Comparative Biochemistry and Physiology, Part C 256 (2022) 109326

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Differential coagulotoxic and neurotoxic venom activity from species of the arboreal viperid snake genus *Bothriechis* (palm-pitvipers)

Nicholas J. Youngman [a,*], Yin-Hsuan Peng [a], Richard J. Harris [a], Lee Jones [a], Joshua Llinas [b], Mark Haworth [c], Amber Gillett [d], Bryan G. Fry [a,*]

[a] Venom Evolution Lab, School of Biological Sciences, University of Queensland, St Lucia, QLD 4072, Australia
[b] Unusual Pet Vets, Jindalee, QLD 4074, Australia
[c] School of Veterinary Science, The University of Queensland, Gatton, QLD 4343, Australia
[d] FaunaVet Wildlife Consultancy, Glass House Mountains, QLD 4518, Australia

ARTICLE INFO

Edited by Martin Grosell

Keywords:
Anticoagulant
Bothriechis
Coagulation
Fibrinogen
Neurotoxicity

ABSTRACT

The viperid snake genus *Bothriechis* consists of eleven species distributed among Central and South America, living across low and high-altitude habitats. Despite *Bothriechis* envenomations being prominent across the Central and South American region, the functional effects of *Bothriechis* venoms are poorly understood. Thus, the aim of this study was to investigate the coagulotoxic and neurotoxic activities of *Bothriechis* venoms to fill this knowledge gap. Coagulotoxic investigations revealed *Bothriechis nigroviridis* and *B. schlegelii* to have pseudo-procoagulant venom activity, forming weak clots that rapidly break down, thereby depleting fibrinogen levels and thus contributing to a net anticoagulant state. While one sample of *B. lateralis* also showed weaker pseudo-procoagulant activity, directly clotting fibrinogen, two samples of *B. lateralis* venom were anticoagulant through the inhibition of thrombin and factor Xa activity. Differential efficacy of PoliVal-ICP antivenom was also observed, with the pseudo-procoagulant effect of *B. nigroviridis* venom poorly neutralised, despite this same activity in the venom of *B. schlegelii* being effectively neutralised. Significant specificity of these fibrinogen cleaving toxins was also observed, with no activity upon model amphibian, avian, lizard or rodent plasma observed. However, upon avian plasma the venom of *B. nigroviridis* exerted a complete anticoagulant effect, in contrast to the pseudo-procoagulant effect seen on human plasma. Neurotoxic investigations revealed *B. schlegelii* to be unique among the genus in having potent binding to the orthosteric site of the alpha-1 postsynaptic nicotinic acetylcholine receptor (with *B. lateralis* having a weaker but still discernible effect). This represents the first identification of postsynaptic nAChR neurotoxic activity for *Bothriechis*. In conclusion this study identifies notable differential activity within the coagulotoxic and postsynaptic neurotoxic activity of *Bothriechis* venoms, supporting previous research, and highlights the need for further studies with respect to antivenom efficacy as well as coagulotoxin specificity for *Bothriechis* venoms.

1. Introduction

Envenomation from snakebites is regarded as one of the most neglected diseases in tropical and subtropical countries in the world, with poor documentation and healthcare systems within these regions further underestimating as well exacerbating the situation. In Central and South America there is approximately 150,000 reported cases of snakebite per year, with 'bothropic envenomation' diagnosed clinically in many cases (Gutiérrez et al., 2011; Kasturiratne et al., 2008). Although a signification proportion of these 'bothropic envenomation' cases are indeed caused by species classified within the genus *Bothrops*, species from other genera such as *Bothriechis* have similar clinical effects of envenoming and are often mistaken as *Bothrops* species.

Eleven species are currently recognized within the monophyletic genus *Bothriechis* (*B. aurifer*, *B. bicolor*, *B. guifarroi*, *B. lateralis*, *B. marchi*, *B. nigroviridis*, *B. nubestris*, *B. rowleyi*, *B. schlegelii*, *B. supraciliaris*, and *B. thalassinus*), ranging from southern Mexico to northern South America (Campbell and Lamar, 2004; Doan et al., 2016; Townsend et al., 2013). Commonly known as palm-pitvipers, they are slender, arboreal, ambush predators. Adult individuals usually grow to less than 80 cm in size,

* Corresponding authors.
E-mail addresses: nicholas.youngman@uqconnect.edu.au (N.J. Youngman), bgfry@uq.edu.au (B.G. Fry).

https://doi.org/10.1016/j.cbpc.2022.109326
Received 19 October 2021; Received in revised form 21 February 2022; Accepted 1 March 2022
Available online 4 March 2022

although *B. aurifer*, *B. bicolor* and *B. lateralis* are known to grow to over 1 m in length (Campbell and Lamar, 2004). Altitude is relatively correlated with their activity period, with species found below 1000 m altitude having a tendency to be nocturnal while species found above 1500 m altitude being diurnal (Campbell and Lamar, 2004). Detailed dietary studies for the majority of species are lacking, however most species are known to be generalists which predate upon a wide range of small vertebrates including amphibians, birds, lizards and mammals (Campbell and Lamar, 2004; Sorrell, 2009). As is typical for other arboreal ambush predators, *Bothriechis* spp. will hold on to their prey while envenomation occurs to avoid losing the prey (Campbell, 1992; Pla et al., 2017; Sorrell, 2009). As such they must feed on smaller, less dangerous prey than is typical of ground dwelling species which can use the 'bite and release' method of envenomation.

Detailed functional investigations into venom activity of *Bothriechis* spp. are also lacking. Clincially, *Bothriechis* envenoming is commonly known to cause a range of local and systematic effects including edema, defibrination, necrosis, haemorrhage, hematuria and hypovolemic shock (Otero et al., 2002). Geographical variation in activity has been shown for *B. schlegelii* venom, including significant variation in different localities ability to induce haemorrhage (Kuch et al., 1996; Prezotto-Neto et al., 2016). Proteomic analysis has also identified major variations between species (Debono et al., 2016), including *B. lateralis* and *B. schlegelii* showing approximately less than 10% similarity in venom proteins (Lomonte et al., 2008). Similarly, although snake venom metalloproteases (SVMP's) are recovered as a major toxin family present in *B. lateralis* and *B. schlegelii* venom, *B. nigroviridis* lacks any detectable SVMP's (Fernández et al., 2010; Lomonte et al., 2008; Pla et al., 2017). Instead, *B. nigroviridis* venom composition is dominated by a high proportion of crotoxin-like PLA_2, named nigroviriditoxin, as well as vasoactive peptides (Fernández et al., 2010). Crotoxin is a heterodimeric β-neurotoxic PLA_2 that targets the presynaptic junction hindering the release of the endogenous acetylcholine (ACh). Crotoxin was first isolated from the South American rattlesnake, *Crotalus durissus terrificus* (Bon et al., 1989; Sampaio et al., 2010). Some North American rattlesnake venoms have been shown to contain a crotoxin homolog, Mojave toxin (French et al., 2004; John et al., 1994).

