Comparative Biochemistry and Physiology, Part C 211 (2018) 7–14

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Does size matter? Venom proteomic and functional comparison between night adder species (Viperidae: *Causus*) with short and long venom glands

Francisco C.P. Coimbra[a], James Dobson[a], Christina N. Zdenek[a], Bianca op den Brouw[a], Brett Hamilton[b], Jordan Debono[a], Paul Masci[c], Nathaniel Frank[d], Lilin Ge[e,f], Hang Fai Kwok[e], Bryan G. Fry[a,*]

[a] *Venom Evolution Lab, School of Biological Sciences, University of Queensland, St Lucia, QLD 4072, Australia*
[b] *Centre for Advanced Imaging & Centre for Microscopy and Microanalysis, University of Queensland, St Lucia, QLD 4072, Australia*
[c] *Princess Alexandra Hospital, Translational Research Institute, University of Queensland, St Lucia, QLD 4072, Australia*
[d] *Mtoxins, 1111 Washington Ave, Oshkosh, WI 54901, USA*
[e] *Faculty of Health Sciences, University of Macau, Avenida da Universidade, Taipa, Macau, China*
[f] *Jiangsu Key Laboratory for Functional Substance of Chinese Medicine, School of Pharmacy, Nanjing University of Chinese Medicine, 138 Xianlin Avenue, Qixia District, Nanjing 215400, China*

A B S T R A C T

Night adders (*Causus* species within the Viperidae family) are amphibian specialists and a common source of snakebite in Africa. Some species are unique in that they have the longest venom glands of any viper, extending approximately 10% of the body length. Despite their potential medical importance and evolutionary novelty, their venom has received almost no research attention. In this study, venoms from a short-glanded species (*C. lichtensteinii*) and from a long-glanded species (*C. rhombeatus*) were compared using a series of proteomic and bioactivity testing techniques to investigate and compare the toxin composition and functioning of the venoms of these two species. Both *C. rhombeatus* and *C. lichtensteinii* were similar in overall venom composition and inhibition of blood coagulation through non-clotting proteolytic cleavage of fibrinogen. While the 1D gel profiles were very similar to each other in the toxin types present, 2D gel analyses revealed isoformic differences within each toxin classes. This variation was congruent with differential efficacy of South African Institute for Medical Research snake polyvalent antivenom, with *C. lichtensteinii* unaffected at the dose tested while *C. rhombeatus* was moderately but significantly neutralized. Despite the variation within toxin classes, the similarity in overall venom biochemistry suggests that the selection pressure for the evolution of long glands served to increase venom yield in order to subjugate proportionally large anurans as a unique form of niche partitioning, and is not linked to significant changes in venom function. These results not only contribute to the body of venom evolution knowledge but also highlight the limited clinical management outcomes for *Causus* envenomations.

1. Introduction

Night Adders (Family *Viperidae,* genus *Causus*) are a clade of small African sub-Saharan batrachophagous (anuran specialist) vipers which contains six well-recognized species, that, as the common name indicates, are primarily nocturnal (Luiselli et al., 2001; Ineich et al., 2006; Toledo et al., 2007; Akani et al., 2012). *C. lichtensteinii* is the slenderest (Ineich et al., 2006) and one of the smallest species in the genus (averaging 30 cm in length), while *C. rhombeatus* is the largest species (95 cm) (Ineich et al., 2006; Mallow et al., 2003).

Viperid snakes typically possess short, robust bodies with a broad head and thin neck, and they are thought to have low metabolic rates due to their ambush predatory strategy and associated low rates of food intake (Ineich et al., 2006). However, night adders stray from this classic viperid profile, as they have slender heads and gracile bodies that are morphologically convergent with many non-venomous snakes or elapids (Mallow et al., 2003; Ineich et al., 2006). Furthermore, as opposed to other vipers, night adders are active pursuit predators that engage in fossorial to semi-fossorial life styles (Mallow et al., 2003; Ineich et al., 2006). They are also reported to prey upon multiple frogs or toads in succession and particularly large ones, digesting meals within 7–10 days (Mallow et al., 2003), which is a relatively short time compared to other vipers, which is indicative of a higher metabolic rate (Ineich et al., 2006). Because of their morphological and ecological

* Corresponding author.
E-mail address: bgfry@uq.edu.au (B.G. Fry).

https://doi.org/10.1016/j.cbpc.2018.05.003
Received 1 January 2018; Received in revised form 3 May 2018; Accepted 8 May 2018

distinction among vipers, *Causus* was thought to be phylogenetically basal and plesiomorphic relative to all other genera within the *Viperidae* family (*Viperinae* subfamily [vipers] and *Crotalinae* subfamily [pit-vipers]) (Janssen et al., 1990; Ineich et al., 2006). However, genetic research revealed strong support for their phylogenetic placement within *Viperinae* subfamily (Wüster et al., 2008; Pook et al., 2009; Pyron et al., 2013; Alencar et al., 2016), although the precise intra-subfamily placement is still unresolved. The morpho-ecological characteristics of *Causus* are therefore derived traits (i.e. apomorphic) rather than representing an ancestral viperid condition (plesiomorphic) (Wüster et al., 2008).

C. rhombeatus (and the recently split sister species *C. maculatus*) are the only representatives of this genus that have elongated venom glands extending beyond the base of the skull and are unique to all *Viperidae* in this regard (Underwood, 1967; Parker, 1977; Mallow et al., 2003; Fry et al., 2015). This confers the ability of delivering very large venom yields (up to 300 mg) relative to their narrow heads (Spawls and Branch, 1995). Proportional to the size of the snakes, these two species also consume much larger anurans than all the others, with prey weight sometimes equaling the snake's own body weight, along with prey diameter dramatically exceeding body diameter and jaw length (Ineich et al., 2006; Mallow et al., 2003). All other *Causus* species prey upon much smaller prey proportional to snake size, with *C. lichtensteinii* having one of the smallest prey to body diameter ratio, where prey diameter rarely exceeds body diameter and never exceeds jaw length (Ineich et al., 2006). These snakes also possess venom glands that, like all other vipers, are confined to the skull and do not extent beyond the base of the neck (Mallow et al., 2003; Ineich et al., 2006). *C. rhombeatus* is known to preferentially feed on robust toads from the genus *Bufo*, while *C. lichtensteinii* preferentially feeds on more gracile frogs from the *Ptychadaena, Chiromantis, Cardioglossa* and *Leptodactylodon* genera (Channing, 2001; Ineich et al., 2006; Mallow et al., 2003; Minter et al., 2004). While the venom appears exceptionally potent to amphibians (with prey items succumbing rapidly – Broadley, 1990) (Broadley, 1990; Mallow et al., 2003) the venom composition and bioactivity characterization remain virtually unknown.

