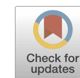
Biochimie 237 (2025) 1–15

Contents lists available at ScienceDirect

Biochimie

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

Venom versatility: Dynamic anticoagulant and procoagulant variations between and within *Bothrocophias* (toad-head) and basal *Bothrops* (lance-head) pit vipers



Lachlan A. Bourke [a,*], David Salazar-Valenzuela [b,c], Marco Mancuso [a,d], Diego R. Quirola [b,e], Amaru Loaiza-Lange [f], Christina N. Zdenek [g], Matthew R. Lewin [h,i], Ernesto Arbeláez-Ortiz [j], Bryan G. Fry [a,**]

[a] Adaptive Biotoxicology Lab, School of the Environment, University of Queensland, St Lucia, QLD, 4072, Australia
[b] Centro de Investigación de la Biodiversidad y Cambio Climático (BioCamb), Universidad Tecnológica Indoamérica, Machala y Sabanilla, Quito, EC170301, Ecuador
[c] Facultad de Ciencias de la Salud y Bienestar Humano, Universidad Tecnológica Indoamérica, Machala y Sabanilla, Quito, EC170301, Ecuador
[d] Department of Biological, Geological and Environmental Sciences, University of Catania, Italy
[e] Department of Biological Sciences, Clemson University, 190 Collings St., Clemson, SC, 29634, USA
[f] Museo de Zoología, Universidad Técnica Particular de Loja, Loja, Ecuador
[g] Australian Reptile Academy, QLD, Australia
[h] California Academy of Science, San Francisco, CA, 94118, USA
[i] Ophirex Inc., Corte Madera, CA, 94925, USA
[j] Amaru Bioparque Cuenca, Autopista Cuenca, Azogues, Ecuador

A R T I C L E I N F O

Article history:
Received 24 April 2025
Received in revised form
17 June 2025
Accepted 1 July 2025
Available online 2 July 2025

Handling Editor: Prof. Xavier Coumoul

A B S T R A C T

Pinpointing the emergence of toxicological evolutionary novelties can be challenging. In American pit vipers, anticoagulant venoms are the paradigm, with a notable exception being the genus *Bothrops*, which are typically procoagulant. A recent study found that the basal *Bothrops* (*B. pictus*) is anticoagulant, raising two competing hypotheses: ancestral *Bothrops* were anticoagulant with procoagulant venom evolving later, or ancestral *Bothrops* were procoagulant with anticoagulant venom in *B. pictus* being a derived trait. To help resolve this, we tested venoms of the sister genus *Bothrocophias* for pathophysiological actions upon blood clotting. The Ecuadorian *Bothrocophias* venoms (*B. campbelli*, *B. lojanus*, and *B. microphthalmus*) were compared to *Bothrops pictus*. Both *Bothrocophias lojanus* and *B. pictus* inhibited various blood clotting enzymes, but *B. pictus* was more potently anticoagulant. Intriguingly, *B. campbelli* and *B. microphthalmus* were procoagulant. Both *B. microphthalmus* populations activated prothrombin, but Zamora Chinchipe locality also activated Factors X and VII. *Bothrocophias campbelli* showed a novel activity, using Factor Va in a calcium-dependent manner as a cofactor to activate prothrombin, the first time this has been shown for any viperid venom. Organismal phylogenetics failed to resolve the relative positions of *B. campbelli* and *B. lojanus*, thus we were unable to ascertain the ancestral trait. To resolve this, more phylogenetic research and venom testing with other *Bothrocophias* species is needed. Neutralisation tests revealed differential efficacy of PoliVal-ICP (Instituto Clodomiro Picado) and Soro Antibotrópico (Instituto Butantan) antivenoms. Together, these findings aid in designing evidence-based clinical-management strategies and provide foundational data for reconstructing venom evolution.

© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

South America was once devoid of front-fanged venomous snakes, while today, pit vipers and coral snakes are highly successful and diverse groups across the continent. In the Miocene

* Corresponding author.
** Corresponding author.
E-mail addresses: lachlan.bourke@uq.edu.au (L.A. Bourke), bgfry@uq.edu.au (B.G. Fry).

https://doi.org/10.1016/j.biochi.2025.07.001
0300-9084/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

(sometime between 10 and 23 million years ago), the last common ancestor of *Bothrocophias* + *Bothrops* pit vipers colonised South America via a small land bridge connecting Central and South America [1]. Following this, *Bothrops* split from *Bothrocophias* and rapidly diversified into the currently recognised 48 species [2]. Wüster [1] postulates that the landscape being devoid of vipers at the time of colonisation allowed *Bothrops* to diversify into unoccupied ambush feeding niches. Today, *Bothrops* are found across South America, Central America, Mexico, and Caribbean islands [3]. It is unclear why *Bothrocophias* did not undergo the same adaptive radiation; there are currently only nine recognised species [2], and they are relatively restricted in range, occupying Andean South America and equatorial Brazil [4–7].

Concerning venom activity, due to their rarity *Bothrocophias* have been the subject of little work on their venom [8]. *Bothrops* venom, on the other hand, have been extensively studied [9,10]. This is reflective of *Bothrops* being considered the most clinically significant genus of venomous snakes in Latin America [10,11]. Notably, the venom of most *Bothrops* species is primarily procoagulant [12,13], with Factor X (FX) and prothrombin activation shown as mechanisms of action [14–18]. The procoagulant trait distinguishes *Bothrops* from other American pit vipers, which are typically dominated by, but not exclusively, anticoagulant or pseudo-procoagulant (aka: thrombin-like) activities [13,19–22]. Our recent study found a notable exception occurring in the venom of *B. pictus*, which we showed to be anticoagulant on human plasma [12]. Because most studies recover *B. pictus* as sister to all other *Bothrops* [23–27], we posited that the procoagulant venom in the Americas is unique to *Bothrops*, with the anticoagulant states as seen in *B. pictus* representing the ancestral trait, and the procoagulant trait seen in other *Bothrops* species being a derived state [12]. This aligns with Nielsen and colleagues' comprehensive study testing many American pit vipers for coagulotoxic activity, revealing most *Bothrops* venoms produced true procoagulant activity (described in the study as thrombin-generating activity) [13]. However, the anticoagulant phenotype of *B. pictus* is not conclusive of this being the ancestral *Bothrops* trait. Indeed, there is the competing hypothesis that the *Bothrops* last common ancestor may have been procoagulant and *B. pictus* evolved the anticoagulant trait as a derived state. A study inferring the evolutionary history of the early diversification of *Bothrops* and its sister genus *Bothrocophias* has been needed to help resolve this question. If all *Bothrocophias* have anticoagulant venom, then this would support the hypothesis that procoagulation has evolved in the *Bothrops* genus.

To our knowledge, only four studies have evaluated the action of *Bothrocophias* venoms upon human plasma. These studies showed that *Bothrocophias campbelli*, *B. colombianus*, and *B. myersi* have low coagulant activity [8,28–30]. However, it must be noted that in the methods, none of the studies included cofactors (calcium and phospholipid) in their assays. Calcium and phospholipid are readily available to venoms *in vivo*. They have been shown to be important cofactors for procoagulant venoms in general [31–33] and are particularly important for complete action by *Bothrops* venoms [14]. Thus, the indication by these studies that the venoms had low coagulant activity can only be regarded as provisional, due to physiological conditions not being replicated in the laboratory methods.

In this study, we aimed to test the coagulotoxic venom action from three species of *Bothrocophias*. We re-evaluated the coagulotoxic activity of *Bothrocophias campbelli* and tested, for the first time, the coagulotoxic activity of *B. lojanus* and *B. microphthalmus* on human plasma. We compared these venoms with the venom of *Bothrops pictus* – thought to be the most basal (earliest diverging) *Bothrops*. We hypothesised that *Bothrocophias* venoms would be anticoagulant and that procoagulation is, consequently, unique among the sister *Bothrops* genus. By investigating these rarely studied venoms, this data set will be important to reveal the evolutionary history of *Bothrops* venom and deliver insights that may be useful to basic scientists, antivenom and drug developers, as well as clinicians treating undifferentiated snakebites presenting unusual features.

2. Methodology

2.1. Venoms and antivenom

Ecuador samples used in the present study were collected under permit MAATE-DBI-CM-2022-0259 from the Ecuadorian Ministry of Environment. Venoms were received under University of Queensland, Animal Ethics Approval March 15, 2021/AE000075 and University of Queensland Biosafety Approval #IBC134BSBS2015. Voucher specimens are housed at MZUTI (Museo de Zoología, Universidad Tecnológica Indoamérica, Quito, Ecuador).

Bothrocophias venom (each species n = 1, single milkings) was collected in the field in Ecuador (Fig. 1)**:**

- *Bothrocophias campbelli*, MZUTI 5638, Mindo, Pichincha province, collected by Jaime Culebras.
- *Bothrocophias microphthalmus*, MZUTI 5635, Mera, Pastaza province, collected by Diego Quirola and Gustavo Hushpa.
- *Bothrocophias microphthalmus*, MZUTI 5670, Cordillera del Cóndor, Río Blanco, Emperador, Zamora Chinchipe province, collected by Nicolás del Castillo.
- *Bothrocophias lojanus*, MZUTI 5569, Las Nieves, Nabón, Azuay province, collected by David Salazar and Gabriel Cabrera.
- *Bothrocophias lojanus*, MZUTI 5559, Road Loja to Tres Leguas, Loja province, collected by Amaru Loaiza-Lange and Juan Cueva.

Supplied by Armando Yarleque, Universidad Nacional Mayor de San Marcos, Lima, Peru:

- *Bothrops pictus* venom sample pooled from n = 7 unsexed adults, locality: Districts of Carabayllo and Comas, Peru.

Venoms were extracted in the lab, immediately frozen in liquid nitrogen, then lyophilised. Venom was resuspended in double deionised water (ddH_2O, hereafter 'water'), centrifuged at 14,000 RCF (4 °C, 10 min), supernatant removed, its concentration measured using a Thermo Fisher Scientific™ NanoDrop 2000 (Waltham, MA, USA) at 280 nm wavelength, and the appropriate amount of venom, water and glycerol added to make a 1 mg/mL 1:1 1:1 water: glycerol solution. After preparation, all venoms were stored at −80 °C until required for experimentation in which they were stored at −20 °C.

Aliquots of antivenom used previously by us [34] study were used (stored at 5 °C). The antivenoms used were: Soro Antibotrópico (SAB) ® (Instituto Butantan, Brazil; Lot: 1305077; Exp: May 2016) and Central American polyspecific antivenom (PoliVal-ICP) ® (Instituto Clodomiro Picado, Universidad de Costa Rica; Lot: 5720416; Exp: April 2021). Before use in the current experiment, the aliquots of antivenoms were centrifuged at 12,00 RCF at 4 °C for 10 min and the supernatant was used. This was to ensure no bacterial contamination was present. Antivenoms were also checked for efficacy to confirm stability. The use of expired antivenoms is acknowledged, but not of concern; this is because previous studies show antivenom to be effective after its expiry date [32,35–37].

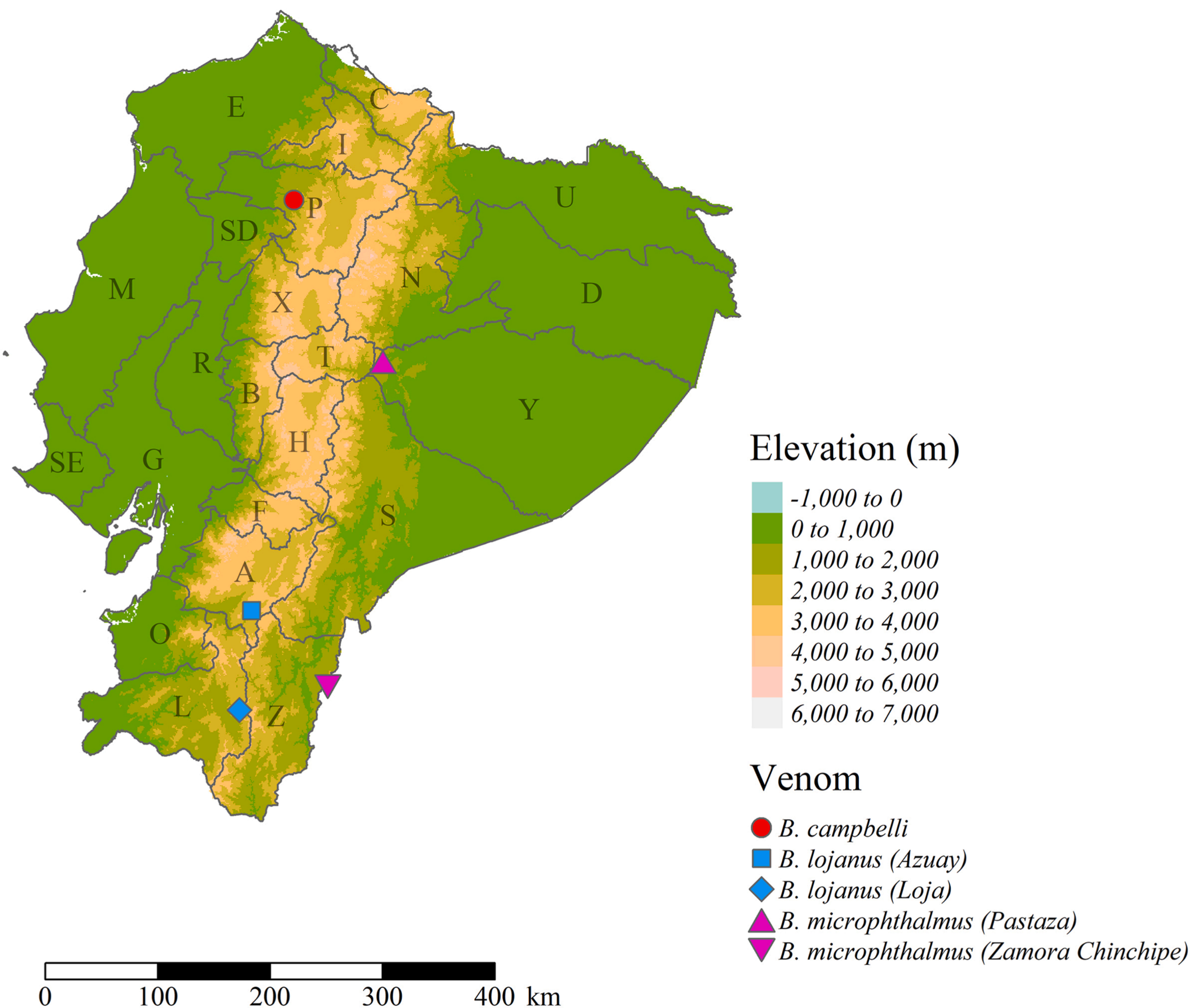


Fig. 1. Map of Ecuador showing elevation and the localities of each *Bothrocophias* venom sample. ISO 3166 codes are used to represent Ecuador's provinces. The R code is available on GitHub (https://github.com/LachlanBourke/Species_location_map).

