounts of venom PLA_2s.

An indirect myotoxic action has been reported for some hemorrhagic metalloproteinases (Queiroz et al., 1985; Gutiérrez et al., 1995), but their relative contribution to myonecrosis, in comparison to directly myotoxic PLA_2s, has not been clearly established. Present results obtained in mice with *B. barnetti* and *B. andianus* venoms suggest that acute muscle damage would be largely driven by PLA_2s, and are in agreement with studies that showed almost complete inhibition of myonecrosis when the venom of *B. asper* was neutralized by PLA_2-specific polyclonal (Lomonte et al., 1990) or monoclonal (Lomonte et al., 1992) antibodies.

In addition to its interest from a medical perspective, from a biological standpoint, the study of inter-species variations might contribute to understanding the evolutionary events that shaped the venom proteomes of extant species. In this work, we attempted to visualize the positions and relationships of bothropoid species from Perú presenting low- or high-PLA_2 venom phenotypes, based on the partial phylogeny generated by Carrasco et al. (2016). Although the interspersed pattern of the two phenotypes precludes reaching a simple phylogenetic deduction, it is clear that bothropoid venoms rich in PLA_2s are more common than those presenting scarce PLA_2s, and that the latter tend to cluster partially in the "microphtalmus" clade (*Bothrocophias* species), and the "taeniata" clade. The basal phylogenetic position of *Bothrops barnetti*
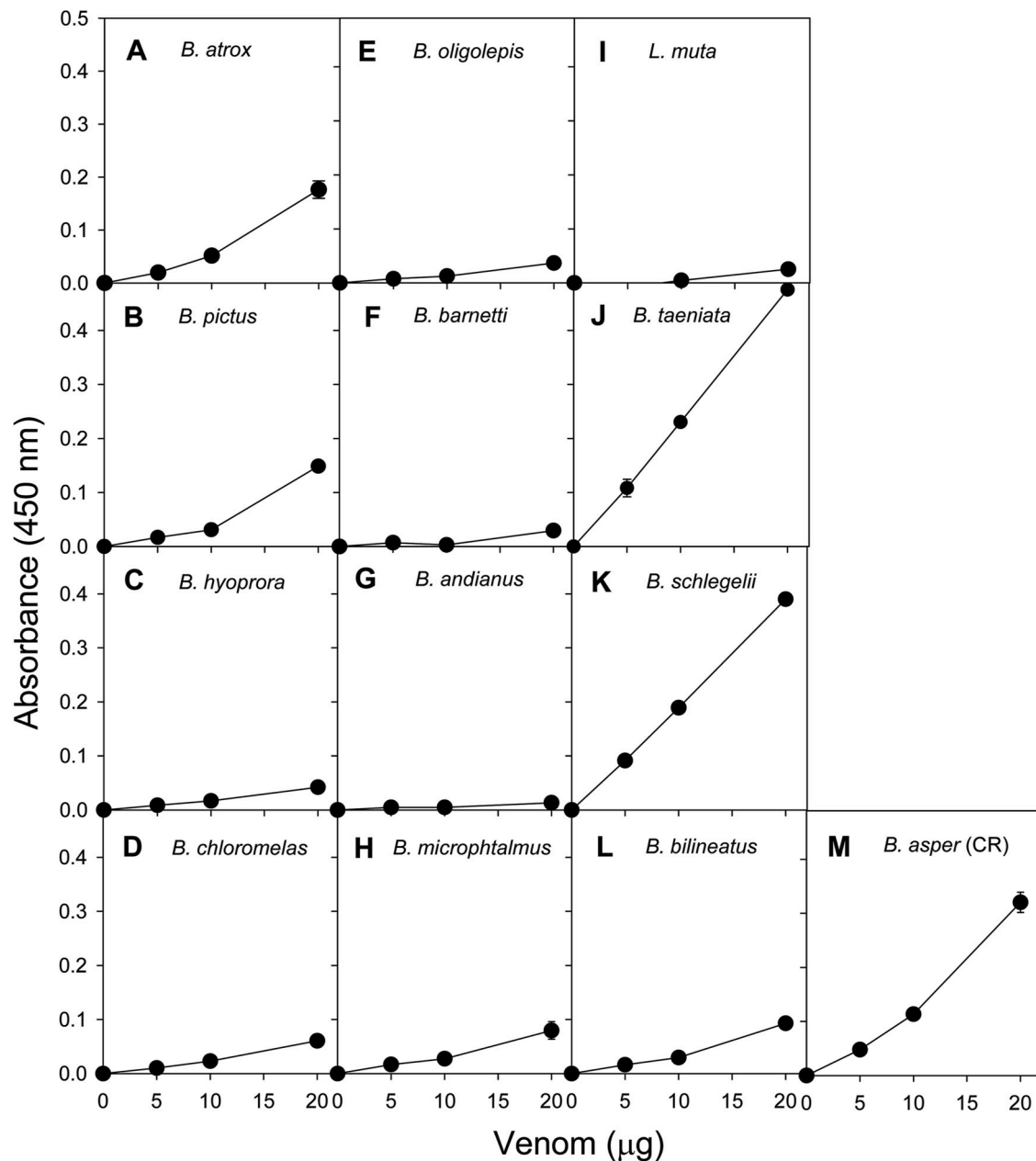
Fig. 4. Phospholipase A_2 activity of venoms from twelve viperid species of Perú on 4-nitro-3-octanoyloxy-benzoic acid. The venom of *Bothrops asper* from Costa Rica was included as a reference. Each point represents the mean $\pm$ SD of three replicates.

(having a "low-PLA$_2$" venom) for the "alternatus" clade is interesting and leads to note that all clades encompassing a combination of the two phenotypes present a "low-PLA$_2$" basal species in these phylogenetic trees. If this observation holds after examining a more significant number of species, it would be tempting to suggest that the increased expression of PLA$_2$s could be an evolutionarily more derived phenotypic feature. However, since the available phylogenetic information is still incomplete and limited in species coverage, further studies will be required to understand the complex relationships between venom phenotypes and phylogeny. Currently enigmatic, understanding the selective forces that originated the highly variable expression of PLA$_2$s in venoms of the *Bothrops* complex anticipates a challenging research task.