Although not usually considered life threatening to humans, night adders should be considered potentially dangerous. They have produced serious symptoms in human bite victims and have killed domestic animals (De Cramer et al., 2012). Documented human envenomations by *C. rhombeatus* report extreme swelling, drowsiness, slight muscle flaccidity, drops in blood pressure and low to moderate bruising, blistering and/or necrosis (Warrell et al., 1976). Fatal canine envenomings have been reported for *C. rhombeatus*, with several systemic symptoms described including swelling, progressive weakness, hemorrhage, hypovolaemia (i.e. decreased blood volume due to plasma loss into extravascular space), depletion or destruction of blood clotting factors leading to inability to clot, tissue necrosis and respiratory failure (De Cramer et al., 2012). These symptoms suggest coagulotoxic activities disrupting hemostasis and vascular integrity. Intravenous LD_{50} experiments in lab mice (*Mus musculus*) showed that *C. rhombeatus* venom is eight times less potent than that of carpet viper (*Echis carinatus*) and twenty six times less potent than that of puff adder (*Bitis arietans*) venom (Warrell et al., 1976), with LD_{50} values of 13.8 mg/kg (intravenous) (Mebs 1978) and 15 mg/kg (subcutaneous) (Minton Jr., 1974) but with these values offset by *C. rhombeatus'* extremely large venom yields relative to the size of the snake. The only component purified from *C. rhombeatus* venom has been an antithrombin inactivating metalloprotease – with hypercoagulative effects (Janssen et al., 1992). However, as this enzyme was a minor component of the venom, its role in envenomation is unclear and thus the mechanisms of *Causus* coagulotoxicity remains to be elucidated. There are no envenomation reports or toxicity assessments available for *C. lichtensteinii*.

Here we investigated and compared the venom composition, functional activity and specific sites of coagulopathic action of venoms from the short-glanded *C. lichtensteinii* and the long-glanded *C. rhombeatus*. A particular focus of the study was to investigate if the substantial differences in venom gland size and prey size are related to substantial variation of the venom composition or functional activity.

2. Materials and methods

2.1. Venoms

Pooled, lyophilized *C. lichtensteinii* (from multiple adults, geographical original unknown) venom was supplied by MToxins (http://www.mtoxins.com/home.html). Pooled, lyophilized *C. rhombeatus* (from multiple adults, geographical origin unknown) venom was purchased from the Miami Serpentarium Laboratories (Punta Gorda, Florida, USA). Lyophilized venoms were resuspended in deionized H_2O before being centrifuged at 14,000 relative centrifugal force (RCF) at 2 °C, for 15 min. The supernatants were collected and protein concentrations (mg/ml) determined using a ThermoFisher Scientific Nanodrop™ 2000c Spectrophotometer. 50% deionized water/50% glycerol (> 99%, Sigma-Alrich) working stocks of both venoms were prepared at 10 mg/ml and 1 mg/ml and stored at −20 °C to preserve enzymatic activity and reduce enzyme degradation.

2.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE)

1 dimensional (1D) and 2 dimensional (2D) SDS-PAGE gels were prepared and MS/MS analysed as previously described by us (Koludarov et al., 2017). MSMS raw results are included in Supplementary File 1.

2.3. Fibrinogen proteolysis

To quantify the proteolytic activity upon human fibrinogen chains we used a modification of our previous protocol (Dobson et al., 2017; Koludarov et al., 2017). This methodology was expanded not only to include a greater number of time periods within a 60-min frame but also to include different venom concentrations and tests for antivenom efficacy. The reaction concentration of fibrinogen was set to a 1 mg/ml constant and a series of experiments were run with venom at concentrations of 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 μg/ml. The designated time periods for sampling each reaction were 8.5, 25.5, 34, 42.5, 51 and 60 min. Each fibrinogen:venom ratio combination was conducted in triplicate and gels run for the time periods of each replicate.

An antivenom assay was also performed to ascertain the relative inhibition of the fibrinogenolytic activity. In one approach the venom was preincubated with antivenom for 2 min prior to addition to fibrinogen, while in the other the antivenom was preincubated with the fibrinogen for 2 min prior to the addition of venom. The reactions were then run as described above. The antivenom used was equine South African Institute for Medical Research (SAIMR) snake polyvalent antivenom (Lot L01146 - South African Vaccine Producers (Pty) Ltd., 1 Modderfontein Road, Edenvale, Gautend, Sandringham 2131, South Africa) developed from and for the treatment against the venoms of *Bitis arietans, Bitis rhinoceros,* as well as the elapid venoms of *Hemachatus haemachatus, Dendroaspis angusticeps, D. jamesoni, D. polylepis, D. viridis, Naja nivea, N. melanoleuca, N. annulifera,* and *N. mossambica*.

2.4. Coagulation analyzer measurement of anticoagulant activity

Anticoagulant activity assessment was performed on human fibrinogen obtained from normal, healthy donors. Human fibrinogen was reconstituted to a concentration of 4 mg/ml in enzyme running buffer (5 mM $CaCl_2$, 150 mM NaCl, 50 mM Tris-HCl at pH 7.4), flash-frozen in liquid nitrogen, and stored at −80 °C until required. A Claussian test for quantitative determination of fibrinogen (Clauss, 1957) was used to

assess the venom's fibrinogenolytic activity and the quality of the human fibrinogen. In this method a vast excess of thrombin is added so that any change in clotting time is directly due to lower levels of intact fibrinogen available for cleavage by the thrombin. Blank control tests were performed by adding 25 µl of OK buffer (Stago Cat# 00360) to 50 µl of $CaCl_2$ (Stago Cat# 00367), 50 µl phospholipids (Stago Cat# 00597), and 75 µl of 2 mg/ml human fibrinogen aliquots. These were then incubated for 60 min at 37 °C. Subsequently, 50 µl thrombin (titrated human calcium thrombin, STA – Liquid FIB standard kit, Stago, Cat#00673) were added and clotting time measured (control values were 5.0–5.6 s at N = 3). To determine the clotting times, two tests were run: 1) Using a fixed venom concentration (0.3 mg/ml) and 8 incubation times (0, 7.5, 22.5, 37.5, 45, 52.5, and 60 min), and 2) using a fixed incubation time (i.e. 60 min) while using 17 venom concentrations (0.00935, 0.0187, 0.0374, 0.0439, 0.0527, 0.0657, 0.0877, 0.1317, 0.26349, 0.28, 0.33, 0.39, 0.4, 0.5, 0.66, 1 and 2 mg/ml). These tests were run N = 3 with the analyzer's clotting time limit being 999 s. If a clot had not formed within 999 s, fibrinogen in the sample was deemed completely cleaved and the formation of a clot no longer possible. Coagulation times (sec) were graphed using Prism 7.0. Data are expressed as mean ± SD.

2.5. Thromboelastography

Clot strength using human fibrinogen was assessed using a using TEG® 5000 Thrombelastograph® Hemostasis Analyzer System. The TEG method consists of a pin suspended from a torsion wire and into a cup connected to a mechanical-electrical transducer (heated at 37 °C). The strength of the developing clot will change the rotation of the pin, which generates the output. Positive control consisted of the addition of thrombin (the final enzyme that functionally cleaves fibrinogen into fibrin to form a clot). Human fibrinogen was reconstituted to a concentration of 4 mg/ml in enzyme running buffer (5 mM $CaCl_2$, 150 mM NaCl, 50 mM Tris-HCl at pH 7.4), flash-frozen in liquid nitrogen, and stored at −80°C until required. 72 µl of 0.025 M CaCl2, 72 µl phospholipids, 20 µl OK buffer and 7 µl of 10 mg/ml venom glycerol stock were initially added together. Then, 189 µl of 4 mg/ml fibrinogen was added, the whole solution pipette-mixed, and the test immediately started. The final reaction concentrations of fibrinogen and venom were 2 mg/ml and 0.2 mg/ml respectively. Tests were run for 30 min. If no clot formed after 30 min, then 7 µl of thrombin was added to perform a Claussian test for quantitative determination of the degree of fibrinogen degradation (Clauss, 1957). The Claussian protocol has thrombin added in a vast excess and therefore any change is due to a depletion of the amount of intact fibrinogen available. Tests were run at N = 3.