2.2. Human plasma and fibrinogen

Human plasma work was performed under University of Queensland Biosafety Approval #IBC134BSBS2015 and Human Ethics Approval #2016000256. Australian Red Cross (44 Musk Street, Kelvin Grove, QLD 4059, Australia) supplied human platelet-poor plasma (3.2 % citrated, O Positive, Label # A550022018621) under research approval #16-04QLD-10. To prepare for experiments, plasma was defrosted from −80 °C in a 37 °C water bath, aliquoted from within a biosafety cabinet into 1.5 mL tubes, flash-frozen in liquid nitrogen, and stored at −80 °C. For tests on the Stago STA-R Max haemostasis analyser (Stago, Asnières sur Seine, France) plasma controls (spontaneous and kaolin, explained in following sections) were performed daily to monitor the health of the plasma. Human fibrinogen (Sigma Aldrich, St. Louis, MO, USA) was solubilised to 4 mg/ml by diluting with buffer (150 mM NaCl, 50 mM Tris-HCl, then dissolved in water, pH 7.4), flash frozen in liquid nitrogen, and stored at −80 °C.

2.3. Thromboelastography

Two TEG® 5000 Thrombelastograph® Haemostasis Analyser systems were used to measure the clotting dynamics of human plasma incubated with venom samples (section 2.1). We followed our previously validated protocols [12,14]: for each run, the analyser temperature was 37.0 °C and disposable cups and pins (Haemonetics®, REF 6211) were loaded. Next, 72 μL $CaCl_2$ (Stago Cat# 00367), 72 μL phospholipid (PL) (Stago Cat# 00597), 20 μL Owren-Koller (OK) buffer (Stago Cat# 00360), and 7 μL 1 mg/ml venom. Lastly, 189 μL human plasma or fibrinogen (thawed for 5 min in a 37 °C water bath) was pipetted into each cup, the entire 360 μL sample pipette-mixed, and the analyser ran for 30 min. For each cup, the time from pipetting the plasma or fibrinogen into the cup until the start of each test (lifting the cup into the test machine and pressing start) was 10 s. All tests were performed in triplicate. A spontaneous clotting time control was performed for human plasma tests, whereby 7 μL 1:1 water: glycerol sample replaced the

venom sample. Two positive controls were also performed, whereby 7 μL thrombin (Stago Cat# 00673) or FXa (Stago Cat# 00311) replaced the venom sample. Only the thrombin control was performed in fibrinogen tests because fibrinogen does not clot spontaneously.

2.4. Clotting time and cofactor dependence

A Stago STA-R Max haemostasis analyser was used to access the coagulotoxicity of each venom. Firstly, all venoms were tested at 20 μg/mL concentration for their ability to clot human plasma. For testing, venom (1 mg/mL) was diluted to 0.1 mg/mL with OK buffer and loaded into the analyser along with the following reagents: human platelet-poor plasma (thawed for 5 min in a 37 °C water bath), $CaCl_2$, PL, and OK buffer. For the 20 μg/mL venom concentration, the analyser pipetted 50 μL $CaCl_2$, 50 μL PL, 25 μL OK buffer, and 50 μL of venom (0.1 mg/mL) into a cuvette. Following 2 min incubation, 75 μL human platelet-poor plasma was added into the cuvette, the cuvette gently shaken by the machine, and clotting automatically determined. Additionally, the dependency of each venom for cofactors (calcium or phospholipid) was accessed, as per the previously validated assay [32]. The same venom protocol as above was used, except for the calcium dependence test 50 μL $CaCl_2$ was replaced with OK buffer, and for the PL dependence test 50 μL PL was replaced with OK buffer. Lastly, using our previous methodology [38], the venom's ability to destroy phospholipids was accessed. The same clotting time protocol as above was used, except 25 μL OK buffer was replaced with 5x concentrated PL, leading to a final PL concentration in the cuvette 3.5x the regular assay. If venom activity decreases compared to the baseline, it suggests the venom potentially destroys phospholipids; the venom is unable to destroy all phospholipids as they are over abundant in this test, thus venom activity is decreased. All venom tests were run in triplicate. Two controls were performed: a spontaneous clotting time test and a modified activated partial thromboplastin time test (kaolin test). For the spontaneous clotting time, the same methodology as above (with both cofactors) was performed, except 50 μL venom was replaced with 1:10 diluted (diluent = OK buffer) 1:1 water: glycerol sample. The kaolin test (positive control) was performed as follows: the positive control as follows: 50 μL plasma, 50 μL kaolin (Stago Cat# 00597), 50 μL PL (solubilised in OK buffer), and 50 μL OK were incubated for 120 s in a cuvette, then 50 μL 0.025 M $CaCl_2$ was added to initiate clotting.

2.5. Antivenom efficacy

A Stago STA-R Max haemostasis analyser was used to measure the ability of antivenoms to neutralise procoagulant *Bothrocophias* venom. Firstly, venoms were tested for their coagulotoxic activity on human plasma across eight different venom concentrations (μg/mL: 20, 10, 4, 1.66, 0.66, 0.25, 0.125, 0.05). The 20 μg/mL venom activity was re-tested using section 2.3 methodology: the analyser pipetted 50 μL $CaCl_2$, 50 μL PL, 25 μL OK buffer, and 50 μL of venom (0.1 mg/mL) into a cuvette, incubated regents for 2 min, added 75 μL plasma, and measured clotting time. Above controls were also performed. To test additional concentrations, the analyser adjusted the volume of venom added to the cuvette, keeping the final volume consistent (250 μL) by adding additional OK buffer. For the antivenom test, the 25 μl OK buffer was replaced with 2.5 % (v/v) antivenom (diluent = OK buffer) (final cuvette concentration of 0.25 %). The effect of antivenom on clotting time was tested by replacing venom with a 1:10 diluted (diluent = OK buffer) 1:1 water: glycerol sample. All tests were run in triplicate. To evaluate antivenom efficacy, percentage shift values were calculated as follows: the clotting time of venom incubated with antivenom was divided by the venom only clotting time, and the total subtracted by 1, so that a value above 0 would indicate neutralisation. To convert to percentage, this value was then multiplied by 100.

2.6. Coagulation factor inhibition assay

A Stago STA-R Max haemostasis analyser was used to access the ability of venom to inhibit coagulation factors. Venom induced inhibition of Factor VIIa (FVIIa), Factor XIa (FXIa), Factor Xa (FXa), Factor IXa (Factor IXa), thrombin, and the prothrombinase complex were tested. The methodology is based off the previously validated assay used in a Zdenek 2020 study [39]. To test for inhibition of factors 25 μL of 0.2 mg/mL venom, 50 μL 0.025 M $CaCl_{2,}$ 50 μL (PL, solubilised in OK buffer), 25 μL Owren-Koller (OK) Buffer, and 25 μL of the active coagulation factor were added to a cuvette and incubated for 120 s at 37 °C. After incubation, 75 μL of human plasma was added to the cuvette, the cuvette gently shaken by the machine, and clotting time recorded. Factors used were FVIIa (Prolytix catalogue number (Cat#) HCVIIA-0031), FXIa (Prolytix Cat# HCXIA-0160), FIXa (Prolytix Cat# HCIXA-0050), FXa (Stago Cat# 00311), and thrombin (Stago Cat# 00611). Prolytix factor working stocks were prepared by diluting with 1:1 water: glycerol, and prior to use in assays were diluted with OK buffer to the following concentrations: FVIIa (1.5 μg/mL), FXIa (15 μg/mL), and FIXa (15 μg/mL). For the prothrombinase complex inhibition assay 25 μL 0.2 mg/mL venom, 50 μL 0.025 M $CaCl_{2,}$ 50 μL PL (solubilised in OK buffer), 25 μL OK buffer, and 75 μL human plasma were incubated for 120 s at 37 °C. After incubation 25 μL FXa was added to the cuvette and clotting time recorded. All venoms were tested in triplicate and alongside negative controls. Negative controls were performed by replacing the venom with a blank (diluted (diluent = OK buffer) 1:1 water: glycerol).

Inhibition occurs when venom clotting time values are higher than the negative control. The prothrombinase complex inhibition assay is based off the fact that this complex consists of FXa and its cofactor FVa. Therefore, if a venom inhibits the formation of this complex during the 120 s incubation, the clotting time recorded after the addition of FXa would be higher than the control. To evaluate inhibition, a percentage shift value was calculated as described in section 2.4 for antivenom.

2.7. Small molecule inhibitor tests

A Stago STA-R Max haemostasis analyser was used to reveal if anticoagulant venom activity is PLA_2 driven. As a baseline, the 20 μg/mL venom activity was re-tested using section 2.3 methodology: the analyser pipetted 50 μL $CaCl_2$, 50 μL PL, 25 μL OK buffer, and 50 μL of venom (diluted to 0.1 mg/mL with OK buffer) into a cuvette, incubated regents for 2 min, added 75 μL plasma, and measured clotting time. To test if anticoagulant venom activity is PLA_2 driven 25 μL OK buffer was replaced with the phospholipid inhibitor varespladib (Company: Chemietek, preparation: dissolved in 10 % DMSO) dissolved in OK buffer to a concentration of 0.04 mM (final reaction concentration = 0.004 mM). All venom tests were run in triplicate. To reveal if procoagulant venom activity is SVMP driven, the same set of tests was performed for procoagulant venoms, except varespladib was replaced with the metalloproteinase inhibitor prinomastat (Cat # PZ0198, preparation: dissolved in 10 % DMSO) dissolved in OK buffer to a concentration of 2 mM (final reaction concentration = 0.2 mM). In these tests, the 50 μL venom samples was diluted to a concentration of 3.33 μg/mL to yield a final venom concentration of 0.67 μg/mL. This was done because of limited venom supplies.

2.8. Zymogen activation assay

A Fluoroskan Ascent™ was used to screen procoagulant venoms for their ability to activate coagulation factors (zymogens) prothrombin (Prolytix Cat# HCP-0010), Factor X (FX) (Prolytix Cat# HCX-0050), and Factor VII (FVII) (Prolytix Cat# HCVII-0030). Prothrombin activation was tested with and without the cofactor FVa (Prolytix Cat# HCVA-0110). Note: Prolytix factor working stocks were prepared by diluting with 1:1 water: glycerol. Fluorogenic peptide substrate (Boc-Val-Pro-Arg-AMC. Boc: t-Butyloxycarbonyl; 7-Amino-4- methylcoumarin; R & D systems, catalogue # ES011, Minneapolis, Minnesota) gives off fluorescence when cleaved by the active form of the above zymogens, thus was used to quantify factor activation. Briefly, a 384-well plate was prepared with a Hamilton Robot to produce quadruplicate wells of differing experimental conditions. Wells testing venom zymogen activation comprised of 10 μL zymogen (10 μg/mL), 10 μL PL, and 10 μL venom (1 μg/mL). For wells testing prothrombin activation in the presence of Va, 10 μL venom was replaced with a 1 μg/mL venom: 4 μg/mL FVa mixture. Positive control wells comprised 10 μL buffer without calcium (150 mM NaCl, 50 mM Tris-HCl, dissolved in water, pH 7.4), 10 μL PL, and 10 μL of active zymogen (10 μg/ml). Wells were also prepared mimicking all above conditions, except 10 μL buffer without calcium replaced the zymogen for venom tests, and active zymogen for positive control test. Lastly, negative control wells comprised of 20 μL of buffer without calcium and 10 μL PL. The 384-well plate was placed in the Fluoroskan Ascent (temperature 37 °C). The assay was started via the automatic pipetting of 70 μL of a mixture of buffer with calcium ((150 mM NaCl, 50 mM Tris-HCl, 50 mM $CaCl_2$, pH 7.4) and fluorogenic peptide substrate. Before each measurement, the plate was shaken for 3 s to homogenise the reagents and venom. The fluorescence levels were measured for 300 min. An excitation wavelength of 390 nm and emission wavelength of 460 nm were used.