5. Concluding remarks

Venom is an intrinsically ecological trophic trait crucial for the foraging success of the organisms that produce it. The evolution of venom phenotypes does not appear to be phylogenetically constrained, but more likely shaped by ecological filtering that permits a small number of optimal solutions involving combinations of only a few major toxin families (Barua and Mikheyev, 2019). Of particular note is the occurrence of congeneric, even conspecific, divergent venom phenotypes, such as the 3FTx-rich/PLA$_2$-predominant venoms described in venoms of the New World genus *Micrurus* (Elapinae) and sea snake (Hydrophiinae) lineages (Sanz et al., 2016; Lomonte et al., 2016); the PIII-SVMP (type-I)/β-neurotoxic heterodimeric PLA$_2$ (type-II) venom compositional dichotomy found within *Crotalus* (Mackessy, 2008; Massey et al., 2012; Calvete, 2017; Strickland et al., 2018) and *Bothriechis* (Pla et al., 2017); and the divergent 3FTx/Kunitz-type dendrotoxins venom phenotypes among *Dendroaspis* (Ainsworth et al., 2018). The high/low PLA$_2$ venom compositional divergence described here among venoms of Peruvian *Bothrops* species represents a new dichotomy. The finding of similar patterns of venom variability among evolutionarily distant snake lineages suggests that dichotomic venom phenotypes may
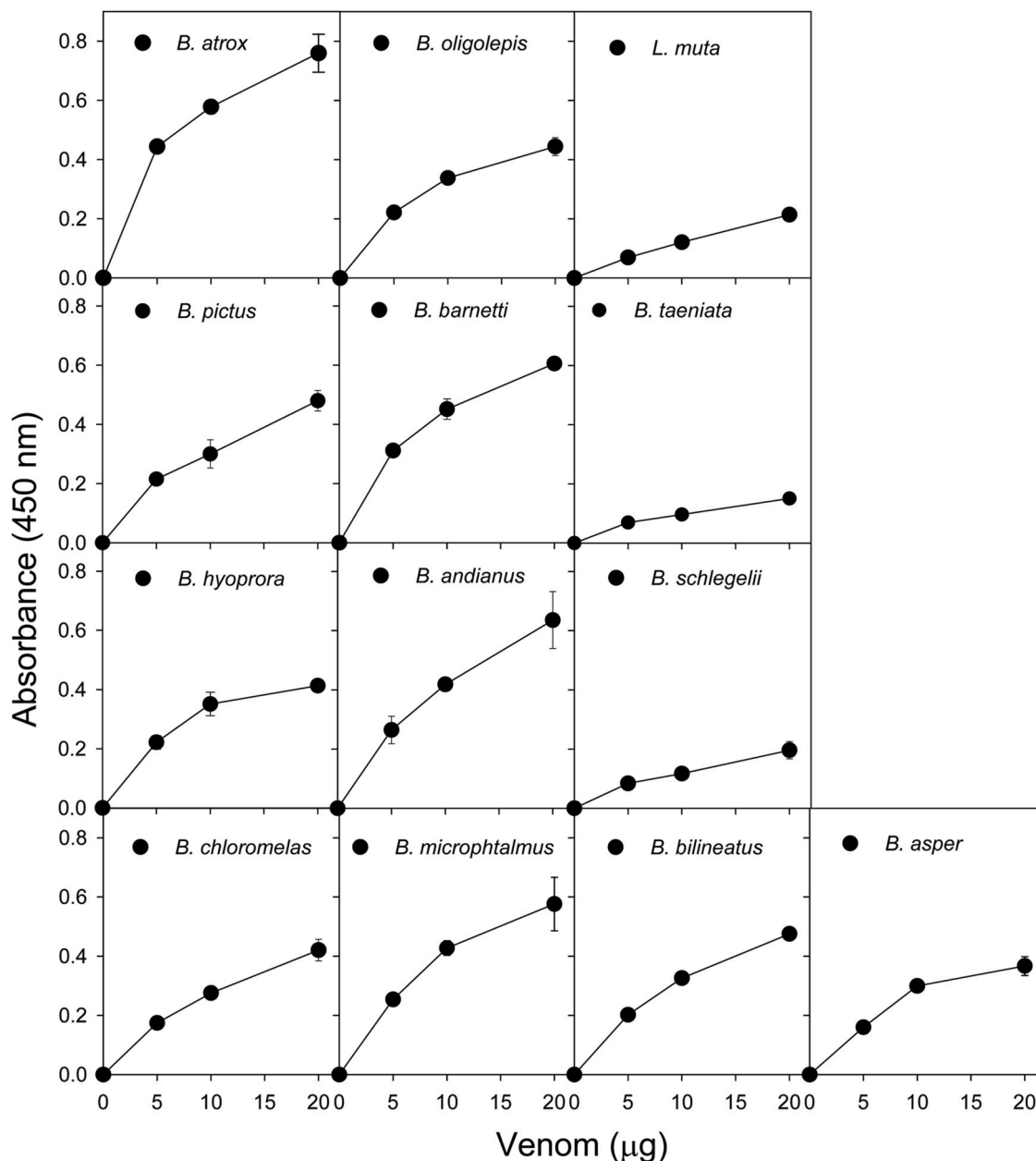
Fig. 5. Proteolytic activity of venoms from twelve viperid species of Perú on azocasein. The venom of *Bothrops asper* from Costa Rica was included as a reference. Each point represents the mean ± SD of three replicates.

constitute a more general trend than previously thought.

Venom dichotomy has different evolutionary origins in Nearctic, Central American, and South American rattlesnakes. Differential expression of type-I or type-II venom phenotypes in North American rattlesnakes appears to be due to recent lineage-independent losses of the genes coding for the subunits of the heterodimeric β-neurotoxin PLA$_2$ (Dowell et al., 2016, 2018). In contrast, in Central American *C. simus* it is ontogenetic and post-transcriptionally modulated by miR-NAs (Durban et al., 2013). It represents a pedomorphic trait driven by the increased gain of neurotoxicity and lethal venom activity to mammals (rodents) in South American *Crotalus durissus* (Calvete et al., 2010). The 3FTx/PLA$_2$ dichotomy among micrurine venoms may have been shaped by balancing selection (Sanz et al., 2019), an evolutionary scenario where local adaptation depends on the frequencies of the two toxin classes, with the hetero- (i.e. 3FTx + PLA$_2$) phenotype having an advantage over the homo-phenotypes (Llaurens et al., 2017; Wang and Mitchell-Olds, 2017). In contrast, the streamlined 3FTx venom of the

aquatic coral snake *M. surinamensis* may have evolved under strong pressure to quickly immobilize aquatic prey (Sanz et al., 2019; Silva et al., 2018). The different evolutionary paths responsible for generating variation in venom composition underscore the importance of considering venom diversification in the proper historical scenario.