Postsynaptic neurotoxicity has been documented only in limited amounts across all viper venoms. Some Old-World viper species, particularly in the genera *Bitis* and *Vipera* have been reported to have venom which contains postsynaptic neurotoxic PLA_2s (Fernandez et al., 2014; Lee et al., 1982; Youngman et al., 2021b). However, the extent to which postsynaptic neurotoxicity was prevalent across *Bitis* went unnoticed until Youngman et al. (2021b) took a genus wide approach in testing crude venoms across postsynaptic nicotinic acetylcholine receptor (nAChR) mimotopes (Youngman et al., 2021b). Within pit vipers, *Calloselasma rhodostoma* and *Tropidolaemus* have been documented to-date as having postsynaptic neurotoxicity (Harris et al., 2020a).

With the exception of rattlesnakes (*Crotalus* and *Sistrurus*), *Mixcoatlus melanurus* (Neri-Castro et al., 2020) and *Ophryacus sphenophrys* (Neri-Castro et al., 2019), neurotoxic molecules such as crotoxin-like toxins seem to be rare across New-World vipers, having only been found in *B. nigroviridis* among *Bothriechis* spp. (Lomonte et al., 2015). However, most venoms from *Bothriechis* species have never been assessed for neurotoxicity, only proteomic venom comparisons for crotoxin-like PLA_2s have been attempted. Thus, the lack of presynaptic neurotoxins within the proteome does not indicate a complete absence of neurotoxicity since postsynaptic toxins may also be prevalent as revealed with *Bitis* (Youngman et al., 2021b).

To address the fundamental knowledge gaps in the literature, our study aimed to investigate the functional activities of five species of *Bothriechis* (*B. aurifer*, *B. lateralis*, *B. marchi*, *B. nigroviridis*, and *B. schlegelii*) upon the vertebrate coagulation cascade using a series of investigative clotting assays. We also aimed to determine the potential postsynaptic neurotoxicity of the venoms for each of the five species by investigating their binding affinity towards the loop-C region of the orthosteric site (ACh binding region) of postsynaptic α-1 nAChRs utilising a range of taxon-specific mimotopes on a biolayer interferometry assay (BLI).

2. Methods and materials

2.1. Venoms and reagents

All venom work was undertaken under the authority of UQ Biosafety Committee approval #IBC134BSBS201 and University of Queensland Animal Ethics approval 2021/AE000075. Venom samples from captive *B. aurifer*, *B. lateralis* (three samples), *B. marchi*, *B. nigroviridis*, and *B. schlegelii* were sourced from the long-term cryogenic collection of the Venom Evolution Laboratory. Except for *B. schlegelii* venom which was known to be from Costa Rica founder stock, the locality information for all other venoms in this study was unknown. Sex information was unknown except for *B. lateralis* sample two was from a female and *B. lateralis* sample three was from a male. The *Crotalus horridus* venom sample was a pooled sample sourced from the long-term cryogenic collection of the Venom Evolution Laboratory. All venom samples were lyophilized and stored at −80 °C. For experiments, lyophilized samples were redissolved with 1:1 (double deionised) ddH_2O:glycerol to make up 1 mg/mL concentration working stocks and stored at −20 °C for the duration of the study. To prevent protein degradation, glycerol was used to inhibit enzymes autocatalysis. Bovine Factor Xa (Stago catalog # 00311), phospholipid (Stago catalog # 00597), kaolin (Stago catalog # 00597), Owren Koller buffer (Stago catalog # 00360), CaCl2 (Stago catalog # 00367) and thrombin (Stago catalog # 00611) were supplied by Stago. Factor XI (Haemonetics Technologies Incorporated (HTI) cat. #HCXIA-0160) and Factor IX (HTI cat. # HCIXA-0050) were supplied by Haemonetics Technologies Incorporated.

Human plasma was supplied by the Red Cross Blood Service (Research Supply Agreement 18-03QLD-09), pooled and stored at −80 °C. *Rhinella marina* (cane toad) plasma was a pooled sample from thirty-five wild caught individuals. *Gallus gallus* (domestic chicken) plasma was pooled from nine individuals from the University of Queensland Gatton Campus chicken farm. *Tiliqua scincoides* (blue tongue skink) plasma was a pooled sample collected from nineteen captive individuals. *Rattus norvegicus* (laboratory rat) plasma was supplied by Animal Resources Centre Western Australia and was a pooled sample from twenty-five individuals. All plasma work was conducted under University of Queensland Biosafety Committee Approval #IBC/149B/SBS/2016 and Animal Ethics Committee Approval #2020/AE000324.

2.2. Coagulation assays

Coagulation assays were conducted on a Stago STA-R Max hemostasis analyser in accordance with previously published protocols (Bittenbinder et al., 2018; Youngman et al., 2019). Pooled frozen human plasma was thawed and warmed to 37 °C in a water bath for 5 min. Venom from a 1 mg/mL glycerol stock solution was diluted with Owren Koller buffer (isotonic saline) to 100 μg/mL. A total 50 μL of diluted venom sample was added by the STA-R Max to 50 μL $CaCl_2$, 50 μL phospholipid and 25 μL Owren Koller buffer, then incubated for 120 s at 37 °C to mix completely before adding 75 μL human plasma. The final concentration of testing venom sample was 20 μg/mL. Clotting time was measured immediately after the addition of the human plasma. Calcium and phospholipid were added to replicate the *in vivo* conditions present in the human body. All experiments at each concentration points were performed in triplicate. Controls were tested with a mixture of 1:1 Owren Koller buffer:glycerol in place of venom sample to represent spontaneous clotting time of healthy human plasma. For any species which showed significant activity directly upon fibrinogen by clotting fibrinogen, dilution assays using an 8-point concentration curve were carried out with and without 5% concentration of Central American polyspecific antivenom (PoliVal-ICP)® (Lot Number 5720416, Instituto

Clodomiro Picado, Universidad de Costa Rica) diluted within 25 μL Owren Koller buffer (the final concentration of antivenom was 0.5% as this was a 1/10 reaction test). In total a series of 8 venom dilutions (1:400, 1:160, 1:80, 1:30, 1: 12, 1:5, 1:2, 1:1) with and without antivenom were conducted for 8-point concentration curves. To further investigate any anticoagulant activity displayed by the venoms, all species were tested upon a series of anticoagulant inhibition assays. Table 1 shows the detailed methodologies of each conducted coagulation assay and anticoagulant inhibition assay included in this study, conducted in accordance with previously published protocols.

Table 1
Coagulation and anticoagulant inhibition assays.