3. Results

3.1. SDS-PAGE

1D non-reduced and 2D reduced SDS-PAGE gels were used to investigate the proteomic variation between the two venoms (Fig. 1). Subsequent tandem-mass spectrometry sequencing performed on the dominant 1D gel bands followed by searching against the UniProt database revealed the presence of L-amino acid oxidases (LAAO), P-III type snake venom metalloproteases (SVMP), kallikrein-type serine proteases, and Type II PLA_2 phospholipases (Fig. 1a). 2D gels revealed that each toxin type was composed of multiple isoforms and with substantial isoformic variation between the two species, indicating that, while the toxin class diversity was low, the intra-class molecular diversity was high (Fig. 1b and c).

3.2. Fibrinogen proteolysis

To quantify the time-dependent fibrinogenolysis, 1 mg/ml fibrinogen was incubated with 10 venom concentrations ranging from to 1.525 to 800 µg/ml and analysed at 6 different time periods over a period of 60 min. All combinations were N = 3. Negative controls were performed at zero minutes of incubation time (C 0′) and sixty minutes of incubation time (C 60′). Each time-period sample was reduced, denatured and subsequently loaded on a 1D gel lane. *C. lichtensteinii* and *C. rhombeatus* venoms showed highly similar fibrinogenolytic activity over the myriad of concentrations and incubation times (Figs. 2 and 3). Both venoms cleaved fibrinogen chains Aα and Bβ, leaving the γ chains intact, hence the exclusion of γ chains from the graphed results in Fig. 4a and b. Additionally, for both venoms, cleavage of the Bβ chains never occurred before the Aα was completely cleaved. While *C. lichtensteinii* was non-significantly faster in Aα chain cleavage (Fig. 4c), the Bβ chain cleavage rates were non-significantly faster for *C. rhombeatus* than for *C. lichtensteinii* (Fig. 4c).

To assess the relative neutralization of the fibrinogenolytic activity by antivenom, South African Institute for Medical Research (SAIMR) polyvalent antivenom (developed for treatment against the viper venoms of *Bitis arietans, Bitis rhinoceros,* as well as the elapid venoms of *Hemachatus haemachatus, Dendroaspis angusticeps, D. jamesoni, D. polylepis, D. viridis, Naja nivea, N. melanoleuca, N. annulifera, and N. mossambica*.) was used. This assay incorporated two different preincubation protocols: 1) the venom with antivenom ((av + v) + fib); and 2) the fibrinogen with antivenom (v + (av + fib)). At the concentration tested, the antivenom demonstrated no inhibition of the fibrinogenolytic effects of *C. lichtensteinii* venom but a 5-fold drop in effect was observed for *C. rhombeatus* venom (Fig. 4d). For *C. rhombeatus*, there was no significant difference between incubating antivenom and venom, prior to adding fibrinogen (5.58 ± 0.63 shift in clotting curve) or incubating antivenom and fibrinogen, prior to the addition of venom (5.71 ± 0.78 shift in clotting curve), indicative of rapid venom binding by the antivenom.

3.3. Coagulation analyzer measurement of anticoagulant activity

Anticoagulant activity assessment was carried out on an automated viscosity-based (mechanical) detection system analyzer (Stago STA-R max) that measures clotting times (with a maximum reading time of 999 s as the analyzer's limit). These anticoagulant activity tests were only performed on *C. rhombeatus* venom due to a limited amount of *C. lichtensteinii* venom in supply. *C. rhombeatus* anticoagulant activity was measured using two different protocols to test non-clotting proteolytic cleavage of 1.2 mg/ml fibrinogen: i) a fixed 0.33 mg/ml venom concentration, tested in 7.5-min step-wise incubation increases up to 60 min (Fig. 4a), and ii) 17 different venom concentrations incubated for a set 60 min incubation (Fig. 4b). Thrombin was added post-incubation in a Claussian method (Clauss, 1957) with immediate measurement of clotting time thereafter. A steady increase in clotting time was observed under both methods – relative to the venom concentration or incubation time used – with the clotting time reaching 999 s in the second scenario (5b) at 0.4 mg/ml and above, as fibrinogen was completely depleted.

3.4. Thromboelastography

Clot strength using human fibrinogen was assessed using a thromboelastograph (TEG) – viscoelastic hemostatic assay where strength of the clot is measured as the maximum amplitude of a clotting curve (i.e. thrombus velocity curve) in millimeters. Fibrinogen cleavage occurred in a non-clotting manner and thrombin was added after 30 min to measure depletion of fibrinogen as indicated by lower clot strength (Fig. 4c). Clot strength was measured at 39 ± 7.7% that of the positive control for *C. lichtensteinii* and 45 ± 3.3% for *C. rhombeatus*, with no significant difference between the two venoms.