Diversification of venoms within *Bothrops* should be understood in the context of the evolution of this clade. The early colonization by the ancestor of all *Bothrops* had its roots in South America during the middle Miocene, 14.07 Mya (CI95% 16.37–11.75 Mya), and was followed much later by *Bothriechis schlegelii* (7–6.6 Mya), *Porthidium* (3.5 Mya), and *Crotalus durissus* (1 Mya) (Wüster et al., 2002; Parkinson et al., 2002; Wüster et al., 2008; Alencar et al., 2016). *Bothrops* represents the most successful South American pitviper radiation, with extant lineages exhibiting extremely diverse morphological and ecological traits (Martins et al., 2002). Except for southwestern South America, the extreme highlands of the Andes, and southernmost Patagonia, extant bothropoid pitvipers are widely distributed in tropical Latin America, from
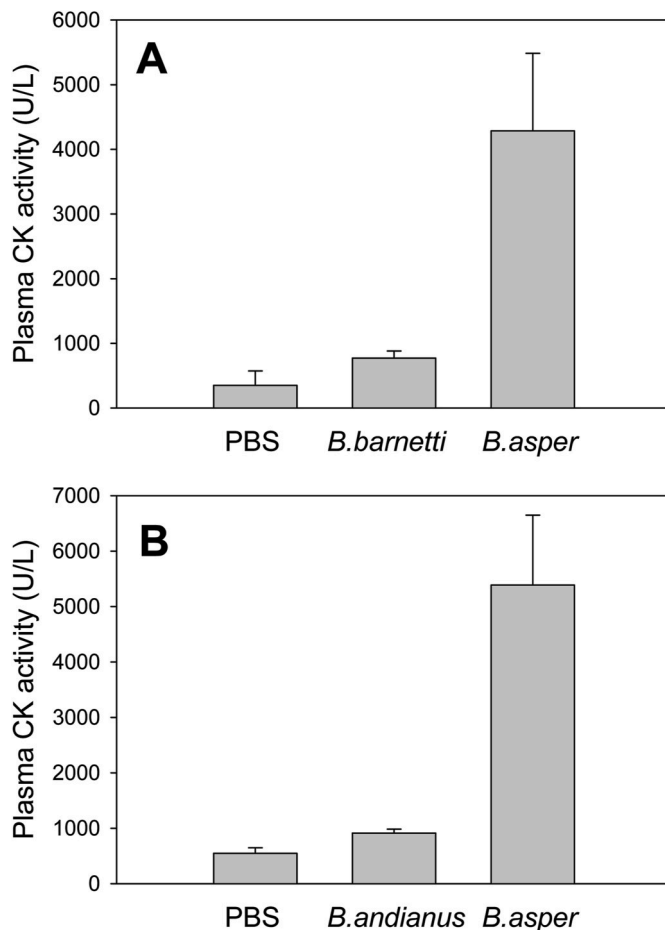
Fig. 6. Comparative evaluation of the myotoxic activity of the venoms of **(A)** *Bothrops barnetti* and **(B)** *Bothrocophias andianus* (Perú), compared to *Bothrops asper* (Costa Rica) in mice. Venoms were injected by the intramuscular route into groups of five mice. A control group received an injection of vehicle (PBS) alone. After 3 h the plasma creatine kinase (CK) activity was determined. Each bar represents the mean ± SD (n = 5). No statistical difference (p > 0.05) was observed between CK levels of PBS and *B. barnetti* venom, or PBS and *B. andianus* venom. *B. asper* venom was significantly different from both groups, in both independent experiments.

northeastern Mexico to Argentina, and the southern parts of the lower Caribbean islands, where they inhabit a broad spectrum of ecoregions, such as lowland evergreen forests, montane semideciduous forests, savannas, and open montane formations, from sea level to 3000 m of elevation (Campbell and Lamar, 1989; 2010*4*; Carrasco et al., 2012).

Diversification of *Bothrops* should be understood in the context of the unique historical contingencies of the evolution of this clade, the absence of other pitvipers and placental carnivores as potential competitors or predators when their lineage radiated throughout South America (Wüster et al., 2002) and the orogenic processes that created vicariant events (Hamdan et al., 2020; Salazar-Valenzuela et al., 2019). For example, in South America the uplift of the Central Andes mountain range (which began in the late Oligocene to early Miocene (23 Mya) with the formation of the highest peaks during the late Middle Miocene 11–12 Mya and continued until around 2–3 Mya, contemporaneous with deformation in the Santa Bárbara system to the east) (Folguera et al., 2011; Hamdan et al., 2020), the uplift of the Guanacaste, Central, and Talamanca mountain ranges in Mesoamerica since the late Miocene or early Pliocene (8-5 Mya) to the Pliocene, and the closure of the Isthmus of Panama 3.2 Mya (Gregory-Wodzicki, 2000; O'Dea et al., 2016; Bergoeing, 2017), are some of the main vicariance events involved in the cladogenesis of the lancehead vipers as a consequence of the fragmentation of populations and allopatric speciation (Kattan et al., 2004; Saldarriaga-Córdoba et al., 2017; Hamdan et al., 2020). Recent phylogeographic analyses have shown that demographic processes and ecological differentiation in montane habitats would also be reliable drivers implicated in the divergence of different taxa along altitudinal gradients (Salazar-Valenzuela et al., 2019).

An increasing number of studies support the idea that snake venom evolution is driven by diet-related selection pressures leading to local adaptations (Daltry et al., 1996; Barlow et al., 2009; Smiley-Walters et al., 2019; Davies and Arbuckle, 2019; Jackson et al., 2019). Adaptive venom variability represents a common phenomenon at all taxonomic levels among individuals of the same or different geographic locations, dietary habits, genders and across the life history stages of an individual (Calvete, 2013, 2017). Although the mechanisms of venom variation remain elusive, ecological release seemingly promotes increased phenotypic variance (Van Valen, 1965; Bolnick et al., 2007). From a functional perspective, venom from a generalist viperid may have the capability to subdue different prey items, although with varying degrees of effectiveness. However, other dimensions, such as non-consumptive habitat use, or dietary decisions, define the trophic niche of the organism and have significant implications on the individual energy budget and the outcomes of interactions between sympatric taxa (Jackson et al., 2004; Vincent and Brown, 2005; Beermann et al., 2018).