Human plasma clotting assay	Step 1: 50 μL 100 μg/mL venom (1 mg/mL 50% glycerol stock diluted with Owren Koller buffer (Stago catalog #00360)) + 50 μL 0.025 M $CaCl_2$ (Stago catalog # 00367) + 50 μL phospholipid (Stago catalog #00597) + 25 μL Owren Koller buffer. Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL human plasma.
Fibrinogen clotting assay	Step 1: 50 μL 100 μg/mL venom (1 mg/mL 50% glycerol stock diluted with Owren Koller buffer) + 50 μL 0.025 M $CaCl_2$ + 50 μL phospholipid +25 μL Owren Koller buffer. Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL 4 mg/mL fibrinogen.
Fibrinogen clotting assay (antivenom assay conditions)	Step 1: 50 μL 100 μg/mL venom (1 mg/mL 50% glycerol stock diluted with Owren Koller buffer) + 50 μL 0.025 M $CaCl_2$ + 50 μL phospholipid +25 μL of 5% concentration of PoliVal-ICP antivenom diluted with Owren Koller buffer. Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL 4 mg/mL fibrinogen.
Thrombin inhibition assay	Step 1: 25 μL 200 μg/mL venom (1 mg/mL 50% glycerol stock diluted with Owren Koller buffer) + 50 μL 0.025 M $CaCl_2$ + 50 μL phospholipid +25 μL Owren Koller buffer +25 μL thrombin. Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL 4 mg/mL fibrinogen.
Factor Xa inhibition assay	Step 1: 25 μL 200 μg/mL venom (1 mg/mL 50% glycerol stock diluted with Owren Koller buffer) + 50 μL 0.025 M $CaCl_2$ + 50 μL phospholipid +25 μL Owren Koller buffer +25 μL Factor Xa (Stago catalog #00311). Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL human plasma.
Prothrombinase complex inhibition assay	Step 1: 25 μL 200 μg/mL venom (1 mg/mL 50% glycerol stock diluted with Owren Koller buffer) + 50 μL 0.025 M $CaCl_2$ + 25 μL Owren Koller buffer +50 μL phospholipid +75 μL human plasma. Step 2: 120 s incubation at 37 °C. Step 3: Addition of 25 μL Factor Xa.
Factor IXa inhibition assay	Step 1: 25 μL 200 μg/mL venom (1 mg/mL 50% glycerol stock diluted with Owren Koller buffer) + 50 μL 0.025 M $CaCl_2$ + 50 μL phospholipid +25 μL Owren Koller buffer +25 μL Factor IXa (15 μg/mL) (HTI catalog #HCIXA-0050). Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL human plasma.
Factor XIa inhibition assay	Step 1: 25 μL 200 μg/mL venom (1 mg/mL 50% glycerol stock diluted with Owren Koller buffer) + 50 μL 0.025 M $CaCl_2$ + 50 μL phospholipid +25 μL Owren Koller buffer +25 μL Factor XIa (15 μg/mL) (HTI catalog #HCXIA-0160). Step 2: 120 s incubation at 37 °C. Step 3: Addition of 75 μL human plasma.

2.3. Thromboelastography assays

Thromboelastography assays were conducted in accordance with previously published protocols to determine the *in vitro* coagulotoxic activity of *Bothriechis* venoms upon the viscoelastic clotting properties of vertebrate plasma's (Grashof et al., 2020; Youngman et al., 2021a; Youngman et al., 2021c). For all assays, 7 μL of either venom (1 mg/mL) or control reagent was added to 72 μL calcium, 72 μL phospholipid, 20 μL Owren Koller buffer and 189 μL plasma. Final reaction concentration of venom is 19.44 μg/mL. To determine the natural activation of the clotting cascade via the intrinsic pathway for each species plasma, positive controls were conducted using 7 μL of Kaolin in replacement of venom for this assay. To determine the natural spontaneous clotting time of each species' plasma, controls were conducted using 7 μL of 50% glycerol and 50% ddH_2O in replacement of venom. The plasma of *R. marina*, *G. gallus*, *T. scincoides* and *R. norvegicus* were used as models for amphibian, avian, reptile and rodent plasma respectively for this study.

2.4. Biolayer interferometry

The biolayer interferometry (BLI) assay was performed on an Octet HTX system (ForteBio™, Fremont, CA, USA). All methodology followed previous research that developed this nAChR binding assay (Harris et al., 2020b; Youngman et al., 2021b; Zdenek et al., 2019). In brief, Streptavidin biosensors were hydrated in assay running buffer for 30–60 min and agitated at 2.0 revolutions per minute (RPM) on a shaker, prior to experimentation. Venom (analyte) samples were diluted to make a final experimental concentration of 50 μg/mL per well and mimotope aliquots were diluted to an experimental concentration of 1 μg/mL per well. 1× DPBS with 0.1% BSA and 0.05% Tween-20 was used for the assay running buffer. Analyte dissociation occurred using a standard acidic solution (glycine buffer), made up of 10 mM glycine (pH 1.5–1.7) in ddH2O. Raw data is provided in supplementary file 1. All experiments were conducted in triplicate across the mimotopes.

2.5. Statistical analysis

GraphPad Prism 9.0.2 (GraphPad Prism Inc., La Jolla, CA, USA) was utilized to analyse and visualise the data. Brown-Forsythe and Welch one-way ANOVA with Dunnett's multiple comparisons were conducted to determine statistical significance for the coagulation cascade factor inhibition assays. For the BLI assays, area under the curve (AUC) values were statistically analysed using a standard *t*-test comparing the values to the control. False-discovery rate (FDR) for all p-values of multiple comparison analysis were corrected using Two-stage step-up (Benjamini, Krieger, and Yekutieli). All raw data is available in Supplementary File 1.

3. Results

Upon human plasma, two samples of *B. lateralis* exhibited significant anticoagulant activity, preventing spontaneous clot formation (Fig. 1A). Minor anticoagulant activity was observed from the venom of *B. marchi* (Fig. 1A). Both *B. nigroviridis* and *B. schlegelii* showed weak coagulant activity upon plasma (Fig. 1A), which was further shown to be direct clotting activity upon fibrinogen (Fig. 1B). One sample of *B. lateralis* also showed coagulant activity upon fibrinogen (Fig. 1B).

Significant inhibition was observed in both the thrombin and FXa inhibition clotting assays for all three *B. lateralis* samples (Fig. 2). Significant delays in clotting time were also observed in the FXa inhibition assay for *B. aurifer*, *B. marchi* and *B. nigroviridis* venom (Fig. 2). No significant inhibition of the prothrombinase complex, FIXa or FXIa was observed (Supplementary File 1). Assays investigating the efficacy of PoliVal-ICP antivenom demonstrated the antivenom had a significant effect at neutralising the fibrinogen clotting pseudo-procoagulant