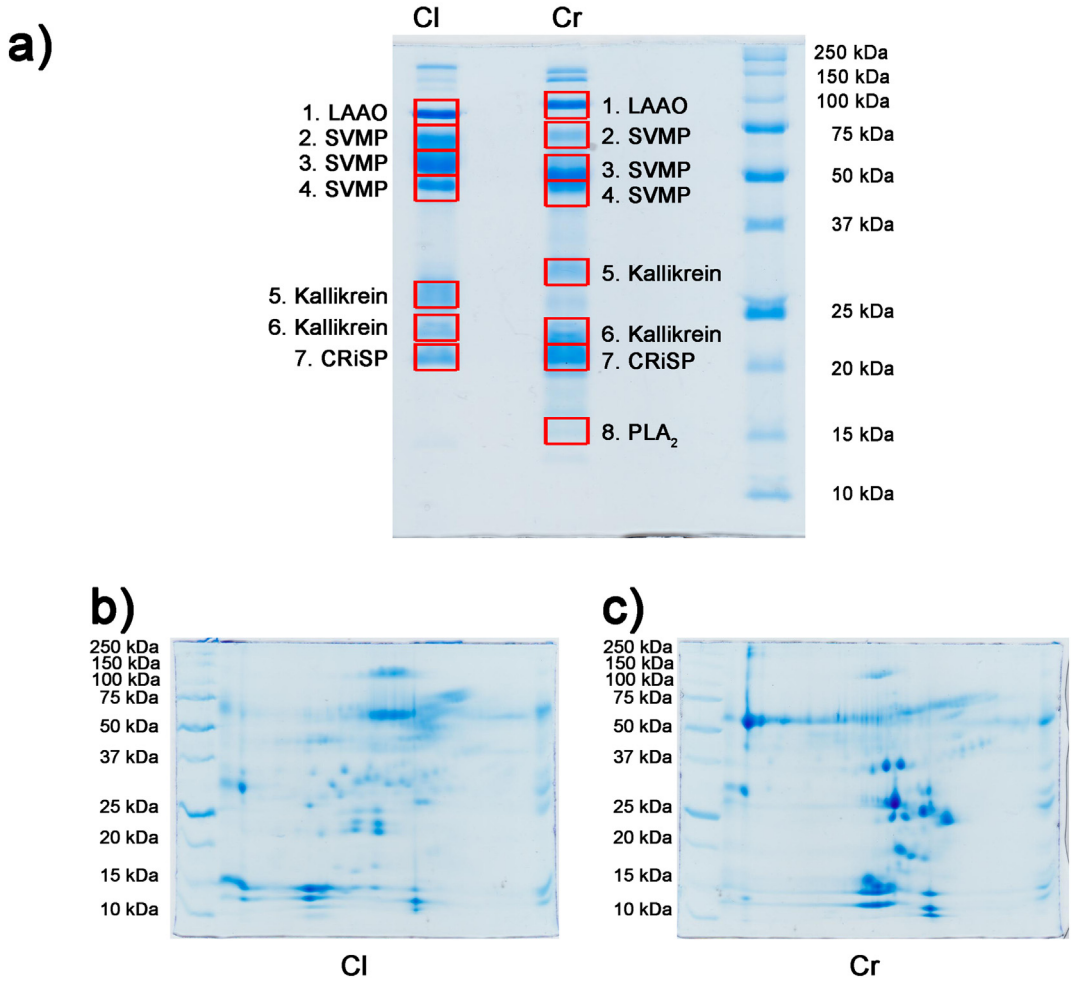

Fig. 1. Proteomic comparison of *C. lichtensteinii* (Cl) and *C. rhombeatus* (Cr) by a) 1D non-reduced and b, c) 2D reduced SDS PAGE electrophoresis.

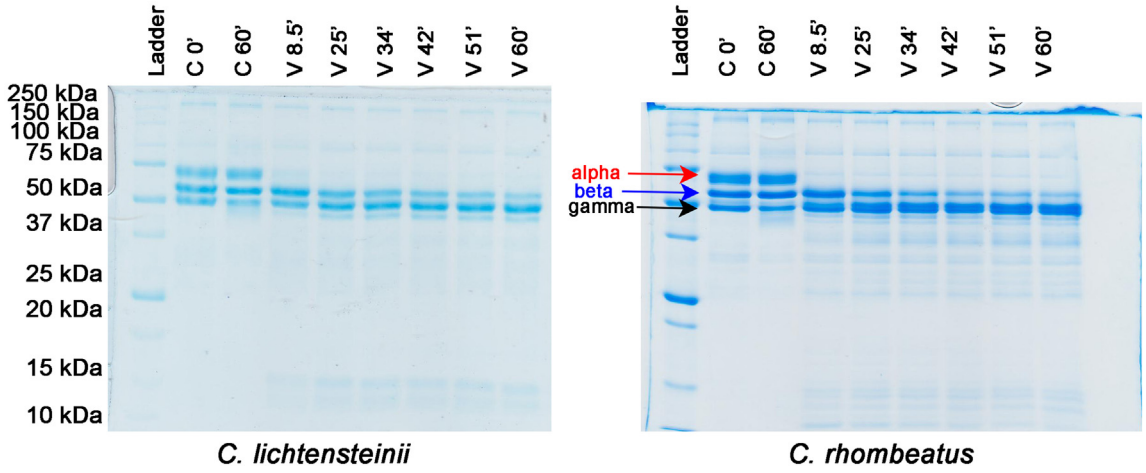

Fig. 2. 100 µg/ml representative gels illustrating the relative chain cleavage of 1 mg/ml fibrinogen for *C. lichtensteinii* and *C. rhombeatus*. Gels were run in triplicate. C = control, V = venom, ′ = time (min).

4. Discussion and conclusion

C. lichtensteinii and *C. rhombeatus* were similar in proteomic composition (Fig. 1), cleavage of fibrinogen chains Aα and Bβ (Figs. 2 and 3), and inhibition of clotting as a consequence of the non-clotting, proteolytic cleavage of fibrinogen (Fig. 4). The 1D gel profiles were similar to each other in the overall toxin classes present, with the venoms of both species containing L-amino acid oxidases (LAAO), snake venom metalloproteases (SVMP), kallikrein-type serine proteases, and Group II phospholipase A_2 (PLA_2) (Fig. 1a). Unlike the LAA0, SVMPs and kallikrein enzymes, only low levels of PLA_2s were present, indicating that they contribute little to the envenomation effects. These findings are consistent with a partial *C. rhombeatus* transcriptome that identified SVMP, kallikrein-type serine protease and PLA_2 (Fry et al.,