Besides providing insights into the selective pressures that resulted in local adaptation and species-level divergence in venoms, understanding the evolutionary processes and the ecological constraints that moulded snake venoms to their present-day variation may also provide compelling insights into venom clinical toxicology. Thus, toxins that have the highest prey incapacitation activity to a mammalian model are often also the most medically important molecules in the context of a human
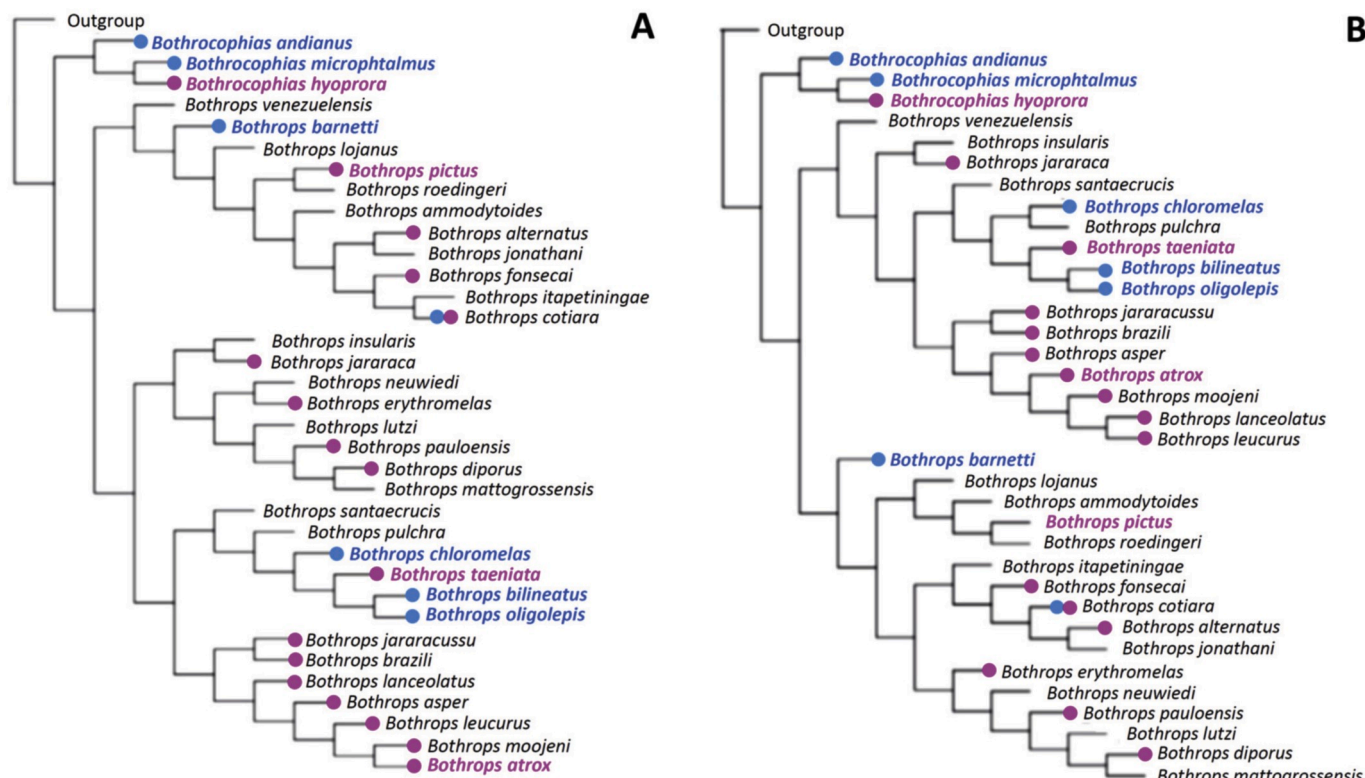
Table 2
Protein families identified in nine venoms from Viperidae species of Perú by bottom-up shotgun proteomics.

Snake species	MP*	SP	PLA$_2$	LAAO	CTL	NUC	GCY	CRiSP	PDE	PLB	NGF	VEGF	HYA	Total N°
Bothrops atrox	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	13
Bothrops oligolepis	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	13
Bothrops chloromelas	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	13
Bothrocophias andianus	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	(−)	12
Bothrops pictus	✓	✓	✓	✓	✓	✓	(−)	✓	✓	✓	✓	✓	(−)	11
Bothrocophias microphtalmus	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	(−)	(−)	11
Bothrocophias hyoprora	✓	✓	✓	✓	✓	✓	✓	✓	✓	(−)	✓	(−)	(−)	10
Bothrops bilineatus	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	(−)	(−)	(−)	10
Bothrops barnetti	✓	✓	✓	✓	✓	✓	✓	✓	(−)	✓	(−)	(−)	(−)	9
Family presence	100%	100%	100%	100%	100%	100%	100%	89%	89%	89%	77%	56%	33%	

* Abbreviations: **MP**: metalloproteinase; **SP**: serine proteinase; **PLA$_2$**: phospholipase A$_2$; **LAAO**: L-amino acid oxidase; **VEGF**: vascular endothelial growth factor; **NGF**: nerve growth factor; **CTL**: C-type lectin/lectin-like; **NUC**: nucleotidase; **PDE**: phosphodiesterase; **GCY**: Glutaminyl cyclotransferase; **PLB**: phospholipase B; **CRiSP**: cysteine-rich secretory protein; HYA: hyaluronidase. For a detailed summary of protein matches refer to Supplemental Table S1.
√: detected; (−) not detected.

Fig. 7. Partial phylogenies of bothropoid viperids showing the relationships of species reported to produce venoms with high (pink dots) and low (blue dots) phospholipase A$_2$ contents. The species from Perú analyzed in the present study are indicated by names in corresponding pink or blue colors. The phylogenetic trees were generated by Carrasco et al. (2016) using a total evidence approach from morphological, ecological, and mtDNA sequences (12 S and 16 S rRNA). Phylogenetic reconstruction included **(A)** or excluded **(B)** sequences from ND4 and cytb genes. For reference to colors, the reader is referred to the web version of this article. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

envenoming. Identifying the molecular basis of venomous snake adaptation to their natural ecosystems may assist in the identification of those toxins that must be neutralized to reverse the effects of venom, thereby guiding the rational development of next-generation snakebite therapeutics.