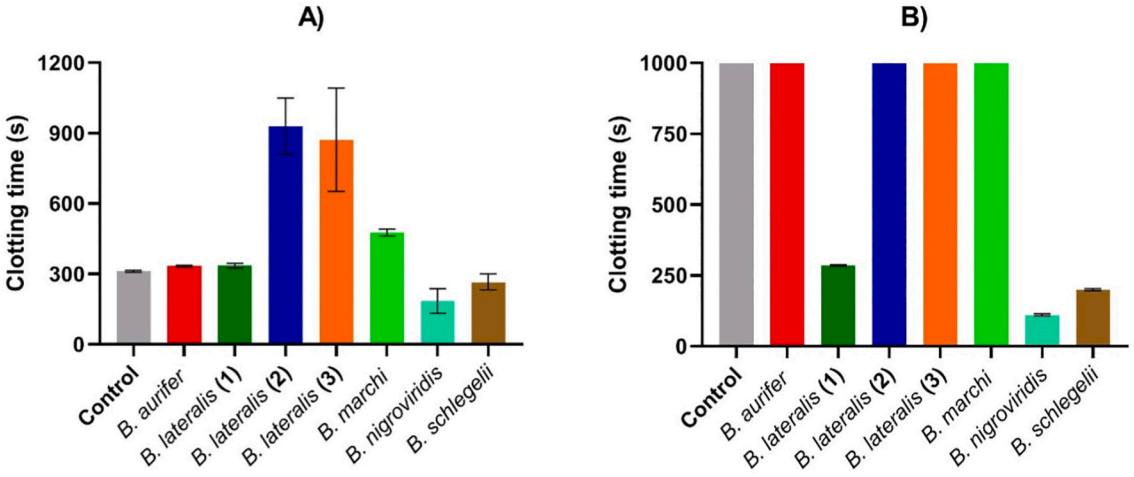

Fig. 1. Coagulant activities of *Bothriechis* venoms upon A) human plasma and B) human fibrinogen. Data represents $n = 3$ mean and standard deviation (SD). Note: for most data points the error bars are smaller than the line.

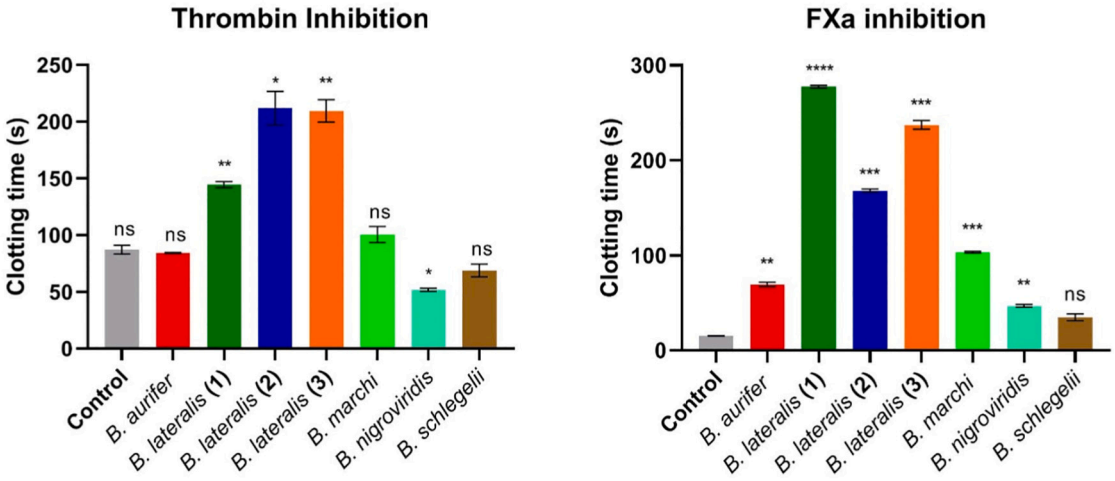

Fig. 2. Thrombin and FXa coagulation cascade factor inhibition assays showing inhibitory effect of *Bothriechis* venoms. Statistical significance shown represents significance relative to the control using Brown-Forsythe and Welch One-Way ANOVA with Dunnett's multiple comparisons. Data represents $n = 3$ mean and SD. A statistical significance is annotated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$) above the corresponding bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

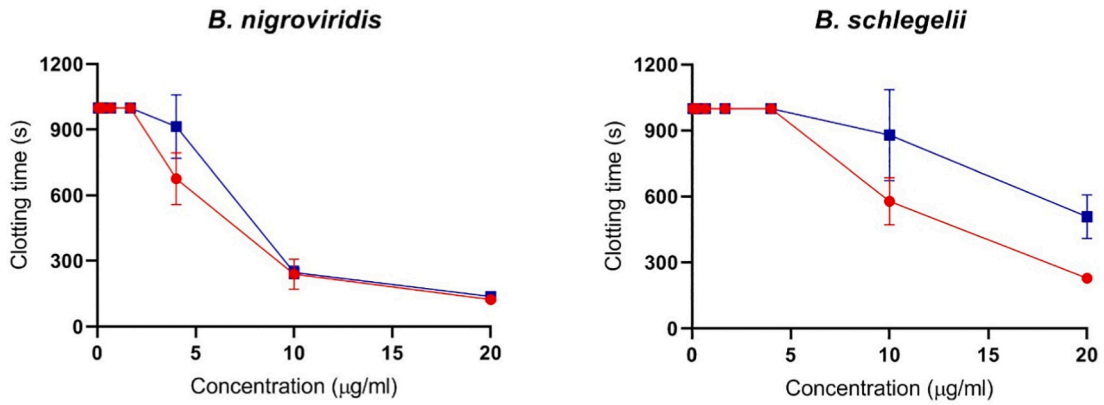

Fig. 3. *Bothriechis nigroviridis* and *B. schlegelii* venom concentration-response curves for fibrinogen clotting activity and PoliVal-ICP antivenom efficacy. Red lines represent venom assays and blue lines represent venom + PoliVal-ICP antivenom assays. Negative control values were 999 ± 0 s. Data points are $n = 3$ mean and SD. Note: for most data points the error bars are smaller than the line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activity of *B. schlegelii* venom, while comparatively possessed poor efficacy at neutralising the same activity in *B. nigroviridis* venom (Fig. 3). No coagulant activity was observed for either *B. nigroviridis* or *B. schlegelii* upon amphibian or lizard plasma (Fig. 4). Although the venom of *B. nigroviridis* directly acted on fibrinogen to rapidly form weak, transient fibrin clots in human plasma, the venom was anticoagulant on avian plasma while being inactive on rat plasma (Fig. 4).

Neurotoxicity tested upon the α-1 nAChR mimotopes of taxa representatives revealed that only *B. schlegelii* had any significantly observed binding in comparison to the control (Fig. 5). *Bothriechis lateralis* (3) showed some potential binding, however the AUC values were particularly low, with the binding curve not too different from the control. This suggests that there might be some very small proportions of nAChR binding toxins. There was no taxa specific binding observed with values varying minutely between taxa mimotopes.