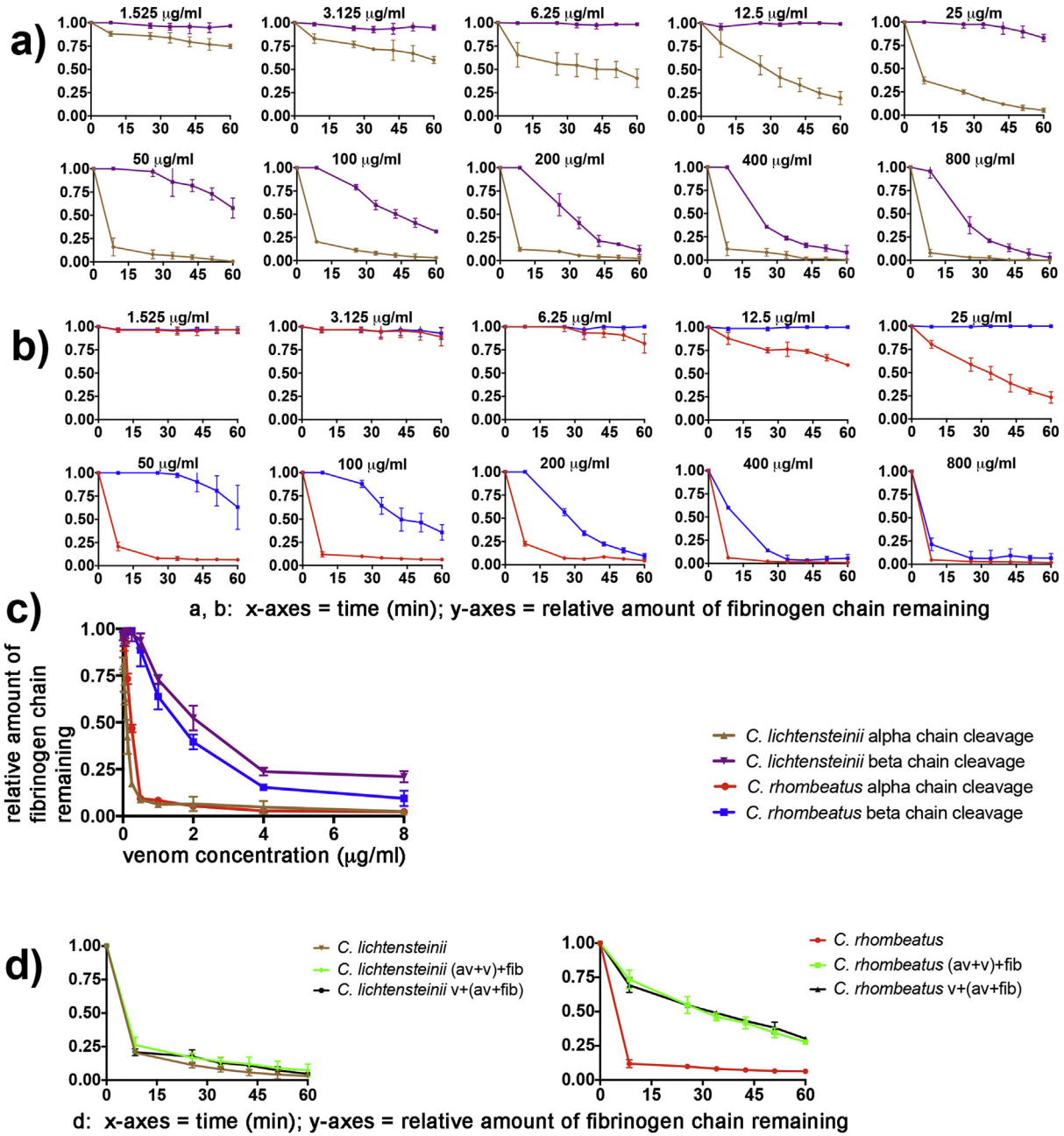

Fig. 3. Relative action upon 1 mg/ml fibrinogen: a, b) by various venom effect upon fibrinogen chains remaining; c) venom concentration effect upon relative fibrinogen chain remaining; and d) effect of SAIMR snake polyvalent antivenom upon the Aα chain cleavage by 100 μg/ml of venom upon 1 mg/ml fibrinogen with (av + v) + fib indicating venom incubated with antivenom prior to addition of fibrinogen and v + (av + fib) indicating fibrinogen incubated with antivenom prior to the addition of venom. Values are N = 3 with error bars showing standard deviation. Note for some values the error bars are smaller than the size of the symbol.

2008). However, the 2D gels differed, with each venom possessing multiple unique isoforms within each toxin class and with little isoformic overlap between venoms (Fig. 1b and c).

SVMP toxins are one of the most highly expressed protein families in viperid venoms, making up, in some cases, as much as 74% of venom composition (Junqueira de Azevedo and Ho, 2002; Bazaa et al., 2005; Wagstaff and Harrison, 2006; Gutiérrez et al., 2008; Casewell et al., 2009; Wagstaff et al., 2009). SVMPs are composed of the three classes, P-I, P-II and P-III, based upon the relative presence of three domains (metalloprotease, disintegrin and cysteine-rich, respectively). P-I lacks the cysteine-rich and disintegrin domains, P-II lacks the cysteine-rich domain, and the tri-domain form (P-III) is the plesiotypic (ancestral) state (Casewell et al., 2015). The large molecular weight of *Causus rhombeatus'* SVMPs (Fig. 1a) suggests they likely belong to the P-III type SVMP. SVMP are associated with a wide range of functional coagulopathic activities, with non-clotting cleavage of fibrinogen being basal (Casewell et al., 2015). The induction of hemorrhage is the result of accompanying proteolytic cleavage of basement membrane components in capillary vessels, resulting in capillary distention (i.e. vasodilation and higher vascular permeability), disruption of endothelial cell integrity, extravasation and swelling (Gutiérrez et al., 2005; Gutiérrez et al., 2006; Gutiérrez et al., 2009). The combination of these activities ultimately induces hemorrhage, myonecrosis, blistering, inflammation and edema (Gutiérrez and Lomonte, 1995; Rucavado et al., 1995; Gutiérrez et al., 2009). Kallikrein-type serine proteases, like SVMP, are also typically found in high concentrations in viperid venoms and, again like SVMP, a basal activity is non-clotting cleavage of fibrinogen (Vaiyapuri et al., 2015). In addition, snake venom kallikreins retain the

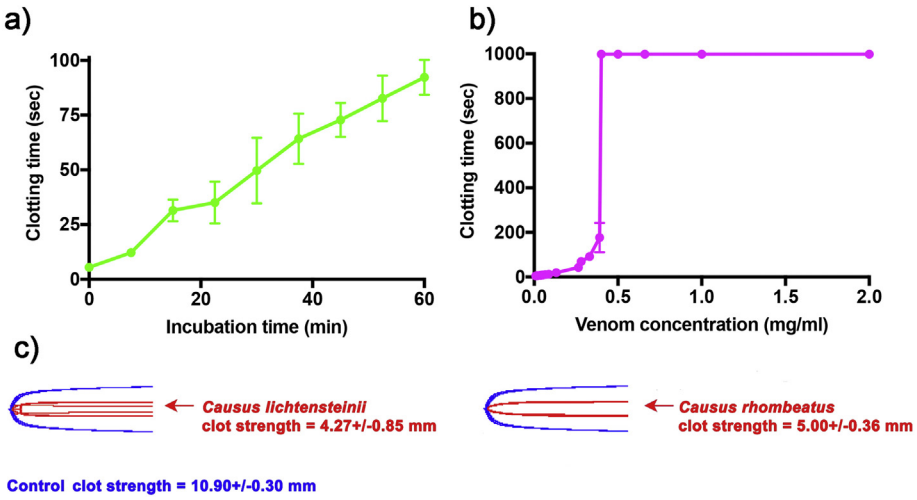

Fig. 4. a) Stago STA-R max measured anticoagulant effect upon 1.2 mg/ml fibrinogen by *C. rhombeatus* venom: a) 0.33 mg/ml venom at eight different incubation times (0, 7.5, 15, 22.5, 37.5, 45, 52.5, and 60 min); and b) 60 min incubation with seventeen different venom concentrations (0.00935, 0.0187, 0.0374, 0.0439, 0.0527, 0.0657, 0.0877, 0.1317, 0.26349, 0.28, 0.33, 0.39, 0.4, 0.5, 0.66, 1, and 2 mg/ml) (999 = maximum measuring time). N = 3 with error bars showing standard deviation. Note for some values the error bars are smaller than the symbols. c) TEG5000 thromboelastography 30 min measured decreases of clot strength of 2.1 mg/ml fibrinogen by 0.2 mg/ml venom concentration after 30 min incubations. N = 3 with the traces overlaid.

ancestral kallikrein ability to cleave kininogen to release bradykinin but are also able to cleave low molecular weight kininogen into kallidin in addition to angiotensin-converting activity (Hendon and Tu, 1981; Bhoola et al., 1992; Hung and Chiou, 2001; Siigur et al., 2011; Vaiyapuri et al., 2012; Kuniyoshi et al., 2012).