Credit author statement

Conceptualization: BL, AZ, MSalas, MSasa. Data curation and Formal analysis: BL, CD, FCH, JF, JJC. Funding acquisition: BL, AZ. Investigation and Methodology: CD, FCH, JF, MR. Resources: AZ, MSalas. Writing and editing: BL, CD, JJC, MS.

Declaration of competing interest

The authors declare that they have no competing interests related to this work.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxcx.2020.100044.

Ethical statement

The authors declare that they have no competing interests related to this work.

References

Ainsworth, S., Petras, D., Engmark, M., Süssmuth, R.D., Whiteley, G., Albulescu, L.O., Kazandjian, T.D., Wagstaff, S.C., Rowley, P., Wüster, W., Dorrestein, P.C., Arias, A.S., Gutiérrez, J.M., Harrison, R.A., Casewell, N.R., Calvete, J.J., 2018. The medical threat of mamba envenoming in sub-Saharan Africa revealed by genus-wide analysis of venom composition, toxicity and antivenomics profiling of available antivenoms. J. Proteomics 172, 173–189.

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

Alencar, L.R.V., Quental, T.B., Grazziotin, F.G., Alfaro, M.L., Martins, M., Venzon, M., Zaher, H., 2016. Diversification in vipers: phylogenetic relationships, time of divergence and shifts in speciation rates. Mol. Phylogenet. Evol. 105, 50–62.

Barlow, A., Pook, C.E., Harrison, R.A., Wüster, W., 2009. Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. Proc. Biol. Sci. 276, 2443–2449.

Barua, A., Mikheyev, A.S., 2019. Many options, few solutions: over 60 My snakes converged on a few optimal venom formulations. Mol. Biol. Evol. 36, 1964–1974.

Beermann, J., Boos, K., Gutow, L., Boersma, M., Peralta, A.C., 2018. Combined effects of predator cues and competition define habitat choice and food consumption of amphipod mesograzers. Oecologia 186, 645–654.

Bergoeing, J.P., 2017. Geomorphology and Volcanology of Costa Rica. Elsevier.

Bolnick, D.I., Svanback, R., Araujo, M.S., Persson, L., 2007. Comparative support for the niche variation hypothesis that more generalized populations also are more heterogeneous. Proc. Natl. Acad. Sci. U.S.A. 104, 10075–10079.

Calvete, J.J., Sanz, L., Cid, P., de la Torre, P., Flores-Díaz, M., Dos Santos, M.C., Borges, A., Bremo, A., Angulo, Y., Lomonte, B., Alape-Girón, A., Gutiérrez, J.M., 2010. *Crotalus simus* and the South American *Crotalus durissus* complex points to neurotoxicity as an adaptive paedomorphic trend along Crotalus dispersal in South America. J. Proteome Res. 9, 528–544.

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

Calvete, J.J., 2017. Venomics: integrative venom proteomics and beyond. Biochem. J. 474, 611–634.

Campbell, J.A., Lamar, W.W., 2014. 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. Zool. Scripta 41, 109–124.

Carrasco, P.A., Venegas, P.J., Chaparro, J.C., Scrocchi, G.J., 2016. Nomenclatural instability in the venomous snakes of the *Bothrops* complex: implications in toxinology and public health. Toxicon 119, 122–128.

Carrillo de Espinoza, N., Icochea, J., 1995. Lista taxonómica preliminar de los reptiles vivientes del Perú. Publ Museo Historia Nat UNMSM 49, 1–27.

Chippaux, J.P., 2017. Incidence and mortality due to snakebite in the Americas. PLoS Neglected Trop. Dis. 11, e0005662.

Chong, H.P., Tan, K.Y., Tan, N.H., Tan, C.H., 2019. Exploring the diversity and novelty of toxin genes in Naja sumatrana, the equatorial spitting cobra from Malaysia through de novo venom-gland transcriptomics. Toxins 11, E104.

Daltry, J.C., Wüster, W., Thorpe, R.S., 1996. Diet and snake venom evolution. Nature 379, 537–540.

Davies, E.L., Arbuckle, K., 2019. Coevolution of snake venom toxic activities and diet: evidence that ecological generalism favours toxicological diversity. Toxins 11, E711.

de Roodt, A., Fernández, J., Solano, D., Lomonte, B., 2018. A myotoxic Lys49 phospholipase A$_2$-homologue is the major component of the venom of *Bothrops cotiara* from Misiones, Argentina. Toxicon 148, 143–148.

Dowell, N.L., Giorgianni, M.W., Kassner, V.A., Selegue, J.E., Sanchez, E.E., Carroll, S.B., 2016. The deep origin and recent loss of venom toxin genes in rattlesnakes. Curr. Biol. 26, 2434–2445.

Dowell, N.L., Giorgianni, M.W., Griffin, S., Kassner, V.A., Selegue, J.E., Sanchez, E.E., Carroll, S.B., 2018. Extremely divergent haplotypes in two toxin gene complexes encode alternative venom types within rattlesnake species. Curr. Biol. 28, 1016–1026.

Durban, J., Pérez, A., Sanz, L., Gómez, A., Bonilla, F., Rodríguez, S., Chacón, D., Sasa, M., Angulo, Y., Gutiérrez, J.M., Calvete, J.J., 2013. Integrated 'omics' profiling indicates that miRNAs are modulators of the ontogenetic venom composition shift in the Central American rattlesnake, *Crotalus simus simus*. BMC Genom. 14, 234.

Estevao-Costa, M.I., Gontijo, S.S., Correia, B.L., Yarleque, A., Vivas-Ruiz, D., Rodrigues, E., Chávez-Olortegui, C., Oliveira, L.S., Sanchez, E.F., 2016. Neutralization of toxicological activities of medically-relevant *Bothrops* snake venoms and relevant toxins by two polyvalent bothropic antivenoms produced in Peru and Brazil. Toxicon 122, 67–77.

Fenwick, A.M., Gutberlet, 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, J., Lomonte, B., Sanz, L., Angulo, Y., Gutiérrez, J.M., Calvete, J.J., 2010. Snake venomics of *Bothriechis nigroviridis* reveals extreme variability among palm viper venoms: different evolutionary solutions for the same trophic purpose. J. Proteome Res. 9, 4234–4241.