4. Discussion

Significant variation in the coagulotoxic venom activity was observed both between and within species of *Bothriechis*. Of the five species we tested, only *B. lateralis* displayed strong anticoagulant activity upon human plasma (Fig. 1A). However, only *B. lateralis* samples two and three displayed this strong anticoagulant activity, with the other sample instead acting directly upon fibrinogen to produce weak, transient fibrin clots that would have a net anticoagulant effect through the depletion of fibrinogen levels (Fig. 1B). Further testing revealed inhibition of thrombin and FXa to be responsible for the anticoagulant activity of the *B. lateralis* venoms (Fig. 2). The minor delays in clotting time for the FXa inhibition assay caused by *B. aurifer*, *B. marchi* and *B. nigroviridis* venom may be indicative of proportionally low levels of FXa inhibiting toxins (Fig. 2). Unfortunately, due to the locality and age data being unknown for our three *B. lateralis* samples we cannot deduce if this was due to geographical or ontogenetic venom variation, which future studies should also endeavour to further investigate. This research however provides a platform upon which to build such future studies to investigate whether this variation is due to changes in ecological niche as the snakes change from neonate to adult, or if there is geographical variation in the venom biochemistry of this species.

Bothriechis nigroviridis and *B. schlegelii* showed significant coagulant activity upon human fibrinogen (Fig. 1). We identified this as a pseudo-procoagulant activity, a functional trait commonly seen in viperid venoms. Thromboelastography testing on human plasma confirmed this result, showing that although the venoms were not rapidly clotting human plasma, they are directly clotting fibrinogen to form weak clots indicative of a pseudo-procoagulant action, thereby reducing fibrinogen levels to ultimately contribute to a net anticoagulant state (Fig. 4). The regional antivenom PoliVal-ICP was tested to determine its efficacy at neutralising the fibrinogen clotting activity of both venoms, with stark differences observed. For *B. schlegelii* venom, PoliVal-ICP antivenom showed a strong ability to neutralise the fibrinogen clotting toxins within the venom (Fig. 3). However, PoliVal-ICP antivenom showed no ability to neutralise the fibrinogen clotting activity of *B. nigroviridis* venom at either the 20 μg/mL or 10 μg/mL concentrations in the assay (Fig. 3). This indicates that although the venoms are exerting the same functional activity, the toxins responsible vary significantly in their surface biochemistry at key sites bound by the antivenom. This is supported by previous research which has found the toxin composition of *Bothriechis* to be highly variable between species (Fernández et al., 2010; Pla et al., 2017). The functional activities observed in our study by both *B. nigroviridis* and *B. schlegelii* are also supported by previous research which has identified serine proteinases, known for possessing fibrinogen clotting activities, to be present in the venom of both species (Fernández

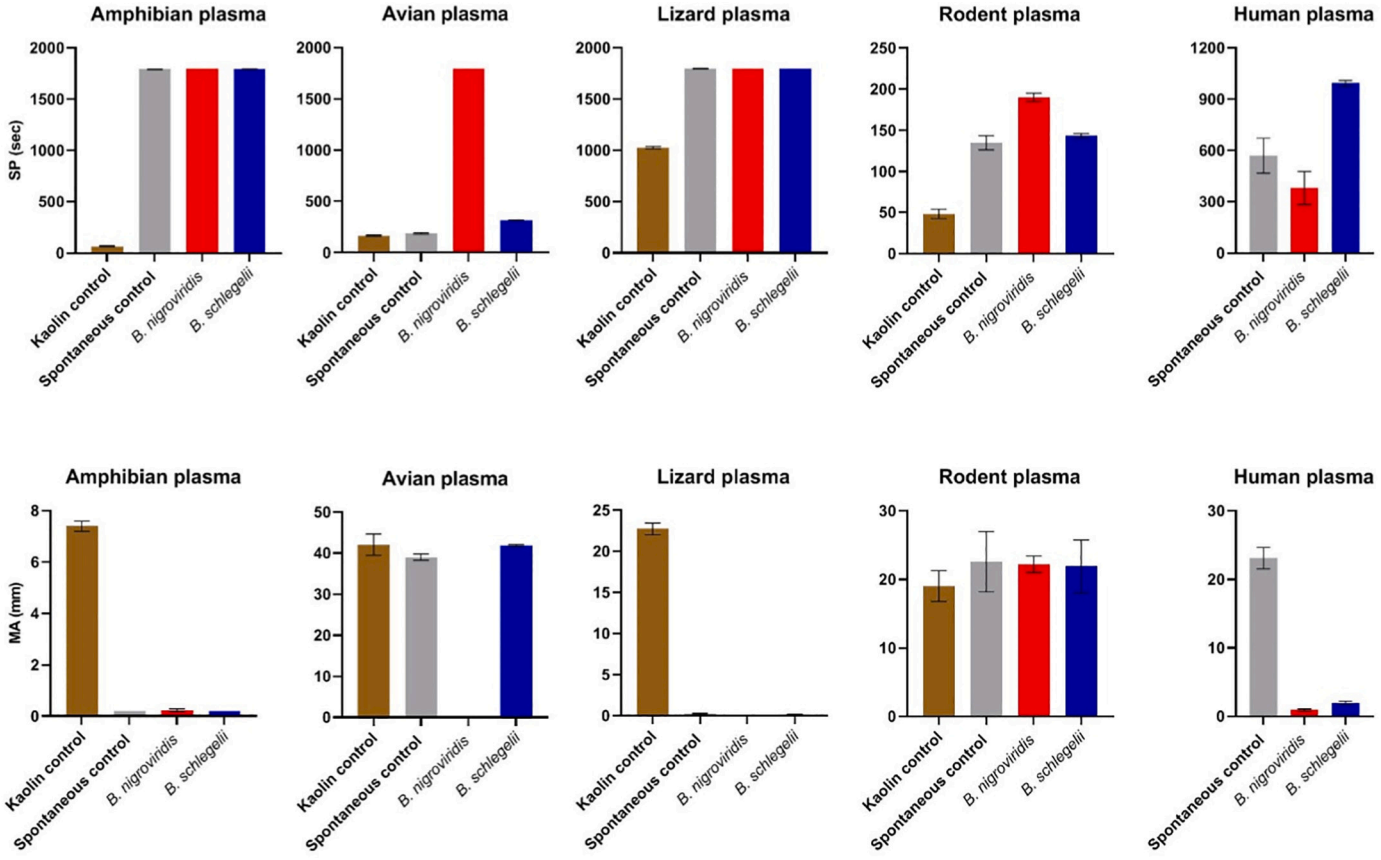

Fig. 4. Taxon-specific coagulotoxic activity of *B. nigroviridis* and *B. schlegelii* venom upon the clotting time of amphibian, avian, lizard, rodent and human plasma. Data points are $n = 3$ mean (except *B. schlegelii* upon lizard plasma is $n = 2$) and SD.