In this study, the abundant presence of SVMP and kallikrein enzymes (Fig. 1) was consistent with the non-clotting cleavage activity on fibrinogen (Figs. 2 and 3). Non-clotting, anticoagulant cleavage of fibrinogen as seen in this study is a well-characterized viper venom activity, with kallikrein-type serine proteases and SVMPs convergently evolving this function (Vaiyapuri et al., 2015; Casewell et al., 2015; Dobson et al., 2017). In this study, fibrinogen samples incubated with venom and then clotted with thrombin using a Claussian protocol (Clauss, 1957) formed weak clots indicative of degraded and depleted fibrinogen levels (Fig. 4d). The non-clotting cleavage of Aα-chains took place first and only then were Bβ-chains degraded (Figs. 2 and 3), suggesting that either 1) the cleavage site used on the Bβ-chain is conformationally obscured by the Aα-chain or 2) there is a much greater enzymatic affinity for the cleavage of the Aα-chains. Fibrinogenolytic kalliikreins and metalloproteases are both reported to degrade preferentially the Aα-chains of fibrinogen followed by the Bβ-chains, while the enzymes degrading the Bβ-chains without fibrinolysis belong to the kallikrein-type serine protease group (Cortelazzo et al., 2010). Cleavage of the Aα-chain followed by the Bβ-chain is a pattern also seen in lizard venom fibrinogenolysis and is reflective of the shared evolutionary history of the Toxicoferan reptile venom system (Koludarov et al., 2017).

The relative contribution by kallikrein and SVMP toxins to fibrinogenolysis in *Causus* venoms should be the subject of future work. However, regardless of the relative contributions, the anticoagulant function as documented here would facilitate prey capture by preventing the plugging of damage to the vascular wall, thus facilitating fluid loss into the extravascular space. This would result in hypotension which would be potentiated by kinin release from kininogen (a likely activity from kallikrein rich venoms such as these, but one that needs to be confirmed in future studies). The combination of anticoagulation and hypotension would result in hemorrhagic shock to prey items.

Consistent with the 2D gel variation, the two venoms differed in the response to SAIMR polyvalent antivenom. At the dose test, the antivenom did not impede the fibrinogenolytic effects of *C. lichtensteinii,* but did moderately neutralize the *C. rhombeatus* venom (Fig. 4d). 1 ml of this antivenom is sufficient to neutralize 53 *B. arietans* LD_{50}s (1 LD50 = 8–14 μg) and 80 *B. gabonica* LD_{50}s (1 LD50 = 16–24 μg) (Priscilla Fleischer, South African Institute for Medical Research personal communication). Thus while the antivenom effects were tested under an ideal scenario (preincubation) the results are indicative of a moderate but significant level of cross reactivity with *C. rhombeatus* venom. This is in contrast to the World Health Organisation statement that no antivenom is available (World Health Organization, 2010). However, this statement is an evidence-free statement though as it does not cite any testing showing inefficiency of the South African polyvalent antivenom. A similar evidence-free statement was found on an online clinical toxinology resource page (Clinical Toxinology Resources, 2018). We could not find any published references regarding antivenom testing for this genus, and the lack of *Causus* venom testing prior to this study was confirmed by the antivenom producing institute (Priscilla Fleischer, South African Institute for Medical Research personal communication). In contrast to the efficacy against *C. rhombeatus* venom at the dose tested, the lack of activity against *C. lichtensteinii* under such idealized circumstances suggests that larger doses may be needed for the antivenom to work, if at all.

In addition to the role of serine proteases or metalloproteases in degrading fibrinogen, the hemorrhagic and inflammatory pathologies clinically noted in *C. rhombeatus* envenomations may be due in part to the presence of LAAO toxins. LAAO range from 110 kDa to 140 kDa in size (Chen et al., 2012b; Franca et al. 2007) and catalyze the oxidative deamination of an L-amino acid to produce alpha-keto acid – releasing hydrogen peroxide (H_20_2) in the process (Tan et al., 2015). The release of hydrogen peroxide causes a painful cytotoxic effect and is reportedly used defensively by *Ophiophagus hannah* (Panagides et al., 2017). Additionally, hydrogen peroxide can trigger or potentiate inflammatory reactions (Tan et al., 2015). It has been suggested that LAAO-induced apoptosis could occur as a result of oxidative stress induced by hydrogen peroxide generated by snake venom LAAOs (Tempone et al., 2001).

While the specimen composition of the pooled venoms for each species was unknown other than being of multiple adults for both species, the highly similar overall venom composition and function between each species makes it a reasonable inference that these are the generalized patterns for each species and, using phylogenetic bracketing, the genus as a whole. As these snakes are amphibian specialists, and there is no ontogenetic variation known for the diets, age related changes are less likely than in other genera which have significant ontogenetic variation in diet such as the switch from lizard specialism (and neurotoxic venom) in neonates to mammal specialism (and coagulotoxic venom) in *Pseudonaja* (Cipriani et al., 2017; Jackson et al., 2016).

The findings reported here shed light on the evolutionary context of the night adder's enigmatic venom gland phenotype. Since both venoms are not functionally different, the spectacular elongation of the venom glands, present in only the sister species *C. rhombeatus* and *C. maculatus*, appears to be the result of positive selection for increased venom

production subsequent to an evolutionary shift in morphology to an active predatory strategy. The drift from the typical viper ambush-feeding morphology is presumed to be prior to elongation of venom glands due to nested nature of *Causus* within *Viperinae* (Wüster et al., 2008). This is consistent with the observation that proportionally larger venom glands are correlated with proportionally larger relative prey sizes (Ineich et al., 2006; Mallow et al., 2003). Relating prey size to snake size, *C. lichtensteinii* feeds on much smaller frogs than *C. rhombeatus,* which feeds preferentially on toads proportionally much larger and is documented to cease feeding upon small amphibians (Ineich et al., 2006; Mallow et al., 2003). Thus a *C. lichtensteinii* of the same size as a *C. rhombeatus* would take much smaller sized prey items.

The elongation of an ancestral venom gland system, and dramatic increase in venom production, has evolved at least once independently in each of the front-fanged venomous snake clades: once in the genus *Atractaspis* within the Lamprophiidae (clade consisting of *Atractaspis engaddensis, A. microlepidota, A. micropholis* and *A. scortecci*), twice in Elapidae (common ancestor of *Calliophis bivirgata* and *Calliophis intestinalis* and again in *Toxicocalamus longissimus*) and once in viperid snakes (common ancestor of *C. rhombeatus* and *C. maculatus*) (Fry et al., 2008; Fry et al., 2015). All species with elongated venom glands share the morpho-ecological aspects of: 1) they are the largest of their genus and feed on the largest proportional prey items; 2) they exhibit active and agile pursuit predatory behaviors; and 3) all are semi-fossorial or fossorial. This suggests long glands are an adaptation that facilitates increased venom production and yield in the presence of slender, agile body morphology. In the case of *C. bivirgatus* the larger venom yield offsets the lower toxicity of their novel sodium channel toxins (Yang et al., 2016).