Following the completion of the run the values from the blank were subtracted from all other well values. To account for venom directly cleaving the substrate, resulting in fluorescence, values obtained from well containing venoms without zymogen were subtracted from values obtained from well containing venoms with zymogen. Venom-activity values were then normalised so that the value was a percentage relative to the active factor positive control.

2.9. Phylogenetic reconstruction of organismal relationships

Mitochondrial gene sequences from Cytochrome B and ND4 belonging to 13 viperid species (*Atropoides picadoi*, *Bothrocophias andianus*, *B. campbelli*, *B. hyoprora*, *B. lojanus*, *B. microphthalmus*, *B. myrringae*, *Bothrops ammodytoides*, *B. asper*, *B. pictus*, *B. taeniatus*, and *Porthidium lansbergii*) were downloaded from NCBI Genbank. We are aware that the presence of additional genetic markers, especially nuclear DNA, would further strengthen our phylogenetic analysis, but, unfortunately, no viable sequences were available for our taxa of interest on any online database. On the other hand, our dataset features an extremely low percentage of missing data, standing at roughly 4 %, which is largely inflated by the only missing sequence (ND4 from *B. andianus*). Therefore, we have a fairly complete dataset that can correctly infer phylogenetic patterns at the species level. Sequences were aligned in mafft (v.7.490.) [40], and subsequently edited and partitioned in AliView (v.1.28) [41]. Our phylogenetic analysis was carried out in MrBayes (v.3.27.) (Ronquist et al., 2012). It consisted of two runs of 200 million generations running on 4 independent chains each. Sampling occurred every 1000 generations, and chain swapping was implemented to ensure equilibrium between tree search and topology refinement. We set a relative burn-in of 25 % and the consensus tree was built under the 50 % Majority Rule Consensus criterion. Convergence was first assessed by ensuring that the average standard deviation of split frequencies (ASDSF) had fallen below the 0.01 threshold and the potential scale reduction factor (PSRF) had approached 1. These results were subsequently further confirmed by analyzing the MrBayes output files in Tracer (v.1.72.) [42]. *Atropoides picadoi* and *Porthidium lansbergii* were set as outgroup organisms.

2.10. Data analysis

Thromboelastography traces, exported from the TEG 500 software, were coloured, annotated, and overlapped, using Adobe Photoshop (version 25.1.0). Fig. 1 was produced in the statistical software R (version 4.3.3) [43] using the "geodata" [44], "tmap" [45], "sf" [46], and "dplyr" [47] packages. Additionally, "extrafont", and "colorspace" packages were used to stylise the figure. The R code is available on GitHub (https://github.com/LachlanBourke/Species_location_map). Other figures were produced using GraphPad PRISM 9.5.1 software (GraphPad Prism Inc., La Jolla, CA, USA).

GraphPad Prism was used to perform statistical analyses on the data, with 0.05 set as the significance threshold. All statistical analyses used are described in the figure captions. Normality and homogeneity of variance assumptions were tested. The supplementary material file 1 provides additional information on the statistics used in the study. All raw data is provided in supplementary file 2.

3. Results

3.1. Thromboelastography

3.1.1. Plasma thromboelastography

Thromboelastography was used to compare the values for: SP (split point), the time at which the clot starts forming and the first measurable viscoelastic properties are detected; R (reaction time), the time from the start of the test until the initial formation of a clot, typically defined as the time it takes to achieve an amplitude of 2 mm; and A (amplitude), the maximum strength and stability of the clot. Both *B. lojanus* populations were strongly anticoagulant, as was *B. pictus* (Fig. 2). In contrast, *B. campbelli* and both *B. microphthalmus* populations were strongly procoagulant (Fig. 2). Rank order of procoagulant potency based on A values was *B. campbelli* > *B. microphthalmus* (Pastaza) > *B. microphthalmus* (Zamora Chinchipe).

3.1.2. Fibrinogen thromboelastography

To test for pseudo-procoagulant (aka: thrombin-like) direct clotting effects upon fibrinogen, the thromboelastography studies were repeated using purified human fibrinogen and the venom effects compared with that of thrombin (Fig. 3). The procoagulant *Bothrocophias* species (*B. campbelli* and *B. microphthalmus)* were more potent than the anticoagulant *Bothrocophias* (*B. lojanus*) and *Bothrops* (*B. pictus*) species, forming faster and stronger clots. However, all species formed weak clots, consistent with a pseudo-procoagulant action.

3.2. Anticoagulant mechanism of actions

Consistent with the thromboelastography results, the anticoagulant *Bothrocophias* (*B. lojanus*) and *Bothrops* (*B. pictus*) venoms significantly extended the clotting times relative to the spontaneous control (*B. lojanus* (Azuay) ($p = 0.0028$), *B. lojanus* (Loja)

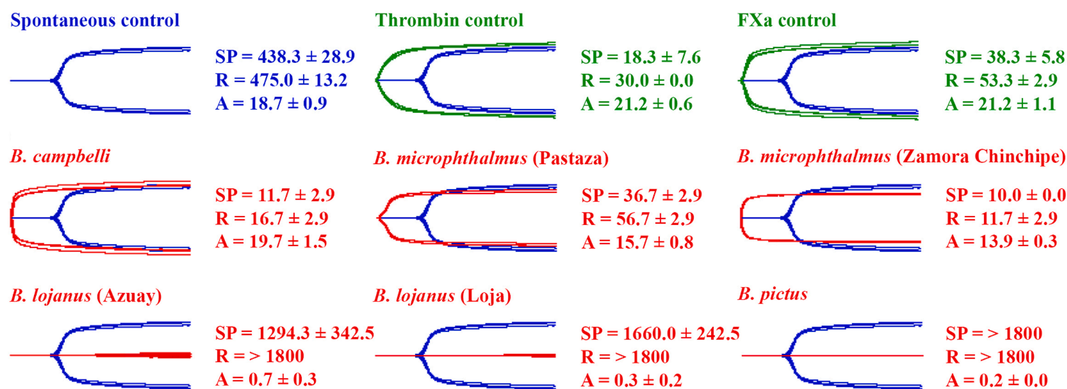


Fig. 2. Thromboelastography traces of *Bothrocophias* spp. and *Bothrops pictus* venoms on human platelet-poor plasma. All venom traces (red) and positive controls (green, thrombin and FXa control) are overlaid with the spontaneous control trace (blue) for comparison. Three clotting parameters (mean ± standard deviation, n = 3) are presented: split point (SP – time until trace splits, representing clot initiation), reaction time (R – time until amplitude = 2 mm, representing time until detectable clot), and amplitude (A – width of tracing at latest time point, representing clot strength). All tests lasted 1800 s, thus "> 1800 s" indicates the clotting parameter was not recorded within this time.

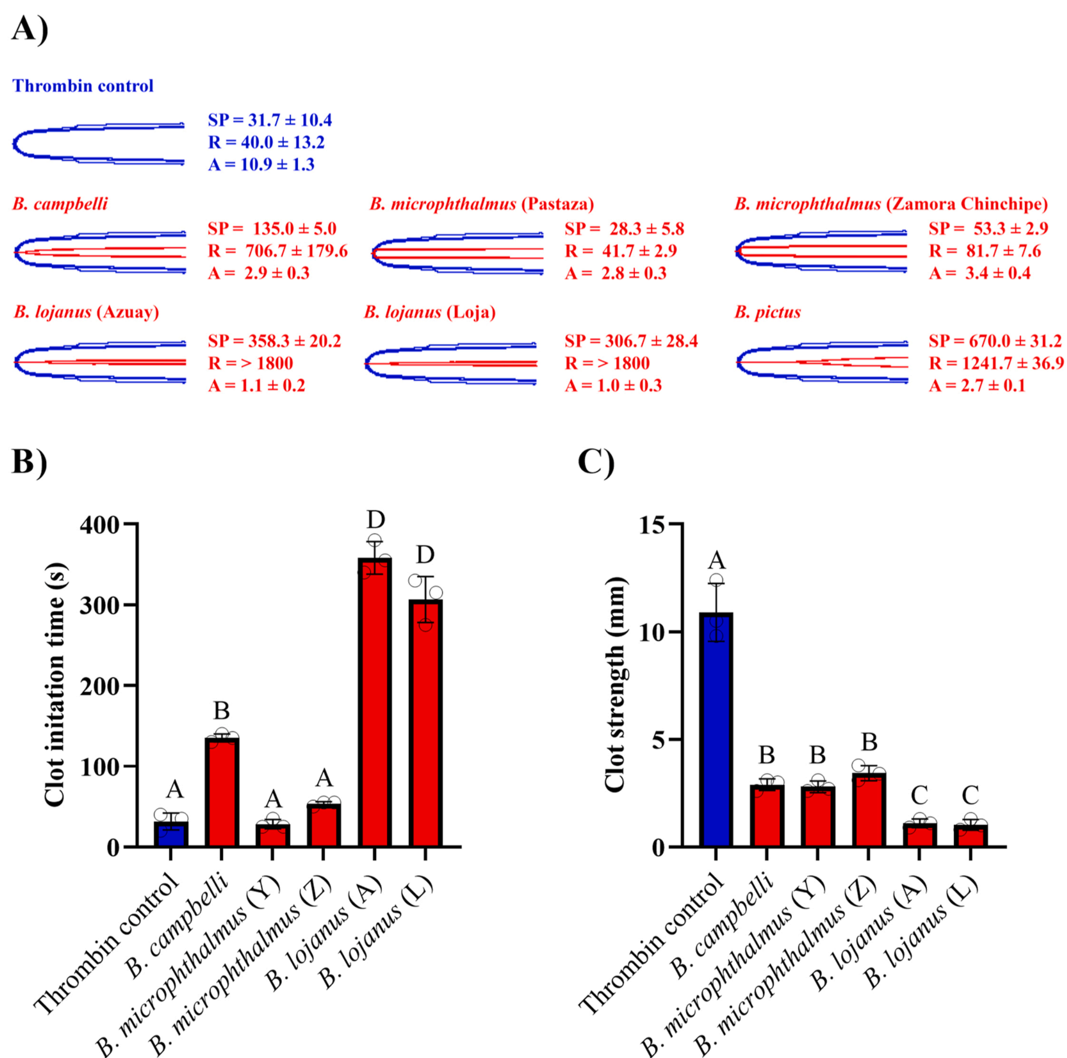


Fig. 3. **A)** Thromboelastography traces of *Bothrocophias* spp. and *Bothrops pictus* venoms on human fibrinogen. Thrombin control is shown in blue, while the venom traces are shown in red. Three clotting parameters (mean ± standard deviation, n = 3) are presented: split point (SP – time until trace splits, representing clot initiation), reaction time (R – time until amplitude = 2 mm, representing time until detectable clot), and amplitude (A – width of tracing at latest time point, representing clot strength). All tests lasted 1800 s, thus "> 1800 s" indicates the clotting parameter was not recorded in this time. **B)** Bar graph of clot initiation time (clotting parameter = SP) and **C)** clot strength (clotting parameter = A). Bars are mean ± standard deviation (n = 3). Circles on bars represent individual data points. Different letters above bars indicate statistical significance (if bars have the same letters, this indicates no significant difference). The statistical test used for B) and C) was a Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons test.

($p = 0.0148$), and *B. pictus* ($p = 0.0067$) (Fig. 4A). Tests using the phospholipase A_2 inhibitor varespladib revealed differential modes of action. The anticoagulant activity of *B. pictus* was completely abolished, while *B. lojanus* (Loja) was only marginally affected, and *B. lojanus* (Azuay) was unaffected (Fig. 4C). This reveals that while the anticoagulant activity of *B. pictus* is driven by PLA_2 toxins, those of *B. lojanus* venoms are exerted using other toxin types.

All anticoagulant *Bothrocophias* (*B. lojanus*) and *Bothrops* (*B. pictus*) venoms were also screened for their ability to inhibit coagulation factors (Fig. 5A). *Bothrops pictus* was the strongest inhibitor of the three tested venoms, with a target rank order of FVIIa (205±7 % increase) > prothrombinase complex (194±4 % increase) > FXIa (116±7 % increase) > FXa (125±8 % increase) > FXIa (143±18 % increase) > thrombin (74±5 % increase). The two populations of *B. lojanus* differed significantly from each other. *Bothrocophias lojanus* (Loja) was the second most potent inhibitor, significantly inhibiting the following coagulation factors to a greater extent than *B. lojanus* (Azuay): FVIIa ($p < 0.0001$), FXIa ($p = 0.0009$), FIXa ($p = 0.0131$), and the prothrombinase complex ($p = 0.0094$). Similar levels of inhibition between both *B. lojanus* venoms were observed for FXa ($p > 0.9999$) and thrombin ($p = 0.0606$). No inhibitory activity of FXIa and FIXa, and little inhibitory activity of FXa, was observed for *B. lojanus* (Azuay) venom. Similarly, negligible inhibition of FXIa, FXa, and FIXa was observed for *B. lojanus* (Loja) venom.