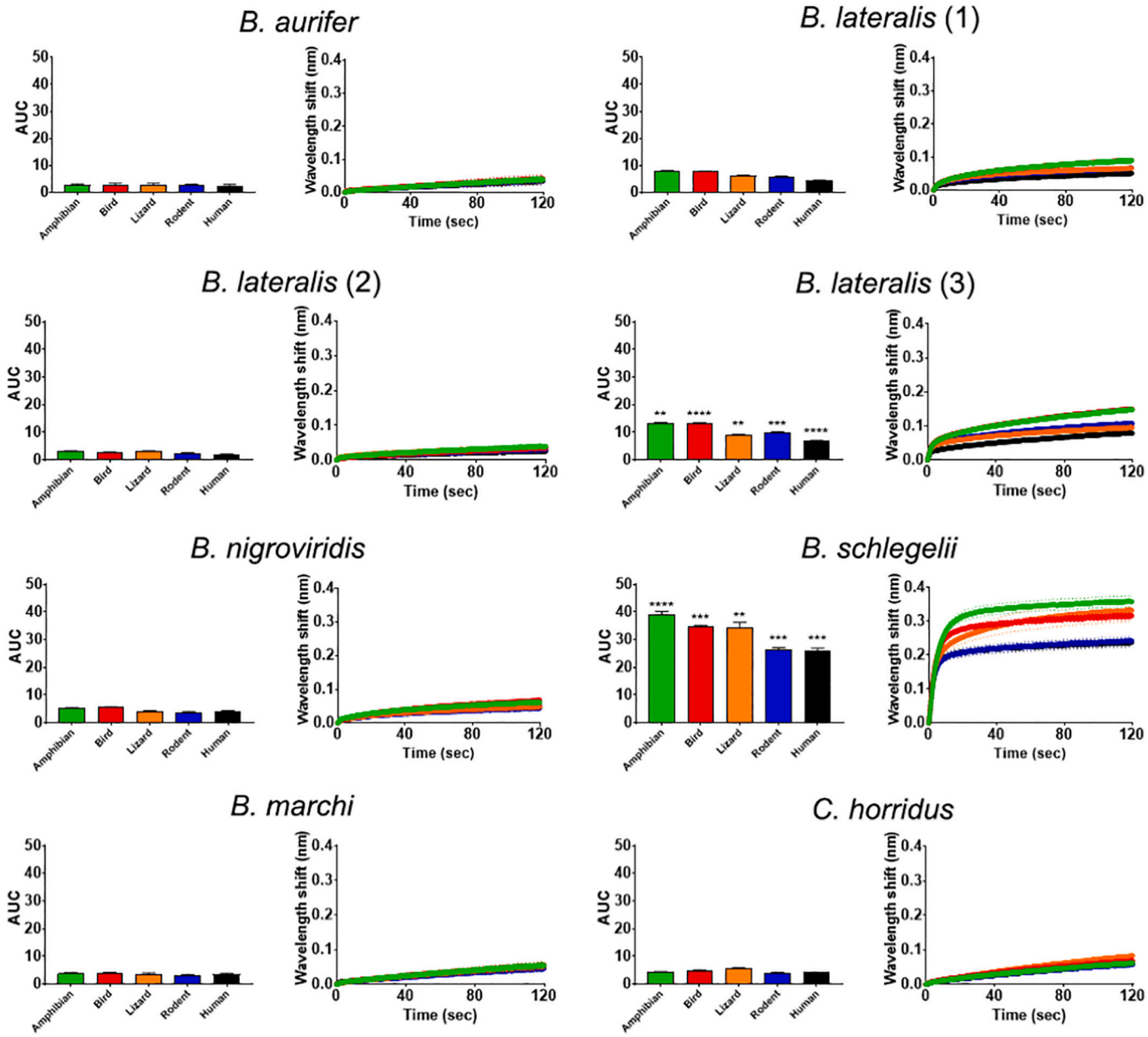

Fig. 5. The taxon-specific effects of crude venom from *Bothriehis aurifer*, *B. lateralis*, *B. nigroviridis*, *B. schlegelii*, *B. marchi* and the control *Crotalus horridus* against amphibian, bird, lizard, rodent and human representative α-1 nAChR mimotope sequences on the BLI assay. Bar graphs represent the mean AUC values of the adjacent curve graphs. Curve graphs show the mean wavelength (nm) shift in light associated with binding over 120 s. The venom was tested in triplicate ($n = 3$). Error bars on all graphs represent the standard error of the mean (SEM) (they are represented by dots surrounding the lines on the curve graphs). AUC values were statistically analysed using a standard *t*-test comparing the values to the control. False-discovery rate (FDR) for all p-values of multiple comparison analysis were corrected using Two-stage step-up (Benjamini, Krieger, and Yekutieli). A statistical significance is annotated by ** ($p < 0.05$), *** ($p < 0.01$) and **** ($p < 0.001$) above the corresponding bar.

et al., 2010; Pla et al., 2017).

Variability in coagulotoxic activity of *B. nigroviridis* and *B. schlegelii* venom was also observed upon plasma from different vertebrate model representatives of model prey types. Both species were chosen to investigate their clotting abilities upon a selection of vertebrate plasma's due to the clotting ability observed upon human plasma. However, upon amphibian and lizard plasma neither species venom showed any coagulant activity (Fig. 4). On avian plasma *B. schlegelii* showed a very slight increase in time until clot formation, while *B. nigroviridis* completely inhibited clotting of avian plasma (Fig. 4). This indicates that the fibrinogen cleaving toxins responsible for the observed clotting upon human plasma have no specificity towards avian fibrinogen. Furthermore, *B. nigroviridis* possesses toxins which are inhibiting coagulation factors within the avian coagulation cascade to cause an anticoagulant effect. This is further supported by the minor FXa inhibition observed on human plasma (Fig. 2), however further work is needed to determine if *B. nigroviridis* venom possess additional anticoagulant toxins with specificity towards avian plasma, as these results tentatively indicate. These results indicate that the fibrinogen clotting toxins have specificity towards mammalian fibrinogen, however the venom of both *B. nigroviridis* and *B. schlegelii* did not have similar effects upon rodent plasma in comparison to human plasma (Fig. 4). These incongruous results between rodent and human plasma raise further questions about the specificity of the fibrinogen clotting toxins present within *Bothriechis* and warrant future investigation. It is important to note, that the plasma was used from locally available species as models for amphibian, lizard,

avian and rodent plasma and thus are not true representatives of natural prey. Due to the variability of factors within the coagulation cascade, including that of fibrinogen, future testing using known prey species of *Bothriechis* to reconstruct evolutionary selection pressures would be preferential.

Postsynaptic targeting of nAChR mimotopes was revealed in two species, *B. schlegelii* and one location of *B. lateralis* (Fig. 5). For both of these species, there was no prey specific targeting of the venom towards any taxa-specific representative mimotopes tested. This data is previously supported by prey-specific venom being mediated by dietary breadth (Harris et al., 2020b; Lyons et al., 2020) since dietary data for *Bothriechis* spp. reveal a range of prey types such as amphibians, bird, lizards and mammals (Campbell and Lamar, 2004; Sorrell, 2009). Although *B. schlegelii* venom showed significant binding, the binding of *B. lateralis* (3) venom was relatively low in comparison and differed marginally from the *C. horridus* control, albeit still statistically significant (Fig. 5). Considering that the other samples of *B. lateralis* showed no binding, we tentatively hypothesise that *B. lateralis* (3) has some very low proportions of postsynaptic nAChR targeting toxins and this low proportion is either due to a reduction in these toxins as they are no longer ecologically necessary or the beginnings of evolving and refining of these toxin types. Unfortunately, we are uncertain of where specifically these locations are and thus cannot infer any tentative hypotheses based on geographical niches. This data supports previous data showing how differential trophic strategies of *Bothriechis* spp. leads to differences in venom composition (Fernández et al., 2010).