Fernández, J., Vargas, N., Pla, D., Sasa, M., Rey-Suárez, P., Sanz, L., Gutiérrez, J.M., Calvete, J.J., Lomonte, B., 2015. Snake venomics of *Micrurus alleni* and *Micrurus mosquitensis* from the Caribbean region of Costa Rica reveals two divergent compositional patterns in New World elapids. Toxicon 107, 217–233.

García, P.J., Yarlequé, A., Bonilla-Ferreira, C., Pessah, S., Vivas, D., Sandoval, G.A., Lazo, F., 2008. Características bioquímicas y evaluación preclínica de un antiveneno botrópico liofilizado contra el veneno de la serpiente *Bohtrops atrox*. Rev. Peru. Med. Exp. Salud Pública 25, 386–390.

Göçmen, B., Heiss, P., Petras, D., Nalbantsoy, A., Süssmuth, R.D., 2015. Mass spectrometry guided venom profiling and bioactivity screening of the Anatolian Meadow Viper, *Vipera anatolica.* Toxicon 107, 163–174.

Gregory-Wodzicki, K.M., 2000. Uplift history of the central and northern Andes: a review. Geol. Soc. Am. Bull. 112, 1091–1105.

Guerra-Duarte, C., Lopes-Peixoto, J., Fonseca-de-Souza, B.R., Stransky, S., Oliveira, D., Schneider, F.S., Lopes-de-Souza, L., Bonilla, C., Silva, W., Tintaya, B., Yarleque, A., Chávez-Olórtegui, C., 2015. Partial *in vitro* analysis of toxic and antigenic activities of eleven Peruvian pitviper snake venoms. Toxicon 108, 84–96.

Gutiérrez, J.M., Lomonte, B., 2013. Phospholipases A$_2$: unveiling the secrets of a functionally versatile group of snake venom toxins. Toxicon 62, 27–39.

Gutiérrez, J.M., Romero, M., Núñez, J., Chaves, F., Borkow, G., Ovadia, M., 1995. Skeletal muscle necrosis and regeneration after injection of BaH1, a hemorrhagic metalloproteinase isolated from the venom of the snake Bothrops asper (Terciopelo). Exp. Mol. Pathol. 62, 28–41.

Hamdan, B., Guedes, T.B., Carrasco, P.A., Melville, J., 2020. A complex biogeographic history of diversification in Neotropical lancehead pitvipers (Serpentes, Viperidae), *Zool.* Scr 49, 145–158.

Jackson, A.C., Rundle, S.D., Attrill, M.J., Cotton, P.A., 2004. Ontogenetic changes in metabolism may determine diet shifts for a sit-and-wait predator. J. Anim. Ecol. 73, 536–545.

Jackson, T.N.W., Jouanne, H., Vidal, N., 2019. Snake venom in context: neglected clades and concepts. Front. Ecol. Evol. 7, 332. https://doi.org/10.3389/fevo.2019.00332.

Jimenez, K.L., Zavaleta, A.I., Izaguirre, V., Yarleque, A., Inga, R.R., 2010. Clonaje y caracterización molecular in silico de un transcrito de fosfolipasa A$_2$ aislado del veneno de la serpiente peruana *Lachesis muta*. Rev. Peru. Med. Exp. Salud Pública 27, 532–539.

Jiménez-Charris, E., Montealegre-Sánchez, L., Solano-Redondo, L., Mora-Obando, D., Camacho, E., Castro-Herrera, F., Fierro-Pérez, L., Lomonte, B., 2015. Proteomic and functional analyses of the venom of *Porthidium lansbergii lansbergii* (Lansberg's

hognose viper) from the Atlantic Department of Colombia. J Proteomics 115, 287–299.

Kattan, G.H., Franco, P., Rojas, V., Morales, G., 2004. Biological diversification in a complex region: a spatial analysis of faunistic diversity and biogeography of the Andes of Colombia. J. Biogeogr. 31, 1829–1839.

Kazandjian, T., Petras, D., Robinson, S., Undheim, E., Arbuckle, K., Whiteley, G., Albulescu, L.-O., Ainsworth, S., Wagstaff, S., Wüster, W., Harrison, R., Vetter, I., Calvete, J., Casewell, N., 2019. Convergent Evolution of Defensive Venom Components in Spitting Cobras. 20th World Congress of the International Society on Toxinology. Buenos Aires, Argentina, Wednesday September 11, Session 5B: Student Invited Presentations.

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.

Laing, G.D., Yarlequé, A., Marcelo, A., Rodriguez, E., Warrell, D.A., Theakston, R.D., 2004. Preclinical testing of three South American antivenoms against the venoms of five medically-important Peruvian snake venoms. Toxicon 44, 103–106.

Llaurens, V., Whibley, A., Joron, M., 2017. Genetic architecture and balancing selection: the life and death of differentiated variants. Mol. Ecol. 26, 2430–2448.

Lomonte, B., Rangel, J., 2012. Snake venom Lys49 myotoxins: from phospholipases A$_2$ to non-enzymatic membrane disruptors. Toxicon 60, 520–530.

Lomonte, B., Calvete, J.J., 2017. Strategies in 'snake venomics' aiming at an integrative view of compositional, functional, and immunological characteristics of venoms. J. Venom. Anim. Toxins Incl. Trop. Dis. 23, 26.

Lomonte, B., Gutiérrez, J.M., Carmona, E., Rovira, M.E., 1990. Equine antibodies to *Bothrops asper* myotoxin II: isolation from polyvalent antivenom and neutralizing ability. Toxicon 28, 379–384.

Lomonte, B., Gutiérrez, J.M., Ramírez, M., Díaz, C., 1992. Neutralization of myotoxic phospholipases A$_2$ from the venom of the snake *Bothrops asper* by monoclonal antibodies. Toxicon 30, 239–245.

Lomonte, B., Escolano, J., Fernández, J., Sanz, L., Angulo, Y., Gutiérrez, J.M., Calvete, J. J., 2008. Snake venomics and antivenomics of the arboreal neotropical pitvipers *Bothriechis lateralis* and *Bothriechis schlegelii*. J. Proteome Res. 7, 2445–2457.