Tests for phospholipid destruction as a mode of anticoagulation were undertaken by increasing the phospholipid levels by 3.5x. Consistent with *B. pictus* being inhibited by the PLA_2-inhibitor varespladib (Fig. 4B), the addition of excess phospholipids

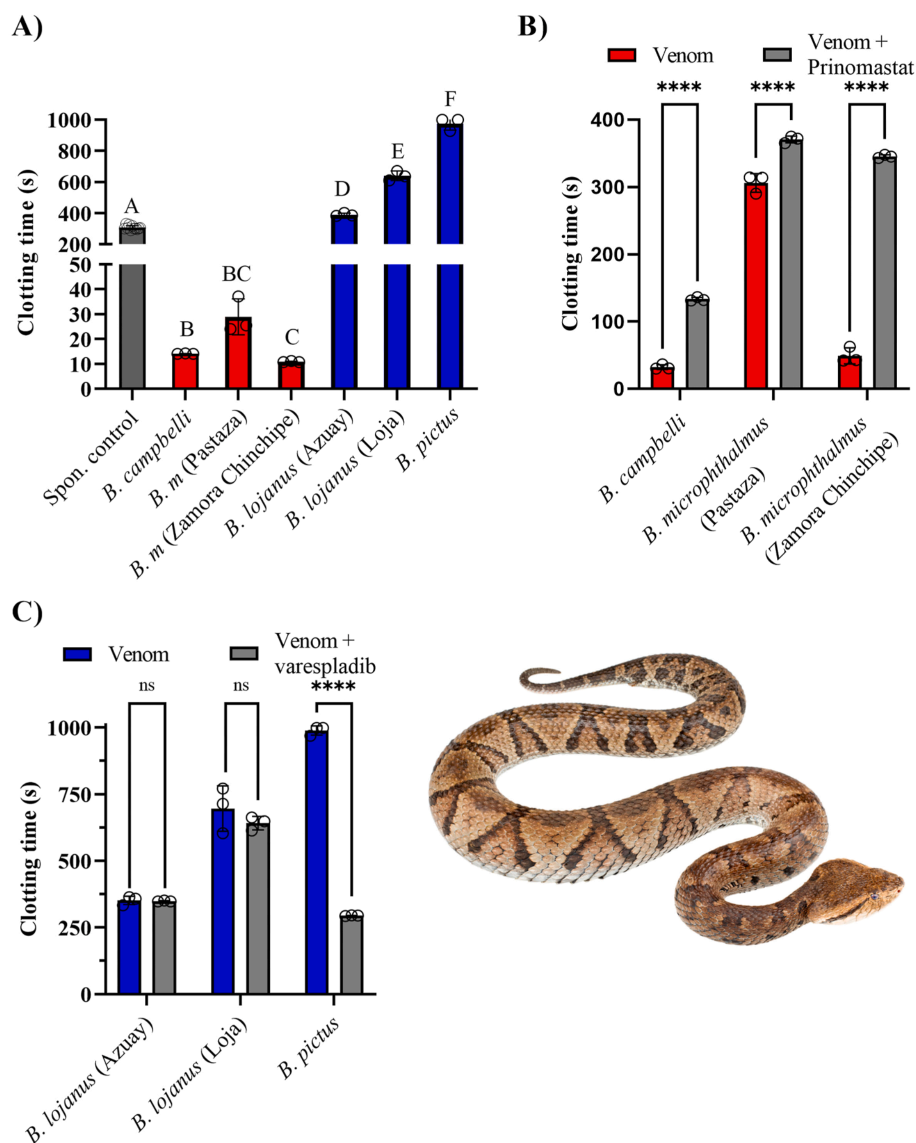


Fig. 4. A) Mean clotting time of human platelet-poor plasma incubated with a blank (spontaneous control), *Bothrocophias* venoms (20 μg/mL), and *Bothrops pictus* venom (20 μg/mL). Bars are: spontaneous control (grey), procoagulant venoms (red), and anticoagulant venoms (blue). Data analysed with an ordinary one-way ANOVA with Tukey's multiple comparisons. Different letters above bars indicate statistical significance (if bars have the same letters, this indicates no significant difference). **B)** Clotting time of procoagulant venoms (0.67 μg/mL) incubated with and without prinomastat, coloured according to legend. **C)** Clotting time of anticoagulant venoms (20 μg/mL) incubated with and without varespladib, coloured according to legend. Graphs in B) and C) analysed with an ordinary one-way ANOVA with Sidak's multiple comparisons. Significance is shown with asterisks (**** = $p \leq 0.0001$, and n.s. = not significant). In B) and C) spontaneous controls were also performed (supplementary material 2). In all graphs, bars are mean ± standard deviation (n = 3, except for spontaneous control bar n = 11). Circles on bars represent individual data points. Photo: *Bothrocophias microphthalmus*, © Jose Vieira.

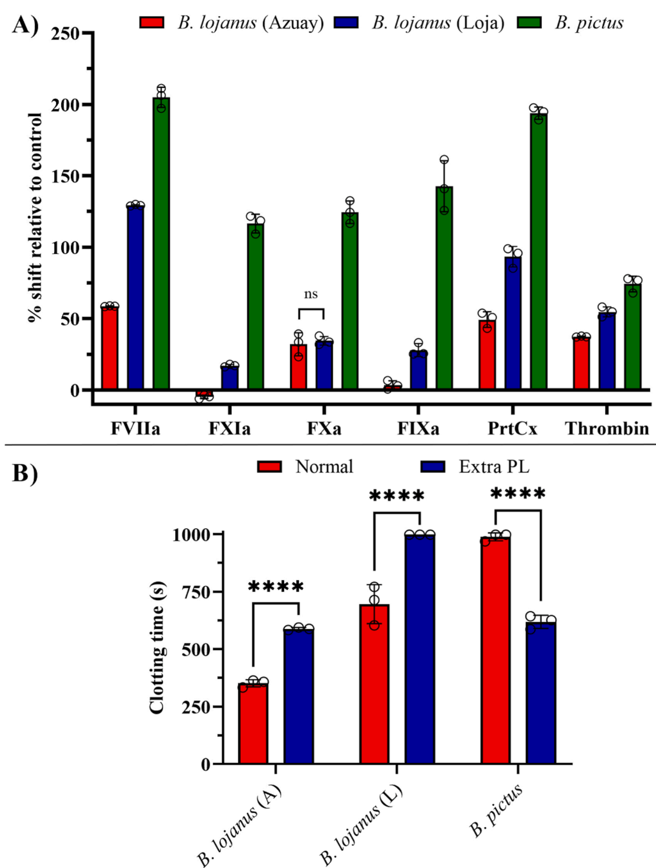


Fig. 5. **A)** Ability of anticoagulant *Bothrocophias* venoms and *Bothrops pictus* venom to inhibit clotting factors. A % shift value above 0 indicates inhibition. PrtCx = Prothrombinase complex. A two-way ANOVA with Tukey's multiple comparisons was used to compare differences between venoms within each clotting factor. All venoms are statistically significant from each other, except bars labelled with ns (not significant). **B)** Clotting time (x – axis) of human platelet-poor plasma incubated with anticoagulant venoms (y – axis) under different experimental conditions (see legend). The experimental conditions are "normal": venom incubated with cofactors, and "extra PL": venom incubated with cofactors and 3.5x extra phospholipids. Within each venom, the extra PL experimental condition was statistically compared to the normal condition. The statistical test used was an ordinary one-way ANOVA with Šídák's multiple comparisons test. Significance is shown with asterisks (**** = P ≤ 0.0001). In all graphs, bars are mean ± SD (n = 3), and circles on bars represent individual data points.

significantly reduced the anticoagulant activity ($p < 0.0001$) (Fig. 5B). This confirmed that *B. pictus* anticoagulant toxicity is driven by PLA_2 toxins depleting phospholipid levels. Intriguingly, the converse was observed for both *B. lojanus* venom samples. In the presence of extra phospholipids, clotting time was significantly prolonged (anticoagulant activity increased) by *B. lojanus* (Azuay) ($p < 0.0001$) and *B. lojanus* (Loja) ($p < 0.0001$) (Fig. 5B), suggestive that phospholipids are used as a binding cofactor by the anticoagulant toxins in these venoms, rather than their molecular target of physiological disruption, and that phospholipid levels are a rate-limiting step for the anticoagulant actions of these responsible toxins.

3.3. Procoagulant mechanism of actions

The procoagulant clotting tests upon human plasma were also congruent with Fig. 2 thromboelastography results, with *B. campbelli* and both *B. microphthalmus* populations significantly procoagulant ($p < 0.0001$) compared to the spontaneous clotting control (Fig. 4A). In the tests for dependence upon the cofactors calcium and phospholipid (Table 1), *B. campbelli* was the most dependent on calcium, with venom-induced clotting time slowed by 1443.2 %. *Bothrocophias campbelli* was also the most dependent on phospholipid, with venom-induced clotting time slowed by 78.5 %. No evidence of phospholipid dependence was observed for either *B. microphthalmus* venoms, but a small degree of calcium dependence was observed for *B. microphthalmus* (Pastaza), with venom-induced clotting time slowed by 52.1 %.

When *B. microphthalmus* (Zamora Chinchipe) venom was incubated with the metalloproteinase prinomastat, clotting time was significantly extended ($p = <0.0001$). Significant extension of clotting time was also observed for *B. campbelli* ($p = <0.0001$) and *B. microphthalmus* (Pastaza), although the overall shift in clotting time was less than *B. microphthalmus* (Zamora Chinchipe) (Fig. 4B). Due to a limited availability of venom, and to better observe the effects of prinomastat, a venom concentration of 0.67 μg/ml was used for this test.

Procoagulant *Bothrocophias* venoms were also screened for their ability to activate coagulation enzyme zymogens into their active forms. Both *B. microphthalmus* venom samples directly activated prothrombin, with *B. microphthalmus* (Zamora Chinchipe) inducing significantly higher activation than *B. microphthalmus* (Pastaza). In contrast, *B. campbelli* venom only activated prothrombin in the presence of Va (Fig. 6A). Intriguingly, *B. microphthalmus* venoms had slightly lower rates of reaction in the presence of FVa; however, this difference was minimal and may be due to binding to FVa without subsequent zymogen catalysis.

A similar pattern of potency was observed for FX activation: *B. microphthalmus* (Zamora Chinchipe) showed the highest activation of FX (38.00 ± 0.04 % relative to control), *B. campbelli* was only weakly active (5.67 ± 0.05 % relative to control), and *B. microphthalmus* (Pastaza) had no FX activation activity (Fig. 6B). Similarly, for FVII activation, only *B. microphthalmus* (Zamora Chinchipe) was strongly active (66.65 ± 0.007 % relative to control), while *B. microphthalmus* (Pastaza) had a very weak effect (1.76 ± 0.13 % relative to the control), and *B. campbelli* had no negligible activation (0.19 ± 0.14 %) (Fig. 6C).

To test antivenom efficacy, concentration curves were first performed. *Bothrocophias campbelli*, *B. microphthalmus* (Pastaza), and *B. microphthalmus* (Zamora Chinchipe) exhibited potent concentration-dependent procoagulant activity on human plasma (Fig. 7A). *Bothrocophias campbelli* was the most potent (fastest clotting time) at lower venom concentrations (Fig. 7A), however, when potency was assessed over multiple concentrations using the area under the curve (AUC), *B. campbelli* was slightly less potent than *B. microphthalmus* (Zamora Chinchipe) (AUC = 359.4 ± 2.2 and 348.3 ± 2.6, respectively) (Fig. 7B). While these AUC values are statistically significant ($p = 0.012$), such a small difference is not biologically meaningful. In contrast, *B. microphthalmus* (Pastaza) was dramatically less potent (AUC = 1389 ± 29.7) than both *B. campbelli* ($p < 0.0001$) and *B. microphthalmus* (Zamora Chinchipe) ($p = 0.0006$).

When venom was incubated with antivenom, the clotting times increased compared to the venom only treatment (Fig. 8). This shift in the curve towards spontaneous control values indicates neutralisation. *Bothrocophias campbelli* was significantly more neutralised by SAB antivenom than PoliVal-ICP antivenom: SAB antivenom neutralisation values were 529 % higher than PoliVal-ICP values (165.0 ± 1.9 % and 26.2 ± 1.4 % shifts in area under the curve, respectively) ($p < 0.0001$). The opposite was observed for both *B. microphthalmus* (Pastaza) and *B. microphthalmus* (Zamora Chinchipe) venoms; however, the difference between antivenoms was less extreme. *Bothrocophias microphthalmus*

Table 1
Cofactor dependence tests.

Species	Standard test	Phospholipid dependence test	Calcium dependence test
Bothrocophias campbelli	14.17±0.12	25.3±0.2 *** (78.5 % increase)	218.67±2.12 **** (1443.2 % increase)
B. microphthalmus (Pastaza)	28.93±7.19	27.43±0.99$^{n.s.}$	44±0.69 **** (52.1 % increase)
B. microphthalmus (Zamora Chinchipe)	10.93±0.23	10.47±0.25$^{n.s.}$	13.4±0.44$^{n.s.}$

Statistical test: Ordinary one-way ANOVA with Sidak's multiple comparisons test. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, and n.s. = not significant.