In *Causus,* the elongation of the venom glands would increase the venom yield without the morphological burden of a broad viper-like head. Thus, both *C. rhombeatus* and *C. maculatus*—the biggest of all the *Causus* species—can produce larger venom yields which allows them to adequately subjugate proportionally larger prey items without compromising their morphology and ecological niches, thereby retaining the slender body type selected for an active and agile predatory strategy. Therefore while both *C. rhombeatus* and *C. lichtensteinii* venoms are functionally similar, each snake is able to occupy a unique ecological niche regarding relative prey size and type, conferred by appropriately sized venom glands and yields. Thus the differential venom size and relationship to discrete prey preferences represents a novel form of niche partitioning.

In conclusion, this study represents the first comparative venom analysis between short-glanded and a long-glanded species within a genus. The results reveal a similar venom composition and activity in *C. rhombeatus* and *C. lichtensteinii*, suggesting a greater advantage in larger venom yields rather than greater venom toxicity in order to subjugate proportionally larger amphibian prey items. Further research is needed, such as the functional characterization of each venom fraction, to assess the relative contribution to the coaogulotoxic function by each toxin type. As such, it is impossible to here accredit any of the reported physiopathologies solely to one specific toxin class, as various toxins synergistically work to induce and potentiate the same functional outcome. However, these results also underscore the fundamental premise that even very closely related snakes with seemingly similar venom composition can differ in responsiveness to antivenom, and this may have important clinical implications.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2018.05.003.

Acknowledgements

BodB, CNZ and JD were the recipients of University of Queensland PhD scholarships. HFK was supported by the Science and Technology Development Fund of Macau SAR (FDCT) [019/2017/A1].

Conflict of interest

The authors declare no conflict of interest.

References

Akani, G.C., Luiselli, L., Tooze, Z., Angelici, F.M., Corti, C., Zuffi, M.A.L., 2012. The ecological distribution of *Causus* Wagler 1830 (*Viperidae*) in Nigeria, with special reference to *C. resimus* (Peters 1862) and *C. lichtensteini* (Jan 1859), two species rarely recorded from this country. Trop. Zool. 14 (1), 185–195. http://dx.doi.org/10.1080/03946975.2001.10531151.

Alencar, L.R.V., Quental, T.B., Grazziotin, F.G., Alfaro, M.L., Martins, M., Venzon, M., Zaher, H., 2016. Molecular Phylogenetics and Evolution Diversification in Vipers: Phylogenetic Relationships, Time of Divergence and Shifts in Speciation Rates, Molecular Phylogenetics and Evolution. 105. Elsevier Inc., pp. 50–62. http://dx.doi.org/10.1016/j.ympev.2016.07.029.

Bazaa, A., Marrakchi, N., El Ayeb, M., Sanz, L., Calvete, J.J., 2005. Snake venomics: comparative analysis of the venom proteomes of the Tunisian snakes *Cerastes cerastes, Cerastes vipera* and *Macrovipera lebetina*. Proteomics 5 (16), 4223–4235.

Bhoola, K.D., Figueroa, C.D., Worthy, K., 1992. Bioregulation of kinins: Kallikreins, kininogens, and kininases. Pharmacol. Rev. 44 (1), 1–80.

Broadley, D.G., 1990. FitzSimons' Snakes of Southern Africa. J Ball & AD Donker Publishers, Parklands (South Africa) (387 pp.).

Casewell, N.R., Harrison, R.A., Wüster, W., Wagstaff, S.C., 2009. Comparative venom gland transcriptome surveys of the saw-scaled vipers (Viperidae: *Echis*) reveal substantial intra-family gene diversity and novel venom transcripts. BMC Genomics 10, 564.

Casewell, N.R., Sunagar, K., Takacs, Z., Calvete, J.J., Jackson, T.N.W., Fry, B.G., 2015. Snake Venom Metalloprotease Enzymes. In: Fry, B.G. (Ed.), Venomous Reptiles and Their Toxins: Evolutionary, Pathophysiological and Biodiscovery Implications. Oxford University Press, New York, pp. 347–364.

Channing, A., 2001. Amphibians of Central and Southern Africa. Cornell University Press, Ithaca NY.

Chen, H.S., Wang, Y.M., Huang, W.T., Huang, K.F., Tsai, I.H., 2012. Cloning, characterization and mutagenesis of Russell's viper venom L-amino acid oxidase: insights into its catalytic mechanism. Biochimie 94 (2), 335–344.

Cipriani, V., Debono, J., Goldenberg, J., Jackson, T.N.W., Arbuckle, K., Dobson, J., Koludarov, I., Li, B., Hay, C., Dunstan, N., Allen, L., Hendrikx, I., Kwok, H.F., Fry, B.G., 2017. Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*). Comp. Biochem. Physiol. Toxicol. Pharmacol. 197, 53–60.

Clauss, A., 1957. Rapid physiological coagulation method in determination of fibrinogen. Acta Haematol. 17, 237–246.

Clinical Toxinology Resources, 2018. http://www.toxinology.com/fusebox.cfm?fuseaction=main.snakes.display&id=SN0263, Accessed date: 5 December 2018.

Cortelazzo, A., Guerranti, R., Bini, L., Hope-Onyekwere, N., Muzzi, Chiara, Leoncini, R., Pagani, R., 2010. Effects of snake venom proteases on human fibrinogen chains. Blood Transfus. 8 (3), s120–s125. http://dx.doi.org/10.2450/2010.019S.

De Cramer, K.G.M., Van Bart, G.A., Huberts, F., 2012. Morbidity and mortality following envenomation by the common night adder (*Causus rhombeatus*) in three dogs. J. S. Afr. Vet. Assoc. 83 (1) (Art. #205, 4 pages). https://doi.org/10.4102/jsava.v83i1.205.

Dobson, J., Yang, D.C., op den Brouw, B., Cochran, C., Huynh, Tam., Kurrupu, Sanjaya., Sánchez, E.E., Massey, J.D., Baumann, K., Jackson, N.W.T., Nouwens, A., Josh, P., Neri-Castro, E., Alagón, A., Hodgson, W.C., Fry, B.G., 2017. Rattling the border wall: Pathophysiological implications of functional and proteomic venom variation between Mexican and US subspecies of the desert rattlesnake *Crotalus scutulatus*. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. (17), 30192–30198. 2017 Oct 23. pii: S1532-0456. https://doi.org/10.1016/j.cbpc.2017.10.008.

Fry, B.G., Scheib, H., van der Weerd, L., Young, B., McNaughtan, J., Ramjan, S.F., Vidal, N., Poelmann, R.E., Norman, J.A., 2008. Evolution of an arsenal: structural and functional diversification of the venom system in the advanced snakes (Caenophidia). Mol. Cell. Proteomics 7 (2), 215–246.

Fry, B.G., Sunagar, K., Casewell, N.R., Kochva, E., Roelants, K., Scheib, H., Wuster, W., Vidal, N., Young, B., Burbrink, F., Pyron, R.A., Vonk, F.J., Jackson, T.N.W., 2015. The origin and evolution of the toxicofera reptile venom system. In: Fry, B.G. (Ed.), Venomous Reptiles and Their Toxins: Evolutionary, Pathophysiological and Biodiscovery Implications. Oxford University Press, New York, pp. 1–32.