Lomonte, B., Tsai, W.C., Bonilla, F., Solórzano, A., Solano, G., Angulo, Y., Gutiérrez, J.M., Calvete, J.J., 2012. Snake venomics and toxicological profiling of the arboreal pitviper *Bothriechis supraciliaris* from Costa Rica. Toxicon 59, 592–599.

Lomonte, B., Fernández, J., Sanz, L., Angulo, Y., Sasa, M., Gutiérrez, J.M., Calvete, J.J., 2014. 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.

Lomonte, B., Rey-Suárez, P., Fernández, J., Sasa, M., Pla, D., Vargas, N., Bénard-Valle, M., Sanz, L., Corrêa-Netto, C., Núñez, V., Alape-Girón, A., Alagón, A., Gutiérrez, J.M., Calvete, J.J., 2016. Venoms of *Micrurus* coral snakes: evolutionary trends in compositional patterns emerging from proteomic analyses. Toxicon 122, 7–25.

Mackessy, S.P., 2008. Venom composition in rattlesnakes: trends and biological significance. In: Hayes, W.K., Beaman, K.R., Cardwell, M.D., Bush, S.P. (Eds.), The Biology of Rattlesnakes. Loma Linda University Press, pp. 495–510.

Mackessy, S.P., 2010. Evolutionary trends in venom composition in the western rattlesnakes (*Crotalus viridis sensu lato*): toxicity vs. tenderizers. Toxicon 55, 1463–1474.

Malih, I., Rusmili, M.R., Tee, T.Y., Saile, R., Ghalim, N., Othman, I., 2014. Proteomic analysis of Moroccan cobra *Naja haje legionis* venom using tandem mass spectrometry. J. Proteomics 96, 240–252.

Martins, M., Marques, O.A.V., Sazima, I., 2002. Ecological and phylogenetic correlates of feeding habits in Neotropical pitvipers of the genus Bothrops. In: Schuett, G.W., Höggren, M., Douglas, M.E., Greene, H.W. (Eds.), Biology of the Vipers. Eagle Mountain Publishing, Eagle Mountain, Utah, pp. 307–328, 13: 978-0972015400.

Massey, D.J., Calvete, J.J., Sánchez, E.E., Sanz, L., Richards, K., Curtis, R., Boesen, K., 2012. Venom variability and envenoming severity outcomes of the *Crotalus scutulatus scutulatus* (Mojave rattlesnake) from Southern Arizona. J. Proteomics 75, 2576–2587.

Ministerio de Salud, 2017. Mapa de Ofidismo por distritos, Perú. Centro Nacional de Epidemiología, Prevención y Control de Enfermedades. MINSA, Perú. Consulted at: www.dge.gob.pe/portal/docs/vigilancia/sala/2017/SE27/ofidismo.pdf.

Mora-Obando, D., Díaz, C., Angulo, Y., Gutiérrez, J.M., Lomonte, B., 2014a. Role of enzymatic activity in muscle damage and cytotoxicity induced by *Bothrops asper* Asp49 phospholipase A$_2$ myotoxins: are there additional effector mechanisms involved? Peer J 2, e569.

Mora-Obando, D., Guerrero-Vargas, J.A., Prieto-Sánchez, R., Beltrán, J., Rucavado, A., Sasa, M., Gutiérrez, J.M., Ayerbe, S., Lomonte, B., 2014b. 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.

O'Dea, A., Lessios, H.A., Coates, A.G., Eytan, R.I., Restrepo-Moreno, S.A., Cione, A.L., Collins, L.S., De Queiroz, A., Farris, D.W., Norris, R.D., 2016. formation of the Isthmus of Panama. Sci. Adv. 2, e1600883.

Parkinson, C., Campbell, J., Chippindale, P., 2002. Multigene phylogenetic analyses of pitvipers; with comments on the biogeographical history of the group. In: Schuett, M.H.G.W., Greene, H.W., Douglas, M.E. (Eds.), Biol. Vipers. Eagle Mountain Publishing, Eagle Mountain, Utah, pp. 93–110.

Petras, D., Sanz, L., Segura, A., Herrera, M., Villalta, M., Solano, D., Vargas, M., León, G., Warrell, D.A., Theakston, R.D., Harrison, R.A., Durfa, N., Nasidi, A., Gutiérrez, J.M., Calvete, J.J., 2011. Snake venomics of African spitting cobras: toxin composition and assessment of congeneric cross-reactivity of the pan-African EchiTAb-Plus-ICP antivenom by antivenomics and neutralization approaches. J. Proteome Res. 10, 1266–1280.

Pla, D., Sanz, L., Sasa, M., Acevedo, M.E., Dwyer, Q., Pérez, A., Rodríguez, Y., Lomonte, B., Calvete, J.J., 2017. Proteomic analysis of venom variability and ontogeny across the arboreal palm-pitvipers (genus *Bothriechis*). J Proteomics 152, 1–12.

Prezotto-Neto, J.P., Kimura, L.F., Alves, A.F., Gutiérrez, J.M., Otero, R., Suárez, A.M., Santoro, M.L., Barbaro, K.C., 2016. Biochemical and biological characterization of Bothriechis schlegelii snake venoms from Colombia and Costa Rica. Exp. Biol. Med. 241, 2075–2085.

Queiroz, L.S., Santo-Neto, H., Assakura, M.T., Reichl, A.P., Mandelbaum, F.R., 1985. Pathological changes in muscle caused by hemorrhagic and proteolytic factors from Bothrops jararaca snake venom. Toxicon 23, 341–345.

Rodrigues, C.R., Teixeira-Ferreira, A., Vargas, F.F.R., Guerra-Duarte, C., Costal-Oliveira, F., Stransky, S., Lopes-de-Souza, L., Dutra, A.A.A., Yarlequé, A., Bonilla, C., Sanchez, E.F., Perales, J., Chávez-Olórtegui, C., 2018. Proteomic profile, biological activities and antigenic analysis of the venom from *Bothriopsis bilineata smaragdina* ("loro machaco"), a pitviper snake from Peru. J. Proteomics 187, 171–181.

Rojas, E., Quesada, L., Arce, V., Lomonte, B., Rojas, G., Gutiérrez, J.M., 2005. Neutralization of four Peruvian *Bothrops* sp. snake venoms by polyvalent antivenoms produced in Perú and Costa Rica: preclinical assessment. Acta Trop. 93, 85–95.