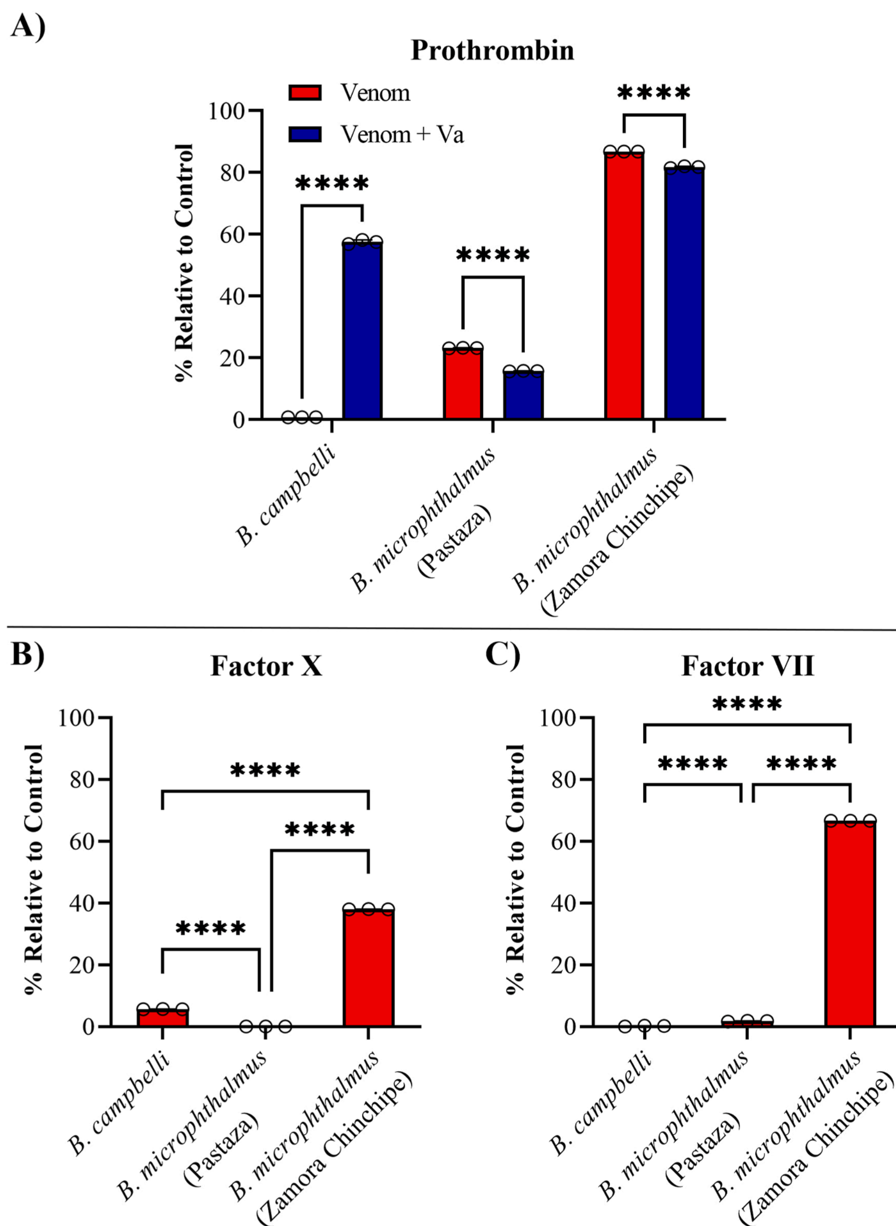


Fig. 6. Activation of coagulation factors by *Bothrocophias* procoagulant venoms on a fluorescent cleavage assay. Coagulation factors tested are **A)** prothrombin, **B)** FX, and **C)** FVII. Prothrombin activation was tested with and without the cofactor FVa. Each bar is a mean ± SD (n = 3) and circles on bars represent individual data points. Some error bars are too small to see. In A) a two-way ANOVA with Tukey's multiple comparisons test was used to compare differences within and between venoms, while in B) and C) an ordinary one-way ANOVA with Tukey's multiple comparisons was used to compare differences between venoms. Significance shown with asterisks (**** = $p \leq 0.0001$) (not all comparisons shown for A) – see supplementary material 1).

(Pastaza) was slightly significantly more neutralised by PoliVal-ICP antivenom than SAB antivenom: PoliVal-ICP antivenom neutralisation values were 46 % higher than SAB values (65.8 ± 3.5 % and 45.1 ± 3.4 % shifts in area under the curve, respectively) ($p = 0.0019$). *Bothrocophias microphthalmus* (Zamora Chinchipe) was also slightly significantly more neutralised by PoliVal-ICP antivenom than SAB antivenom: PoliVal-ICP antivenom neutralisation values were 54 % higher than SAB values (35.6 ± 5.5 % and 23.1 ± 3.0 % shifts in area under the curve, respectively) ($p = 0.0258$).

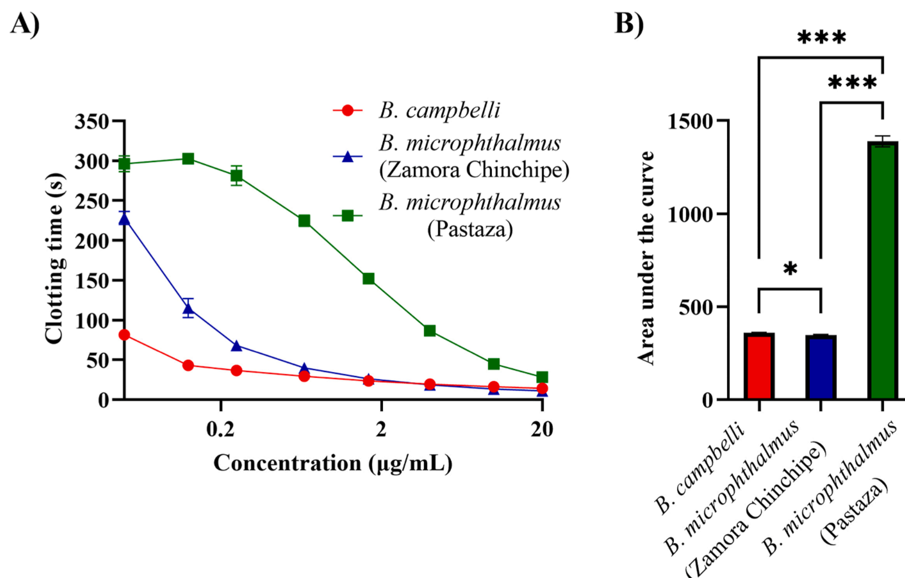


Fig. 7. A) Clotting time in seconds of three *Bothrocophias* venoms on human plasma, across multiple concentrations (µg/ml) (the x-axis is displayed in logarithmic view). Data points are mean ± SD (n = 3). Some error bars are too small to see. **B)** Area under the curve (AUC) values calculated from the data in A). Bars = mean ± SD (n = 3). To compare bars a Brown-Forsythe and Welch ANOVA with Dunnet's T3 multiple comparisons was performed. Significance shown with asterisks (*** = $p \leq 0.001$, * = $p \leq 0.05$).

4. Discussion

The present study tested venom coagulation activities from representatives of the understudied pit viper genus *Bothrocophias*. In addition, we aimed to build upon and extend results relating to earlier works by us [12] and Nielsen [13], providing evolutionary insights into the phylogenetic position of the procoagulant venom trait seen in *Bothrops*. As such, we also investigated the venom activity of the most basal *Bothrops* member: *B. pictus*. It was hypothesised that the *Bothrocophias* venoms tested would be anticoagulant, since *B. pictus* has been shown to have this trait [12]. Our data partially supports this hypothesis, reaffirming the anticoagulant activity of *B. pictus*, and revealing both *B. lojanus* venom samples tested were anticoagulant on human plasma but were less potent than the basal *Bothrops* (*B. pictus*) (Figs. 2 and 4A). To our knowledge, this is the first time *B. lojanus* venom activity has been tested in a laboratory setting. Intraspecific variation in clotting time was observed for *B. lojanus* samples, but we refrain from making interpretations of why this difference has arisen, due to our small sample size. Future work should investigate whether these differences are due to geographical, individual, sexual, or ontogenetic variations.

To ascertain the anticoagulant mechanism of action, we tested *B. pictus* and *B. lojanus* venoms for their ability to inhibit isolated clotting enzymes (Fig. 5A). *Bothrops pictus* venom significantly inhibited all the coagulation factors to a greater extent than either *Bothrocophias* venoms. Within *Bothrocophias*, *B. lojanus* (Loja) induced significantly more inhibition than *B. lojanus* (Azuay) venom. *Bothrocophias lojanus* (Azuay), also did not inhibit FXIa or FIXa. This data is congruent with the plasma clotting tests, which also showed *B. pictus* to be the most potently anticoagulant venom, followed by *B. lojanus* (Loja) and, lastly, *B. lojanus* (Azuay). Other than factor inhibition, hydrolysis of phospholipids by venom PLA_2s can also cause an anticoagulant effect [48,49]. Further tests revealed *B. pictus* clotting time decreases in the presence of excess phospholipid (Fig. 5B), potentially indicating phospholipid destruction, which is consistent with its anticoagulant activity being driven by PLA_2s (Fig. 4C). However, neither *B. lojanus* venom was shown to have this activity nor were driven by PLA_2s, indicating divergent venom strategies between the two genera. This suggests that clotting factor inhibition is a basal trait shared in the anticoagulant last common ancestor of the *Bothrocophias*/*Bothrops* clade, but phospholipid destruction is a derived trait present in *Bothrops pictus*. An alternative explanation for this result, however, is that toxins are competing with phospholipids for binding to clotting factors, thus in the presence of excess phospholipid fewer clotting factors are bound and inhibited by *B. pictus* venom. Regardless, our results show differences between the basal *Bothrops* and *Bothrocophias* venoms.

Surprisingly, we also found divergent venom phenotypes in procoagulant *Bothrocophias*, with *B. campbelli* and *B. microphthalmus* being procoagulant on human plasma. This appears to contradict previous research that found Ecuadorian *B. campbelli* venom had low coagulant activity on human plasma [8]. A more recent study found higher coagulant activity for Colombian *B. campbelli*, but still deemed this activity as weak [29]. However, Salazar-Valenzuela [8] and Sevilla-Sánchez [29] did not use calcium or phospholipid in their coagulation assay, which can be important cofactors for some venoms [31–33], and this likely contributed to their results. Indeed, we found *B. campbelli* was dependent on both calcium and phospholipid for its venom action (Table 1). In the absence of calcium, *B. campbelli* clotted citrated human plasma in 219 s; this is deemed weakly coagulant in our assay. Considering this, previous research showing other *Bothrocophias* species (*B. colombianus* and *B. myersi*) are weakly coagulant [28,30], should be reappraised with cofactors included. On the other hand, to our knowledge, no literature is available on the coagulant venom effects of *B. microphthalmus*. Here, we present the first evidence showing the procoagulant action of these venoms. Interestingly, while the *B. microphthalmus* (Zamora Chinchipe) locality was similar in potency to *B. campbelli,* the *B. microphthalmus* (Pastaza) locality was less potent on human plasma (Figs. 2, 4A and 7). We again refrain from making interpretations regarding geography as each of our *Bothrocophias* venom samples was from just one individual.