Given the dynamicity of PLA_2 toxins in their neurotoxic targeting of both the pre- and postsynaptic junction and the fact that PLA_2 neurotoxins have been revealed across many viperid species (Sampaio et al., 2010; Su and Chang, 1984; Viljoen et al., 1982; Vulfius et al., 2011; Vulfius et al., 2014), it is highly likely that the PLA_2 postsynaptic toxins are utilized by *B. schlegelii* and *B. lateralis* (3). Proteomic data has revealed that both *B. schlegelii* and *B. lateralis* contain venom that are rich in PLA_2 toxins. This composition also changes heavily with ontogenetic shifts (Pla et al., 2017) and that the nAChR binding across *B. lateralis* might have been due to venom extraction from different ontogenetic stages since we have no further information on the age or sexual maturity of these venoms.

The lack of neurotoxic binding in some of the species tested also corroborates previous research. For example, the lack of nAChR binding in *B. nigroviridis* is expected since it has been revealed that all its PLA_2 toxins are presynaptic targeting with the crotoxin-like toxin nigroviriditoxin (Lomonte et al., 2015; Pla et al., 2017). Given that it is likely PLA_2 toxins that are causing the postsynaptic nAChR activity (Fig. 5), then any venom devoid of PLA_2s such as that of *B. aurifer* (Pla et al., 2017) would likely not elicit any nAChR activity, which was shown by the BLI assay (Fig. 5). Interestingly, *B. supraciliaris* is a sister species to *B. schlegelii* and has venom containing a large proportion of PLA_2 toxin types, similarly to *B. schlegelii*, thus it would be interesting if the venom from this species also contains postsynaptic nAChR binding toxins.

5. Conclusion

Our study identifies the first evidence of postsynaptic nAChR neurotoxicity for *Bothriechis* venoms as well as identifying significant variation in coagulotoxic venom activity across the five species included within the study. Future work should investigate additional species to determine how widespread postsynaptic neurotoxins are across *Bothriechis*. Notably we also identify the venoms of *B. nigroviridis* and *B. schlegelii* to have a pseudo-procoagulant function upon human plasma (by directly clotting fibrinogen), while having no pseudo-procoagulant effects upon amphibian, avian, lizard or rodent plasma. This indicates specificity within the fibrinogen cleaving toxins which warrants future investigation, in particular to determine at what level of specificity these toxins are acting upon mammalian plasma due to the functional difference observed upon the human and rodent plasma. Thrombin and FXa, inhibition are also distinguished to be responsible for the anticoagulant activity of *B. lateralis*. Overall, our data supports previous proteomic and functional studies conducted on *Bothriechis* venoms, and further expands our understanding of the functional complexity of venom from *Bothriechis* spp. which is critical in understanding the evolutionary ecology of these species as well as informing clinical management of envenomation.

Declaration of competing interest

The authors declare no conflict of interests.

Acknowledgements

This work was funded by ARC Discovery Project DP190100304. N.J.Y. and R.J.H were supported by PhD scholarships funded from the University of Queensland.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2022.109326.

References

Bittenbinder, M.A., Zdenek, C.N., Op Den Brouw, B., Youngman, N.J., Dobson, J.S., Naude, A., Vonk, F.J., Fry, B.G., 2018. Coagulotoxic cobras: clinical implications of strong anticoagulant actions of African spitting Naja venoms that are not neutralised by antivenom but are by LY315920 (Varespladib). Toxins 10.

Bon, C., Bouchier, C., Choumet, V., Faure, G., Jiang, M.S., Lambezat, M.P., Radvanyi, F., Saliou, B., 1989. Crotoxin, half-century of investigations on a phospholipase A2 neurotoxin. Acta Physiol. Pharmacol. Latinoam. 39, 439–448.

Campbell, J., 1992. The distribution, variation, and natural history of the Middle American montane pitviper,Porthidium godmani. In: Biology of the Pitvipers, pp. 223–250.

Campbell, J.A., Lamar, W.W., 2004. The Venomous Reptiles of the Western Hemisphere. Comstock Pub. Associates, Ithaca New York.

Debono, J., Cochran, C., Kuruppu, S., Nouwens, A., Rajapakse, N.W., Kawasaki, M., Wood, K., Dobson, J., Baumann, K., Jouiaei, M., Jackson, T.N.W., Koludarov, I., Low, D., Ali, S.A., Smith, A., Barnes, A., Fry, B.G., 2016. Canopy venom: Proteomic comparison among new world arboreal pit-viper venoms. Toxins 8 (7), 210. https://doi.org/10.3390/toxins8070210.

Doan, T.M., Mason, A.J., Castoe, T.A., Sasa, M., Parkinson, C.L., 2016. A cryptic palm-pitviper species (Squamata: Viperidae: Bothriechis) from the Costa Rican highlands, with notes on the variation within B. nigroviridis. Zootaxa 4138, 271–290.

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

Fernandez, S., Hodgson, W., Chaisakul, J., Kornhauser, R., Konstantakopoulos, N., Smith, A.I., Kuruppu, S., 2014. In vitro toxic effects of puff adder (Bitis arietans) venom, and their neutralization by antivenom. Toxins 6, 1586.

French, W.J., Hayes, W.K., Bush, S.P., Cardwell, M.D., Bader, J.O., Rael, E.D., 2004. Mojave toxin in venom of Crotalus helleri (Southern Pacific Rattlesnake): molecular and geographic characterization. Toxicon 44, 781–791.

Grashof, D., Zdenek, C.N., Dobson, J.S., Youngman, N.J., Coimbra, F., Benard-Valle, M., Alagon, A., Fry, B.G., 2020. A web of coagulotoxicity: failure of antivenom to neutralize the destructive (non-clotting) fibrinogenolytic activity of loxosceles and sicarius spider venoms. Toxins 12, 91.

Gutiérrez, J.M., León, G., Burnouf, T., 2011. Antivenoms for the treatment of snakebite envenomings: the road ahead. Biologicals 39, 129–142.

Harris, R.J., Zdenek, C.N., Debono, J., Harrich, D., Fry, B.G., 2020a. Evolutionary interpretations of nicotinic acetylcholine receptor targeting venom effects by a clade of asian viperidae Snakes. Neurotoxicity Reserch 38 (2), 312–318. https://doi.org/10.1007/s12640-020-00211-2.

Harris, R.J., Zdenek, C.N., Harrich, D., Frank, N., Fry, B.G., 2020b. An appetite for destruction: detecting prey-selective binding of α-neurotoxins in the venom of Afro-Asian Elapids. Toxins 12.