Salazar-Valenzuela, D., Mora-Obando, D., Fernández, M.L., Loaiza-Lange, A., Gibbs, L.H., Lomonte, B., 2014. Proteomic and toxicological profiling of the venom of *Bothrocophias campbelli*, a pitviper species from Ecuador and Colombia. Toxicon 90, 15–25.

Salazar-Valenzuela, D., Kuch, U., Torres-Carvajal, O., Valencia, J.H., Gibbs, H.L., 2019. Divergence of tropical pitvipers promoted by independent colonization events of dry montane Andean habitats. J. Biogeogr. 46, 1826–1840.

Saldarriaga-Córdoba, M., Parkinson, C.L., Daza, J.M., Wüster, W., Sasa, M., 2017. Phylogeography of the central American lancehead *Bothrops asper* (serpentes: Viperidae). PloS One 12, e0187969.

Sanz, L., Escolano, J., Ferretti, M., Biscoglio, M.J., Rivera, E., Crescenti, E.J., Angulo, Y., Lomonte, B., Gutiérrez, J.M., Calvete, J.J., 2008. Snake venomics of the South and Central American Bushmasters. Comparison of the toxin composition of *Lachesis muta* gathered from proteomic versus transcriptomic analysis. J. Proteomics 71, 46–60.

Sanz, L., Pla, D., Pérez, A., Rodríguez, Y., Zavaleta, A., Salas, M., Lomonte, B., Calvete, J. J., 2016. Venomic analysis of the poorly studied desert coral snake, *Micrurus tschudii tschudii*, supports the 3FTx/PLA$_2$ dichotomy across Micrurus venoms. Toxins 8, E178.

Sanz, L., Quesada-Bernat, S., Ramos, T., Casais-e-Silva, L.L., Corrêa-Netto, C., Silva-Haad, J.J., Sasa, M., Lomonte, B., Calvete, J.J., 2019. New insights into the phylogeographic distribution of the 3FTx/PLA$_2$ venom dichotomy across genus *Micrurus* in South America. J Proteomics 200, 90–101.

Serrano, S.M.T., Zelanis, A., Kitano, E.S., Tashima, A.K., 2018. Analysis of the snake venom peptidome. Methods Mol. Biol. 1719, 349–358.

Silva, F.M., Prudente, ALdaC., Machado, F.A., Santos, M.M., Zaher, H., Hingst-Zaher, E., 2018. Aquatic adaptations in a Neotropical coral snake: a study of morphological convergence. J. Zool. Syst. Evol. Res. 56, 382–394.

Silva-de-França, F., Villas-Boas, I.M., Serrano, S.M.T., Cogliati, B., Chudzinski, S.A.A., Lopes, P.H., Kitano, E.S., Okamoto, C.K., Tambourgi, D.V., 2019. *Naja annulifera* snake: new insights into the venom components and pathogenesis of envenomation. PLoS Neglected Trop. Dis. 13, e0007017.

Smiley-Walters, S.A., Farrell, T.M., Gibbs, H.L., 2019. High levels of functional divergence in toxicity towards prey among the venoms of individual pigmy rattlesnakes. Biol. Lett. 15, 20180876.

Strickland, J.L., Smith, C.F., Mason, A.J., Schield, D.R., Borja, M., Castañeda-Gaytán, G., Spencer, C.L., Smith, L.L., Trápaga, A., Bouzid, N.M., Campillo-García, G., Flores-Villela, O.A., Antonio-Rangel, D., Mackessy, S.P., Castoe, T.A., Rokyta, D.R., Parkinson, C.L., 2018. Evidence for divergent patterns of local selection driving venom variation in Mojave Rattlesnakes (*Crotalus scutulatus*). Sci. Rep. 8, 17622.

Tan, N.H., Fung, S.Y., Tan, K.Y., Yap, M.K., Gnanathasan, C.A., Tan, C.H., 2015. Functional venomics of the Sri Lankan Russell's viper (*Daboia russelii*) and its toxinological correlations. J Proteomics 128, 403–423.

Tan, C.H., Wong, K.Y., Tan, N.H., Ng, T.S., Tan, K.Y., 2019a. Distinctive distribution of secretory phospholipases A$_2$ in the venoms of afro-asian cobras (subgenus: *Naja, afronaja, boulengerina* and *uraeus*). Toxins 11, E116.

Tan, C.H., Wong, K.Y., Chong, H.P., Tan, N.H., Tan, K.Y., 2019b. Proteomic insights into short neurotoxin-driven, highly neurotoxic venom of Philippine cobra (*Naja philippinensis*) and toxicity correlation of cobra envenomation in Asia. J. Proteomics 206, 103418.

Tashima, A.K., Sanz, L., Camargo, A.C., Serrano, S.M., Calvete, J.J., 2008. Snake venomics of the Brazilian pitvipers *Bothrops cotiara* and *Bothrops fonsecai*. Identification of taxonomy markers. J Proteomics 71, 473–485.

Van Valen, L., 1965. Morphological variation and width of ecological niche. Am. Nat. 99, 377–390.

Viala, V.L., Hildebrand, D., Fucase, T.M., Sciani, J.M., Prezotto-Neto, J.P., Riedner, M., Sanches, L., Nishimura, P.J., Oguiura, N., Pimenta, D.C., Schlüter, H., Betzel, C., Arni, R.K., Spencer, P.J., 2015. Proteomic analysis of the rare Uracoan rattlesnake *Crotalus vegrandis* venom: evidence of a broad arsenal of toxins. Toxicon 107, 234–251.

Vincent, T.L., Brown, J.S., 2005. Evolutionary Game Theory, Natural Selection, and Darwinian Dynamics. Cambridge University Press.

Wang, B., Mitchell-Olds, T., 2017. Balancing selection and trans-specific polymorphisms. Genome Biol. 18, 231.

Wüster, W., Salomão, M.G., Quijada-Mascareñas, J.A., Thorpe, R.S., 2002. Origin and evolution of the South American pitviper fauna: evidence from mitochondrial DNA sequence data. In: Schuett, G.W., Höggren, M., Douglas, M.E., Greene, H.W. (Eds.), Biology of the Vipers. Eagle Mountain Publishing, USA, pp. 111–128.

Wüster, W., Peppin, L., Pook, C.E., Walker, D.E., 2008. A nesting of vipers: phylogeny and historical biogeography of the Viperidae (Squamata: serpentes). Mol. Phylogenet. Evol. 49, 445–459.