Prinomastat reduced clotting time in all *Bothrocophias* procoagulant venom samples (Fig. 4B), suggesting metalloproteinases are, in part, responsible for the coagulotoxic effects. *Bothrocophias microphthalmus* (Pastaza) venom was more potently inhibited than

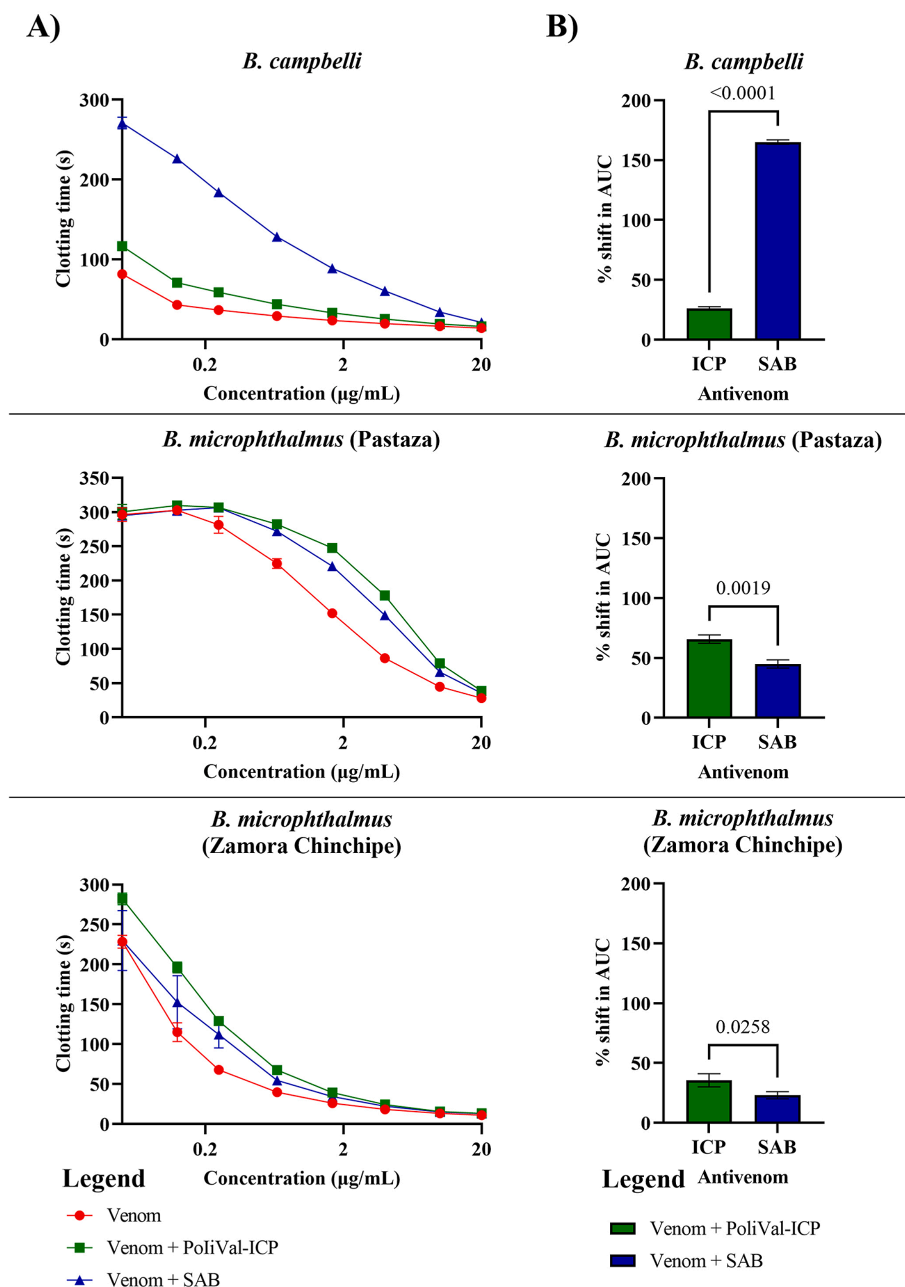


Fig. 8. **A)** Clotting time in seconds of *Bothrocophias* venom on human plasma, across multiple concentrations (μg/ml) (the x-axis is displayed in logarithmic view). Venom was tested without antivenom (red) and with antivenom (PoliVal-ICP: green, SAB: blue). Data points are mean ± SD (n = 3). Some error bars are too small to see. **B)** % shift in area under the curve (AUC) relative to the control (venom only) values for each antivenom. % shift is a proxy for neutralisation. Bars = mean ± SD (n = 3). To compare bars, an unpaired *t*-test (two-tailed) was performed (*p* values are shown between bars).

B. campbelli and *B. microphthalmus* (Zamora Chinchipe), potentially indicative of structural variations in the metalloproteinases present.

After uncovering a procoagulation trait present within *Bothrocophias*, we ascertained what venom mechanisms drives this action (Fig. 6). Both *B. microphthalmus* venom samples directly activated prothrombin. However, *B. campbelli* only activated prothrombin in the presence of FVa. Based on the ability of the metalloproteinase-inhibitor prinomastat to impede the procoagulant activity, this suggests that the prothrombin activating toxin is a metalloproteinase. If so, this is the first time the use of FVa as a cofactor has been documented for a metalloproteinase driven procoagulant activity. The use of FVa as a cofactor has been shown in Australian elapid snakes which differ from *B. campbelli* in the enzyme type that uses FVa as a cofactor. In the case of the Australian elapids, it is a weaponised form of the blood clotting enzyme Factor Xa instead of metalloproteinases [31]. In these elapid snakes, the FXa:FVa complex evolved to activate FVII as the basal trait within this clade, with prothrombin activation observed only for some derived species [50–52]. As the type of toxins which use FVa as a cofactor likely differ between *B. microphthalmos* and Australian elapid snakes (metalloproteinases and Factor Xa, respectively), this therefore represents a case of convergent molecular and functional evolution.

Due to limitations in venom supply, we were not able to test whether *B. campbelli* simply utilized endogenous FVa or if the

venom was also able to directly activate FV into FVa to provide a supply of this cofactor. While *B. campbelli* is the first viper venom documented as using FVa as a cofactor, other snake venoms have been previously shown to activate FV into FVa, with this trait shown for *Daboia*, *Macrovipera*, and *Vipera* within the Palearctic true viper clade, and *Bothrops* from within the pit vipers [53,54] *Daboia* and *Macrovipera* venoms have been previously shown to contain kallikrein-scaffold serine proteinases (RVV-V isoforms) which activate FV by cleaving it at the Arg1545-Ser1546 bond, which is important for the formation of the prothrombinase complex necessary for thrombin generation in hemostasis [53,55]. *Daboia russelii* venom has been shown to facilitate blood clot formation by activating both factors X (through the use of SVMP) and V (using kallikrein-scaffold serine proteinases), with FXa and FVa, along with calcium and phospholipids, subsequently forming the prothrombinase complex to convert prothrombin to thrombin, with the generated thrombin in turn cleaving fibrinogen into fibrin strands [53,55–58]. This swift action often results in a condition known as venom-induced consumption coagulopathy (VICC), characterized by the excessive consumption of clotting factors, including fibrinogen and prothrombin, as coagulopathy progresses [56,59]. However, while the ability of *D. russelii* venom to convert FV into FVa is well described, it has never been tested whether this venom is able to use FVa as a cofactor to activate prothrombin, as was shown to be the case for *B. campbelli* in this study. As such, the lack of documented prothrombin activating activity by *D. russelii* venom may be due to prior work not including FVa as a cofactor in the assays, which may be required for prothrombin activation as per *B. campbelli* venom. Therefore, the attribution of *D. russelii* venom triggering procoagulation soley through the activation of Factor X (but not through direct venom activation of prothrombin), may be a case of absence of evidence being taken as evidence of absence, rather than *D. russelii* truly not being able to activate prothrombin. Thus, the ability to use FVa as an obligate prothrombin-activating cofactor as revealed in the current study, may in fact be widespread in viperid snake venoms and this is therefore a rich area for future research.

Another interesting result was the FVII activation observed in *B. microphthalmus* (Zamora Chinchipe). In contrast, the Pastaza locality of *B. microphthalmus* exhibited negligible FVII activation. Future work should investigate whether these differences are due to geographical, individual, sexual, or ontogenetic variations. This finding represents only the second time FVII activation has been found in a pit viper species, and the fourth in vipers overall. The first documented case of FVII activation by a pit viper venom was the central American pit viper *Porthidium volcanicum* [22]. As *Porthidium volcanicum* is novel within its genus in being the only known procoagulant species [22], with this genus in turn nested amongst an extensive diversity of anticoagulant species, this suggests that FVII activation has evolved on two separate occasions within pit viper snake venom metalloproteinases. However, an alternate scenario exists whereby FVII activation by SVMP is an earlier evolved trait in pit vipers, with the toxins persisting at low levels in anticoagulant species before being amplified in these latter evolving procoagulant species. Expanding on this, is the hypothesis that FVII activation is a basal trait in viperid snake venoms. Supporting this, FVII activation is a trait that has been documented across the full taxonomical diversity of viperid snakes, having also been documented in the venoms of *Echis* [60], *Macrovipera* species [61], and *Vipera* [62]. To resolve whether this is a basal trait for SVMP or if the diversity represents multiple neofunctionalization events would require sequencing the toxins responsible and reconstructing their molecular phylogenetic history. If the FVII activating toxins form a monophyletic group relative to other types of SVMP, then this would support the theory of FVII being an early evolving trait. Conversely, if the toxins do not form a monophyletic group, this would support convergent evolution for the same biochemical action. FVII activation has also been documented in the SVMP-driven venom of *Rhabdophis subminiatus*, a non-viperid snake in the Natricidae snake family [63]. However, as the SVMP toxins in *Rhabdophis* are phylogenetically distinct from those in viperid snakes [64], this suggests that FVII activation evolved convergently in *Rhabdophis* relative to viperid snakes. Intriguingly, FVII activation has also been documented in *Heloderma* lizard venoms, but this trait clearly convergent in this case [65].

Lastly, we tested the efficacy of two regional antivenoms on procoagulant *Bothrocophias* venoms (Fig. 8), and our antivenom results underscore the difference in venom composition between *B. campbelli* and *B. microphthalmus*. To directly compare the antivenom results with previous studies, we used the antivenom concentration as per our previous work [34], except in our study we present neutralisation as a percentage value rather than x-fold shift (percentage equallying x-fold shift x 100). We showed SAB antivenom significantly better neutralised *B. campbelli* venom than PoliVal-ICP antivenom, but PoliVal-ICP antivenom still showed neutralisation capacity. This is consistent with previous research showing PoliVal-ICP cross recognised *B. campbelli* venom and protected mice from its lethal effects [8]. Both *B. microphthalmus* venoms exhibited an opposite pattern of neutralisation, with PoliVal-ICP neutralisation more effective than SAB antivenom. Regardless, our results are still consistent with prior work showing some level of SAB antivenom recognition of Peruvian *B. microphthalmus* venom [66]. Overall, the neutralisation results for *B. microphthalmus* were relatively weak compared to the results of a previous study, in which PoliVal-ICP neutralised *Bothrops asper* venoms and SAB neutralised *B. atrox* venoms to a higher degree than shown in our study [34]. This demonstrates the antivenom has an inherently high neutralisation potential with the right antigenic match. Despite this, we still showed neutralisation, and these results are promising as they provide data that may contribute to the evidence-based design of clinical management strategies for the envenomed patient. These are but the first stage of preclinical studies and require *in vivo* animal studies and clinical validation trials.

To provide further insights into the coagulotoxic venom activity, we also tested venom effects for both the anticoagulant and procoagulant venoms on isolated human fibrinogen (Fig. 3). Direct venom action upon fibrinogen in a pseudo-procoagulant manner (forming transient fibrin clots) has been documented in a wide array of *Bothrops* species [12]; thus, we hypothesised this activity is present in *Bothrocophias* as an ancestral trait. Indeed, when tested on thromboelastography, all venoms produced weak fibrin clots. This is consistent with pseudo-procoagulant activity; weak fibrin clots that are, in turn, friable and easily break down. These results are supported by research showing *B. campbelli* venom consists of a large proportion of kallikrein-scaffold serine proteinases [8] and *B. microphthalmus* has high serine proteinase activity [66]. Interestingly, within *Bothrocophias* venoms, two weak fibrin clot phenotypes emerged, both linked with plasma clot phenotypes. *Bothrocophias* venoms that were procoagulant on human plasma (*B. campbelli*, *B. microphthalmus* (Pastaza), and *B. microphthalmus* (Zamora Chinchipe)) all produced fibrin clots with a quick clot initiation time like the thrombin control, but a relatively weak clot strength compared to the thrombin control. Conversely, *B. lojanus* venoms, which were anticoagulant on human plasma, produced fibrin clots with a slow clot initiation time and very weak clot strengths, compared to the thrombin control. This activity on fibrinogen is likely one of the driving forces behind the net anticoagulant effect observed on human plasma.

The array of assays revealed clear differences between *B. lojanus*, *B. campbelli*, and *B. microphthalmus* venoms, providing evolutionary insights into the venom evolution of this group. To interpret these results in an evolutionary context, we inferred our own phylogenetic tree due to the conflict in the prior literature. Using molecular data, as part of much larger studies into pit viper evolution and containing a vast array of taxa, *B. lojanus* has been recovered within *Bothrocophias* but with an unresolved phylogenetic position. Some studies indicate *B. lojanus* as basal [24,25], with other studies having *B. campbelli* as the most basal derivation [23,26,27]. However, in all these studies, the node support was weak and therefore the phylogenetic placement remained unresolved. A study that combined genetic and morphological data also failed to resolve the position of *B. lojanus* [67]. We constructed our own tree (Fig. 9) using the mitochondrial cytb and ND4 genes available for *Bothrocophias* species, in addition to *Bothrops pictus* and other representative *Bothrops* species. Consistent with the unresolved nature of other phylogenetic analyses, the placement of *B. campbelli* and *B. lojanus* was unresolved, forming a basal polytomy. Future work utilizing more genes would be needed to accurately reconstruct the evolutionary history, especially nuclear genes considering the deep-time level of division.

As the relative organismal phylogenetic placement of *B. lojanus* and *B. campbelli* are unresolved, the molecular evolutionary history of the venom cannot be fully reconstructed at this time, but there are three potential outcomes. Outcome 1, where *B. lojanus* is the more basal *Bothrocophias* species relative to *B. campbelli*; Outcome 2, where *B. campbelli* is the more basal species relative to *B. lojanus;* and Outcome 3, where of *B. lojanus* + *B. campbelli* form a clade that is in turn sister to the *Bothrocophias.*

In Outcome 1, where the anticoagulant *B. lojanus* is the basal *Bothrocophias* species, just as the anticoagulant *B. pictus* is the most basal *Bothrops* species, this would suggest that the last common ancestor of the *Bothrops* + *Bothrocophias* clade was anticoagulant and procoagulation convergently evolved at least two times: once after the split of *B. lojanus* from other *Bothrocophias,* and again subsequent to the split of *B. pictus* from other *Bothrops*. Consistent with this is the extremely unique biochemical nature of *B. campbelli* procoagulation, whereby Factor Va is used as a cofactor to activate prothrombin. As *B. campbelli* and *B. microphthalmus* differ in venom procoagulant biochemistry and are not sister species, this leads to two further competing hypotheses within *Bothrocophias*: hypothesis 1 is that procoagulation is an early evolving trait within *Bothrocophias,* and the use of FVa as a cofactor by *B. campbelli* represents a case of extreme secondary derivation; and hypothesis 2 that procoagulation evolved on two occasions within *Bothrocophias*, which is reflected by the sharp difference in venom biochemistry between *B. campbelli* and *B. microphthalmus*. Overall, Outcome 1 is the most parsimonious explanation supported by the available data.