John, T.R., Smith, L.A., Kaiser, I.I., 1994. Genomic sequences encoding the acidic and basic subunits of Mojave toxin: unusually high sequence identity of non-coding regions. Gene 139, 229–234.

Kasturiratne, A., Wickremasinghe, A.R., de Silva, N., Gunawardena, N.K., Pathmeswaran, A., Premaratna, R., Savioli, L., Lalloo, D.G., de Silva, H.J., 2008. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths (Global Burden of Snakebite). PLoS Med. 5, e218.

Kuch, U., Mebs, D., Gutiérrez, J.M., Freire, A., 1996. Biochemical and biological characterization of Ecuadorian pitviper venoms (genera Bothriechis, Bothriopsis, Bothrops and Lachesis). Toxicon 34, 714–717.

Lee, C.Y., Ho, C.L., Botes, D.P., 1982. Site of action of caudoxin, a neurotoxic phospholipase A2 from the horned puff adder (Bitis caudalis) venom. Toxicon 20, 637–647.

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., Mora-Obando, D., Fernández, J., Sanz, L., Pla, D., Gutiérrez, J.M., Calvete, J.J., 2015. First crotoxin-like phospholipase A2 complex from a New World non-rattlesnake species: nigroviriditoxin, from the arboreal Neotropical snake Bothriechis nigroviridis. Toxicon 93, 144–154.

Lyons, K., Dugon, M.M., Healy, K., 2020. Diet breadth dediates the prey specificity of venom potency in snakes. Toxins 12.

Neri-Castro, E., Lomonte, B., Valdés, M., Ponce-López, R., Bénard-Valle, M., Borja, M., Strickland, J.L., Jones, J.M., Grünwald, C., Zamudio, F.J.J.O.P., 2019. In: Venom Characterization of the Three Species of Ophryacus and Proteomic Profiling of O. sphenophrys Unveils Sphenotoxin, a Novel Crotoxin-like Heterodimeric β-Neurotoxin, 192, pp. 196–207.

Neri-Castro, E., Sanz, L., Olvera-Rodríguez, A., Bénard-Valle, M., Alagón, A., Calvete, J.J. J.J.O.P., 2020. In: Venomics and Biochemical Analysis of the Black-tailed Horned Pitviper, Mixcoatlus melanurus, and Characterization of Melanurutoxin, a Novel Crotoxin Homolog, 225, p. 103865.

Otero, R., Gutiérrez, J., Mesa, M.A.B., Duque, E., Arango, J.L., Gómez, F., Toro, A., Cano, F., Rodríguez, O., Rodríguez, L.M.A., 2002. Complications of Bothrops, Porthidium, and Bothriechis snakebites in Colombia. A clinical and epidemiological study of 39 cases attended in a university hospital. Toxicon 40, 1107–1114.

Pla, D., Sanz, L., Sasa, M., Acevedo, M.E., Dwyer, Q., Durban, J., Pérez, A., Rodriguez, Y., Lomonte, B., Calvete, J.J., 2017. Proteomic analysis of venom variability and ontogeny across the arboreal palm-pitvipers (genus Bothriechis). J. Proteome 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. (Maywood) 241, 2075–2085.

Sampaio, S.C., Hyslop, S., Fontes, M.R., Prado-Franceschi, J., Zambelli, V.O., Magro, A.J., Brigatte, P., Gutierrez, V.P., Cury, Y., 2010. Crotoxin: novel activities for a classic β-neurotoxin. Toxicon 55, 1045–1060.

Sorrell, G., 2009. Diel movement and predation activity patterns of the eyelash palm-pitviper (Bothriechis schlegelii). Copeia 2009, 105–109.

Su, M., Chang, C., 1984. Presynaptic effects of snake venom toxins which have phospholipase A2 activity (β-bungarotoxin, taipoxin, crotoxin). Toxicon 22, 631–640.

Townsend, J.H., Medina-Flores, M., Wilson, L.D., Jadin, R.C., Austin, J.D., 2013. A relict lineage and new species of green palm-pitviper (Squamata, Viperidae, Bothriechis) from the Chortís Highlands of Mesoamerica. ZooKeys 77.

Viljoen, C.C., Botes, D.P., Kruger, H., 1982. Isolation and amino acid sequence of caudoxin, a presynaptic acting toxic phospholipase A2 from the venom of the horned puff adder (Bitis caudalis). Toxicon 20, 715–737.

Vulfius, C.A., Gorbacheva, E.V., Starkov, V.G., Osipov, A.V., Kasheverov, I.E., Andreeva, T.V., Astashev, M.E., Tsetlin, V.I., Utkin, Y.N., 2011. An unusual phospholipase A2 from puff adder Bitis arietans venom – a novel blocker of nicotinic acetylcholine receptors. Toxicon 57, 787–793.

Vulfius, C.A., Kasheverov, I.E., Starkov, V.G., Osipov, A.V., Andreeva, T.V., Filkin, S.Y., Gorbacheva, E.V., Astashev, M.E., Tsetlin, V.I., Utkin, Y.N., Silman, I., 2014. Inhibition of nicotinic acetylcholine receptors, a novel facet in the pleiotropic activities of snake venom phospholipases A2. PLoS ONE 9.

Youngman, N.J., Debono, J., Dobson, J.S., Zdenek, C.N., Harris, R.J., Brouw, B.O.D., Coimbra, F.C.P., Naude, A., Coster, K., Sundman, E., Braun, R., Hendrikx, I., Fry, B. G., 2019. Venomous landmines: clinical implications of extreme coagulotoxic diversification and differential neutralization by antivenom of venoms within the Viperid snake genus Bitis. Toxins 11, 422.

Youngman, N.J., Chowdhury, A., Zdenek, C.N., Coster, K., Sundman, E., Braun, R., Fry, B. G., 2021a. Utilising venom activity to infer dietary composition of the Kenyan horned viper (Bitis worthingtoni). Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 240, 108921.

Youngman, N.J., Harris, R.J., Huynh, T.M., Coster, K., Sundman, E., Braun, R., Naude, A., Hodgson, W.C., Fry, B.G., 2021b. Widespread and differential neurotoxicity in venoms from the Bitis genus of viperid snakes. Neurotox. Res. 39, 697–704.

Youngman, N.J., Llinas, J., Fry, B.G., 2021c. Evidence for resistance to coagulotoxic effects of Australian elapid snake venoms by sympatric prey (blue tongue skinks) but not by predators (monitor lizards). Toxins 13, 590.

Zdenek, C.N., Harris, R.J., Kuruppu, S., Youngman, N.J., Dobson, J.S., Debono, J., Khan, M., Smith, I., Yarski, M., Harrich, D., Sweeney, C., Dunstan, N., Allen, L., Fry, B.G., 2019. A taxon-specific and high-throughput method for measuring ligand binding to nicotinic acetylcholine receptors. Toxins 11.