In the case of Outcome 2, whereby the procoagulant *B. campbelli* is shown to be the most basal *Bothrocophias* species relative to the anticoagulant *B. lojanus*, this would suggest the last common ancestor of *Bothrocophias* + *Bothrops* was procoagulant. *Bothrocophias lojanus*; therefore, would represent a secondary derivation to an anticoagulant state within *Bothrocophias,* and *B. pictus* a secondary derivation to an anticoagulant state within *Bothrops.* As with Outcome 1, the unique use of FVa as a cofactor by *B. campbelli* would represent a lineage specific secondary derivation.

In the case of Outcome 3, anticoagulant or procoagulant toxicity are equally plausible. First, as per Outcome 1 above, anticoagulant toxicity is the basal state, with *B. campbelli* and *B. microphthalmos* independently evolving the procoagulant trait, which is consistent with the sharp difference in venom biochemistry between *B. campbelli* and *B. microphthalmos;* or secondly, as Outcome 2 above, procoagulant toxicity evolved at the base of *Bothrocophias* + *Bothrops,* with the anticoagulant state in *B. lojanus* being a derived trait in *Bothrocophias*, just as *B. pictus* represents an anticoagulant derivation from an ancestral procoagulant state in *Bothrops.*

More individual venom samples from these snakes for testing, and a more refined organismal *Bothrocophias* phylogenetic tree, are required for final resolution of these questions. Indeed, it is also possible *B. campbelli* and *B. lojanus* are not the most basal species. With the addition of more data, *Bothrocophias colombianus* and *B. myersi* (not shown in our phylogeny due to limited data) may prove to be the earliest diverging species However, the most parsimonious explanation of our results is that anticoagulant venom is ancestral to both *Bothrops* and *Bothrocophias*, with procoagulant venom evolving convergently between *Bothrops* and *Bothrocophias* as independent evolutions of this trait. As such, this detailed study builds a foundation for future characterization of their unique pit viper venom activities and phylogenetic placement of individual *Bothrocophias* relative to each other and the most basal members of the *Bothrops* genus. It also provides data essential for evidence-based design of clinical management strategies for the envenomed patient.

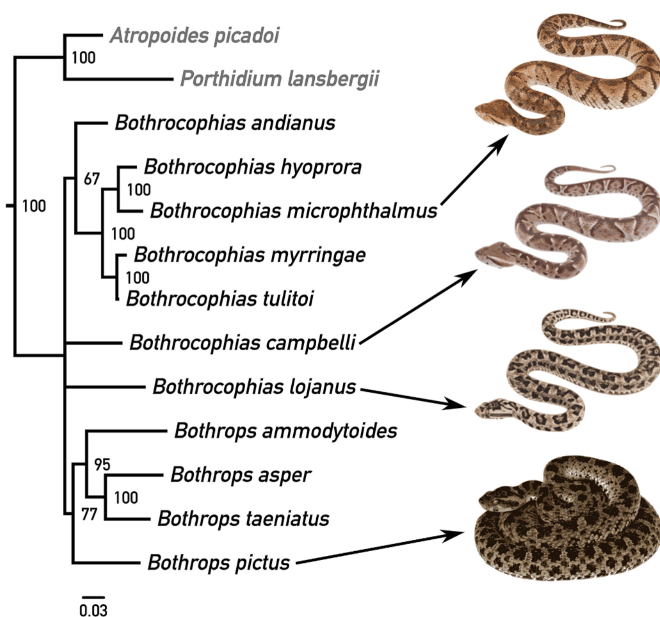


Fig. 9. Organismal relationships of *Bothrocophias* and representative *Bothrops* species. Outgroups are *Atropoides* and *Porthidium.* Posterior probability values above 60 % are displayed at internal nodes. The scale bar at the bottom represents the number of substitutions per site. Photos of *Bothrocophias* were sourced from reptilesofecuador.com with permission from Alejandro Arteaga, and *Bothrops pictus* photo is https://es.mongabay.com/2017/07/fauna-urbana-lima-animales ©P. Venegas.

CRediT authorship contribution statement

Lachlan A. Bourke: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Writing - review & editing, Visualization. **David Salazar-Valenzuela:** Writing – review & editing, Resources. **Marco Mancuso:** Writing – review & editing, Software, Methodology, Formal analysis, Visualization. **Diego R. Quirola:** Writing – review & editing, Resources. **Amaru Loaiza-Lange:** Writing – review & editing, Resources. **Christina N. Zdenek:** Writing – review & editing, Supervision. **Matthew R. Lewin:** Writing – review & editing, Resources.

Ernesto Arbeláez-Ortiz: Resources. **Bryan G. Fry:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

We have a competing interest to declare (please describe below):

No conflicts of interest declared by any author except M.R.L. Director of and owns stock in Ophirex, Inc, a Public Benefit Corporation, however the company did not have any input into experimental design or review of results before publication.

This statement is to certify that all Authors have seen and approved the manuscript being submitted, and agree to the submission to *BIOCHIMIE*. We warrant that the article is the Authors' original work. We warrant that the article has not received prior publication, is not under consideration for publication elsewhere, and will not be submitted for publication elsewhere, in whole or in part, while under consideration for publication in *BIOCHIMIE*. On behalf of all Co-Authors, the corresponding Author shall bear full responsibility for the submission.

We attest to the fact that all Authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and their interpretation, and agree to its submission to *BIOCHIMIE*. We further attest that no other person has fulfilled the requirements for authorship as stated in the Elsevier Authorship-factsheet (**2019_ETHICS_AUTH02 - attached**), but is not included in the list of authors, and that no other person has contributed substantially to the writing of the manuscript but is not included either among the authors or in the acknowledgements.

All authors agree that no modification to the author list can be made without the written acceptance of all authors and the formal approval of the Editor-in-Chief. All authors accept that the Editor-in-Chief's decisions over acceptance or rejection or in the event of any breach of the Principles of Ethical Publishing in *BIOCHIMIE* being discovered, of retraction are final.

On behalf of all authors *(delete line if not appropriate*).

Acknowledgements

This research was funded by Australian Research Council Discovery Project DP190100304 to B.G.F. A PhD Scholarship from the University of Queensland was awarded to L.A.B. L.A.B would like to acknowledge all members of the Adaptive Biotoxicology Lab for their continued support throughout the project, especially Lorenzo Seneci for his expertise on the zymogen activation assay. We thank Jaime Culebras, Nicolás del Castillo, Amalia Espinoza, Gustavo Hushpa, and Gabriel Cabrera, and Juan Cueva for their help in the field and the laboratory. We also thank Armando Yarleque for providing *Bothrops pictus* venom. We would also like to acknowledge and pay our respects to the Indigenous groups from where this work was performed (Meanjin (Brisbane), Australia) and the areas where these venoms were collected (Ecuador and Peru).

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2025.07.001.

References

[1] W. Wüster, M. Salomão, J.A. Quijada-Mascareñas, R. Thorpe, BBBSP, origins and evolution of the South American pitviper fauna: evidence from mitochondrial DNA sequence analysis, Biology of the Vipers (2002) 111–128.

[2] Reptile-Database, 2025. (Accessed 1 May 2025).

[3] C.C. Nogueira, A.J.S. Argôlo, V. Arzamendia, J.A. Azevedo, F.E. Barbo, R. S. Bérnils, B.E. Bolochio, M. Borges-Martins, M. Brasil-Godinho, H. Braz, M. A. Buononato, D.F. Cisneros-Heredia, G.R. Colli, H.C. Costa, F.L. Franco, A. Giraudo, R.C. Gonzalez, T. Guedes, M.S. Hoogmoed, O.A.V. Marques, G. G. Montingelli, P. Passos, A.L.C. Prudente, G.A. Rivas, P.M. Sanchez, F. C. Serrano, N.J. Silva, C. Strüssmann, J.P.S. Vieira-Alencar, H. Zaher, R. J. Sawaya, M. Martins, Atlas of Brazilian snakes: verified point-locality maps to mitigate the wallacean shortfall in a megadiverse snake fauna, South American J. Herpetol. 14 (1) (2019) 274, 274.

[4] J.R.L. Gutberlet, J.A. Campbell, 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 (2001) 1–16.

[5] D.F. Cisneros-Heredia, M. Borja, D. Proano, J. Touzet, Distribution and natural history of the Ecuadorian toad-headed pitvipers of the genus *bothrocophias*, HERPETOZOA 19 (2006) 10.

[6] A.M. Fenwick, R.L. Gutberlet, J.A. Evans, C.L. Parkinson, Morphological and molecular evidence for phylogeny and classification of South American pitvipers, genera *bothrops, bothriopsis*, and *bothrocophias* (serpentes: viperidae), Zool. J. Linn. Soc. 156 (2009) 617–640.

[7] V. Carvalho, R. Fraga, E. Eler, R. Kawashita-Ribeiro, E. Feldberg, R. Vogt, M. Carvalho, J. Noronha, L. Condrati, S. Bittencourt, Toad-headed pitviper *Bothrocophias hyoprora* (amaral, 1935)(Serpentes, viperidae): new records of geographic range in Brazil, hemipenial morphology, and chromosomal characterization, Herpetol. Rev. 44 (2013) 410–414.

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

[9] S. Larréché, J.-P. Chippaux, L. Chevillard, S. Mathé, D. Résière, V. Siguret, B. Mégarbane, Bleeding and thrombosis: insights into pathophysiology of *bothrops* venom-related hemostasis disorders, Int. J. Mol. Sci. 22 (2021) 9643.

[10] W.M. Monteiro, J.C. Contreras-Bernal, P.F. Bisneto, J. Sachett, I. Mendonça da Silva, M. Lacerda, A. Guimarães da Costa, F. Val, L. Brasileiro, M.A. Sartim, S. Silva-de-Oliveira, P.S. Bernarde, I.L. Kaefer, F.G. Grazziotin, F.H. Wen, A. M. Moura-da-Silva, *Bothrops atrox*, the Most important snake involved in human envenomings in the Amazon: how venomics contributes to the knowledge of snake biology and clinical toxinology, Toxicon X 6 (2020) 100037.

[11] R. Otero-Patiño, Epidemiological, clinical and therapeutic aspects of *Bothrops asper* bites, Toxicon 54 (2009) 998–1011.

[12] L.A. Bourke, C.N. Zdenek, A.M. Tanaka-Azevedo, G.P.M. Silveira, S. S. Sant'Anna, K.F. Grego, C.F.B. Rodrigues, B.G. Fry, Clinical and evolutionary implications of dynamic coagulotoxicity divergences in *bothrops* (lancehead pit viper) venoms, Toxins 14 (2022).

[13] V.G. Nielsen, N. Frank, S. Afshar, De Novo Assessment and Review of Pan-American Pit Viper Anticoagulant and Procoagulant Venom Activities via Kinetomic Analyses, Toxins 11 (2019).

[14] L.F. Sousa, C.N. Zdenek, J.S. Dobson, B. Op den Brouw, F. Coimbra, A. Gillett, T. H.M. Del-Rei, H.M. Chalkidis, S. Sant'Anna, M.M. Teixeira-da-Rocha, K. Grego, S.R. Travaglia Cardoso, A.M. Moura da Silva, B.G. Fry, Coagulotoxicity of *bothrops* (lancehead pit-vipers) venoms from Brazil: differential biochemistry and antivenom efficacy resulting from prey-driven venom variation, Toxins 10 (2018) 411.

[15] C.F.B. Rodrigues, C.N. Zdenek, L.A. Bourke, L. Seneci, A. Chowdhury, L. A. Freitas-de-Sousa, F. de Alcantara Menezes, A.M. Moura-da-Silva, A. M. Tanaka-Azevedo, B.G. Fry, Clinical implications of ontogenetic differences in the coagulotoxic activity of *Bothrops jararacussu* venoms, Toxicol. Lett. 348 (2021) 59–72.

[16] H. Hofmann, C. Bon, Blood coagulation induced by the venom of *bothrops Atrox*. 2. Identification, purification, and properties of two factor X activators, Biochemistry 26 (1987) 780–787.

[17] H. Hofmann, C. Bon, Blood coagulation induced by the venom of *bothrops Atrox*. 1. Identification, purification, and properties of a prothrombin activator, Biochemistry 26 (1987) 772–780.

[18] G.D. Loría, A. Rucavado, A.S. Kamiguti, R.D.G. Theakston, J.W. Fox, A. Alape, J. M.a. Gutiérrez, Characterization of 'basparin A,' a prothrombin-activating metalloproteinase, from the venom of the snake *Bothrops asper* that inhibits platelet aggregation and induces defibrination and thrombosis, Arch. Biochem. Biophys. 418 (2003) 13–24.

[19] N.J. Youngman, D.J. Carlsson, L. Jones, E. Neri-Castro, A. Alagon, B.G. Fry, Cloud serpent coagulotoxicity: the biochemical mechanisms underpinning the anticoagulant actions of *mixcoatlus* and *ophryacus* venoms, Toxicon 211 (2022) 44–49.

[20] N.J. Youngman, Y.-H. Peng, R.J. Harris, L. Jones, J. Llinas, M. Haworth, A. Gillett, B.G. Fry, Differential coagulotoxic and neurotoxic venom activity from species of the arboreal viperid snake genus *bothriechis* (palm-pitvipers), Comp. Biochem. Physiol. C Toxicol. Pharmacol. 256 (2022) 109326.

[21] L. Seneci, C.N. Zdenek, L.A. Bourke, C. Cochran, E.E. Sanchez, E. Neri-Castro, M. Benard-Valle, A. Alagon, N. Frank, B.G. Fry, A symphony of destruction: dynamic differential fibrinogenolytic toxicity by rattlesnake (*crotalus* and *sistrurus*) venoms, comparative biochemistry and physiology, Toxicol. Pharmacol.: CBP 245 (2021) 109034.

[22] L. Jones, N.J. Youngman, E. Neri-Castro, A. Guadarrama-Martinez, M.R. Lewin, R. Carter, N. Frank, B.G. Fry, Differential antivenom and small-molecule inhibition of novel coagulotoxic variations in *atropoides, cerrophidion, metlapilcoatlus*, and *porthidium* American viperid snake venoms, Toxins (2022) 14.

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

[24] B. Hamdan, T.B. Guedes, P.A. Carrasco, J. Melville, A complex biogeographic history of diversification in neotropical lancehead pitvipers (serpentes, viperidae), Zool. Scr. 49 (2020) 145–158.

[25] A. Figueroa, A.D. McKelvy, L.L. Grismer, C.D. Bell, S.P. Lailvaux, A species-level phylogeny of extant snakes with description of a new colubrid subfamily and genus, PLoS One 11 (2016) e0161070.

[26] A. Arteaga, R.A. Pyron, N. Penafiel, P. Romero-Barreto, J. Culebras, L. Bustamante, M.H. Yanez-Munoz, J.M. Guayasamin, Comparative phylogeography reveals cryptic diversity and repeated patterns of cladogenesis for amphibians and reptiles in northwestern Ecuador, PLoS One 11 (2016) e0151746.

[27] H. Zaher, R.W. Murphy, J.C. Arredondo, R. Graboski, P.R. Machado-Filho, K. Mahlow, G.G. Montingelli, A.B. Quadros, N.L. Orlov, M. Wilkinson, Y.-P. Zhang, F.G. Grazziotin, Large-scale molecular phylogeny, morphology, divergence-time estimation, and the fossil record of advanced Caenophidian snakes (squamata: serpentes), PLoS One 14 (2019) e0216148.

[28] J.A. Pereañez, L.M. Preciado, J. Fernández, E. Camacho, B. Lomonte, F. Castro, C.A. Cañas, C. Galvis, S. Castaño, Snake venomics, experimental toxic activities and clinical characteristics of human envenomation by *Bothrocophias myersi* (serpentes: viperidae) from Colombia, J. Proteonomics 220 (2020) 103758.

[29] M.-J. Sevilla-Sánchez, J.A. Guerrero-Vargas, S. Ayerbe-González, J.J. Calderón-Leytón, B. Lomonte, D. Mora-Obando, Toxinological profile and histopathological alterations induced by *Bothrocophias campbelli* venom from Colombia, Acta Trop. 250 (2024) 107094.

[30] Y. Moreno, K.L. Ángel-Camilo, M.L. Bueno-Ospina, S. Ayerbe-González, J. A. Guerrero-Vargas, Four physiopathological effects of the venom of *Bothrocophias colombianus* (rendahl & vestergren, 1940) endemic to the Colombian Pacific: toxinological profile of the venom of *Bothrocophias colombianus*, Revista Novedades Colombianas 19 (2024).

[31] R.M. Kini, The intriguing world of prothrombin activators from snake venom, Toxicon 45 (2005) 1133–1145.

[32] C. Lister, K. Arbuckle, T.N. Jackson, J. Debono, C.N. Zdenek, D. Dashevsky, N. Dunstan, L. Allen, C. Hay, B. Bush, Catch a tiger snake by its tail: differential toxicity, co-factor dependence and antivenom efficacy in a procoagulant clade of Australian venomous snakes, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 202 (2017) 39–54.

[33] R.M. Kini, V.S. Rao, J.S. Joseph, Procoagulant proteins from snake venoms, Haemostasis 31 (2001) 218–224.

[34] L.A. Bourke, C.N. Zdenek, E. Neri-Castro, M. Benard-Valle, A. Alagon, J. M. Gutierrez, E.F. Sanchez, M. Aldridge, B.G. Fry, Pan-american lancehead pitvipers: coagulotoxic venom effects and antivenom neutralisation of *Bothrops asper* and *B. atrox* geographical variants, Toxins (2021) 13.

[35] M.A. O'Leary, G.K. Isbister, A turbidimetric assay for the measurement of clotting times of procoagulant venoms in plasma, J. Pharmacol. Toxicol. Methods 61 (2010) 27–31.

[36] S. Soopairin, C. Patikorn, S. Taychakhoonavudh, Preclinical testing of expired antivenoms and its uses in real-world experience: a systematic review, Emerg. Med. J. (2024) emermed-2023-213707.

[37] G. Solano, S. Cunningham, R.J. Edge, G. Duran, A. Sanchez, M. Villalta, R. H. Clare, M.C. Wilkinson, A.E. Marriott, C. Abada, S.K. Menzies, M. Keen, D. G. Lalloo, Y. Stienstra, M. Abouyannis, N.R. Casewell, G. León, S. Ainsworth, African polyvalent antivenom can maintain pharmacological stability and ability to neutralise murine venom lethality for decades post-expiry: evidence for increasing antivenom shelf life to aid in alleviating chronic shortages, BMJ Glob. Health 9 (2024).

[38] L.A. Bourke, C.N. Zdenek, T.M. Huynh, W.C. Hodgson, A. Alagon, E.N. Castro, J. Jones, B.G. Fry, Fangs and foliage: unearthing the haemotoxic secrets of cannabis-dwelling rattlesnakes, Toxicon 244 (2024) 107756.

[39] C.N. Zdenek, N.J. Youngman, C. Hay, J. Dobson, N. Dunstan, L. Allen, L. Milanovic, B.G. Fry, Anticoagulant activity of Black snake (elapidae: *pseudechis*) venoms: potency, mechanisms, and antivenom efficacy, Comp. Biochem. Physiol. C Toxicol. Pharmacol. (320) (2020) S0378–S4274, 30147-30148. doi: 30110.31016/j.toxlet.32020.30105.30014.

[40] K. Katoh, D.M. Standley, MAFFT multiple sequence alignment software version 7: improvements in performance and usability, Mol. Biol. Evol. 30 (2013) 772–780.

[41] A. Larsson, AliView: a fast and lightweight alignment viewer and editor for large datasets, Bioinformatics 30 (2014) 3276–3278.

[42] A. Rambaut, A.J. Drummond, D. Xie, G. Baele, M.A. Suchard, Posterior summarization in Bayesian phylogenetics using tracer 1.7, Syst. Biol. 67 (2018) 901–904.

[43] R Core Team, R, A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2024.

[44] R.J. Hijmans, Geodata: Download Geographic Data, 2024.

[45] M. Tennekes, Tmap: Thematic Maps, 2023.

[46] E. Pebesma, Sf: Simple Features for R, 2024.

[47] H. Wickham, Dplyr: a Grammar of Data Manipulation, 2023.

[48] R.M. Kini, H.J. Evans, A model to explain the pharmacological effects of snake venom phospholipases A2, Toxicon 27 (1989) 613–635.

[49] D. Dashevsky, M. Benard-Valle, E. Neri-Castro, N.J. Youngman, C.N. Zdenek, A. Alagon, J.A. Portes-Junior, N. Frank, B.G. Fry, Anticoagulant micrurus venoms: targets and neutralization, Toxicol. Lett. 337 (2021) 91–97.

[50] U. Chandrasekara, A. Chowdhury, L. Seneci, C.N. Zdenek, N. Dunstan, B.G. Fry, From venom to vein: factor VII activation as a major pathophysiological target for procoagulant Australian elapid snake venoms, Toxins 16 (2024) 430.

[51] C.N. Zdenek, B.O. den Brouw, D. Dashevsky, A. Gloria, N. Youngman, E. Watson, P. Green, C. Hay, N. Dunstan, L. Allen, B.G. Fry, Clinical implications of convergent procoagulant toxicity and differential antivenom efficacy in Australian elapid snake venoms, Toxicol. Lett. 316 (2019) 171–182.

[52] C.N. Zdenek, C. Hay, K. Arbuckle, T.N.W. Jackson, M.H.A. Bos, B. Op den Brouw, J. Debono, L. Allen, N. Dunstan, T. Morley, M. Herrera, J.M. Gutierrez, D. J. Williams, B.G. Fry, Coagulotoxic effects by brown snake (pseudonaja) and taipan (oxyuranus) venoms, and the efficacy of a new antivenom, Toxicol. Vitro 58 (2019) 97–109.

[53] J. Rosing, J.W. Govers-Riemslag, L. Yukelson, G. Tans, Factor V activation and inactivation by venom proteases, Haemostasis 31 (2001) 241–246.

[54] E. Siigur, M. Samel, K. Tonismagi, J. Subbi, T. Reintamm, J. Siigur, Isolation, properties and N-terminal amino acid sequence of a factor V activator from *vipera Lebetina* (levantine viper) snake venom, Biochim. Biophys. Acta 1429 (1998) 239–248.

[55] C.R. Prentice, O.D. Ratnoff, The action of Russell's viper venom on factor V and the prothrombin-converting principle, Br. J. Haematol. 16 (1969) 291–302.

[56] G.K. Isbister, K. Maduwage, F.E. Scorgie, S. Shahmy, F. Mohamed, C. Abeysinghe, H. Karunathilake, M.A. O'Leary, C.A. Gnanathasan, L.F. Lincz, Venom concentrations and clotting factor levels in a prospective cohort of Russell's viper bites with coagulopathy, PLoS Neglected Trop. Dis. 9 (2015) e0003968.

[57] G. Tans, J. Rosing, Snake venom activators of factor X: an overview, Haemostasis 31 (2001) 225–233.

[58] D. Yamada, F. Sekiya, T. Morita, Prothrombin and factor X activator activities in the venoms of viperidae snakes, Toxicon 35 (1997) 1581–1589.

[59] G.K. Isbister, Snakebite doesn't cause disseminated intravascular coagulation: coagulopathy and thrombotic microangiopathy in snake envenoming, Semin. Thromb. Hemost. 36 (2010) 444–451.

[60] A. Barker, L. Jones, L.A. Bourke, L. Seneci, A. Chowdhury, A. Violette, R. Fourmy, R. Soria, M. Aldridge, B.G. Fry, Snake venom makeover: age-Dependent variations in procoagulant biochemistry of Egyptian saw-scaled viper (*Echis pyramidum pyramidum*) venom, Toxins 17 (2025).

[61] K. Kempson, A. Chowdhury, A. Violette, R. Fourmy, R. Soria, B.G. Fry, Age is just a number: ontogenetic conservation in activation of blood clotting factors VII, X, and XII by caucasus blunt-nosed viper (*macrovipera lebetina obtusa*) venoms, Toxins 16 (2024).

[62] Z. Qiao, L. Jones, L.A. Bourke, L. Seneci, A. Chowdhury, A. Violette, R. Fourmy, R. Soria, M. Aldridge, B.G. Fry, Tiny but mighty: *Vipera Ammodytes meridionalis* (eastern long-nosed viper) ontogenetic venom variations in procoagulant potency and the impact on antivenom efficacies, Toxins 16 (2024).

[63] A. Chowdhury, M.R. Lewin, R.W. Carter, N.R. Casewell, B.G. Fry, Keel venom: *rhabdophis subminiatus* (Red-Necked keelback) venom pathophysiologically affects diverse blood clotting pathways, Toxicon 218 (2022) 19–24.

[64] B. Xie, D. Dashevsky, D. Rokyta, P. Ghezellou, B. Fathinia, Q. Shi, M. K. Richardson, B.G. Fry, Dynamic genetic differentiation drives the widespread structural and functional convergent evolution of snake venom proteinaceous toxins, BMC Biol. 20 (2022) 4.

[65] J. Dobson, A. Chowdhury, A.P.J. Tai, H. van der Ploeg, A. Gillett, B.G. Fry, The clot thickens: differential coagulotoxic and cardiotoxic activities of anguimorpha lizard venoms, Toxins 16 (2024).

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

[67] P.A. Carrasco, C. Koch, F.G. Grazziotin, P.J. Venegas, J.C. Chaparro, G.J. Scrocchi, D. Salazar-Valenzuela, G.C. Leynaud, C.I. Mattoni, Total-evidence phylogeny and evolutionary morphology of new world pitvipers (serpentes: viperidae: crotalinae), Cladistics 39 (2023) 71–100.