Gutiérrez, J.M., Lomonte, B., 1995. Phospholipase A2 myotoxins from *Bothrops* snake venoms. Toxicon 33 (11), 1405–1424.

Gutiérrez, J.M., Rucavado, A., Escalante, T., Diaz, C., 2005. Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. Toxicon 45 (8), 997–1011.

Gutiérrez, J.M., Nuñez, J., Escalante, T., Rucavado, A., 2006. Blood flow is required for rapid endothelial cell damage induced by a snake venom hemorrhagic metalloproteinase. Microvasc. Res. 71 (1), 55–63.

Gutiérrez, J.M., Sanz, L., Escolano, J., Fernandez, J., Lomonte, B., Angulo, Y., Rucavado, A., Warrell, D.A., Calvete, J.J., 2008. Snake venomics of the lesser Antillean pit vipers *Bothrops caribbaeus* and *Bothrops lanceolatus*: correlation with toxicological activities and immunoreactivity of a heterologous antivenom. J. Proteome Res. 7 (10), 4396–4408.

Gutiérrez, J.M., Rucavado, A., Escalante, T., Mackessy, S.P., 2009. Snake venom metalloproteinases: Biological roles and participation in the pathophysiolgy of envenomation. In: Handbook of Venoms and Toxins of Reptiles. CRC Press, Boca Raton,

FL, pp. 116–138.
Hendon, R.A., Tu, A.T., 1981. Biochemical characterization of the lizard toxin gilatoxin. Biochemistry 20 (12), 3517–3522.
Hung, C.C., Chiou, S.H., 2001. Fibrinogenolytic proteases isolated from the snake venom of Taiwan habu: serine proteases with kallikrein-like and angiotensin-degrading activities. Biochem. Biophys. Res. Commun. 281 (4), 1012–1018.
Ineich, I., Bonnet, X., Shine, R., Shine, T., Brischoux, F., Lebreton, M., 2006. What, if anything, is a "typical" viper? Biological attributes of basal viperid snakes (genus *Causus* Wagler, 1830). Biol. J. Linn. Soc. 89, 575–588.
Jackson, T.N., Koludarov, I., Ali, S.A., Dobson, J., Zdenek, C.N., Dashevsky, D., 2016. Rapid radiations and the race to redundancy: An investigation of the evolution of Australian elapid snake venoms. Toxins (Basel) 8 (11), 309. http://dx.doi.org/10.3390/toxins8110309.
Janssen, M., Freyvogel, A.T., Meier, J., 1990. Antigenic relationship between the venom of the night adder *Causus maculatus* and venoms of other viperids. Toxicon 28 (8), 975–983.
Janssen, M., Meier, J., Freyvogel, A.T., 1992. Purification and characterization of an venom of the african night adder (*Causus rhombeatus*). Toxicon 9, 985–999.
Junqueira de Azevedo, I.L., Ho, P.L., 2002. A survey of gene expression and diversity in the venom glands of the pitviper snake *Bothrops insularis* through the generation of expressed sequence tags (ESTs). Gene 299 (1–2), 279–291.
Koludarov, I., Jackson, T.N.W., Den Brouw, B., Dobson, J., Dashevsky, D., Arbuckle, K., Clemente, C.J., Stockdale, E.J., Cochran, C., Debono, J., Stephens, C., Panagides, N., Li, B., Louise, M., Manchadi, R., Violette, A., Fourmy, R., Hendrikx, I., Nouwens, A., Clements, J., Martelli, P., Kwok, H.F., Fry, B.G., 2017. Enter the dragon: the dynamic and multifunctional evolution of Anguimorpha lizard venoms. Toxins 9 (242), 1–38. http://dx.doi.org/10.3390/toxins9080242.
Kuniyoshi, A.K., Rocha, M., Cajado Carvalho, D., Juliano, M.A., Juliano Neto, L., Tambourgi, D.V., Portaro, F.C., 2012. Angiotensin-degrading serine peptidase: a new chymotrypsin-like activity in the venom of *Bothrops jararaca* partially blocked by the commercial antivenom. Toxicon 59 (1), 124–131.
Luiselli, L., Akani, G.C., Angelici, F.M., Politano, E., Ude, L., Wariboko, S.M., 2001. Short notes aspects of the ecology of a population of *Causus maculatus* (Reptilia: Viperidae) from southern Nigeria. Amphibia-Reptilia 25, 99–104.
Mallow, D., Ludwig, D., Nilson, G., 2003. True Vipers: Natural History and Toxinology of Old World Vipers. Original Edition. Krieger Publishing, Malabar (Florida), pp. 19–34.
Minter, L.R., Burguer, M., Harrison, J.A., Braack, H.H., Bishop, P.J., Knoepfer, D., 2004. Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland. SI/MAB Series No. 9. Washington D.C., USA.
Minton Jr., S.A., 1974. Venom Diseases. CC Thomas Publ, Springfield (IL), pp. 386.
Panagides, N., Jackson, T.N., Ikonomopoulou, M.P., Arbuckle, K., Pretzler, R., Yang, D.C., Ali, S.A., Koludarov, I., Dobson, J., Sanker, B., Asselin, A., Santana, R.C., Hendrikx, I., 2017. How the cobra got its flesh-eating venom: Cytotoxicity as a defensive innovation and its co-evolution with hooding, aposematic marking, and spitting. Toxins (Basel) 9 (3), E103. http://dx.doi.org/10.3390/toxins9030103.
Parker, H.W., 1977. Snakes: A Natural History, second edition. Cornell University Press, New York (188 pp.).
Pook, C.E., Joger, U., Stümpel, N., Wüster, W., 2009. When continents collide: phylogeny, historical biogeography and systematics of the medically important viper genus *Echis* (Squamata: Serpentes: Viperidae). Mol. Phylogenet. Evol. 53 (3), 792–807. http://dx.doi.org/10.1016/j.ympev.2009.08.002.
Pyron, R.A., Burbrink, F.T., Wiens, J.J., 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC Evol. Biol. 13, 1–53. http://dx.doi.org/10.1186/1471-2148-13-93.
Rucavado, A., Lomonte, B., Ovadia, M., Gutiérrez, J.M., 1995. Local tissue damage induced by BaP1, a metalloproteinase isolated from *Bothrops asper* (Terciopelo) snake venom. Exp. Mol. Pathol. 63 (3), 186–199.
Siigur, E., Tonismagi, K., Trummal, K., Samel, M., Vija, H., Aaspollu, A., Ronnholm, G., Subbi, J., Kalkkinen, N., Siigur, J., 2011. A new tyrosine-specific chymotrypsin-like and angiotensin-degrading serine proteinase from *Vipera lebetina* snake venom. Biochimie 93 (2), 321–330.
Spawls, S., Branch, B., 1995. The Dangerous Snakes of Africa. London: Ralph Curtis Books. Oriental Press, Dubai, pp. 192.
Tan, N.H., Fry, B.G., Sunagar, K., Jackson, T.N.W., Reeks, T., Fung, S.Y., 2015. L-Amino acid oxidase enzymes. In: Fry, B.G. (Ed.), Venomous Reptiles and Their Toxins: Evolutionary, Pathophysiological and Biodiscovery Implications. Oxford University Press, New York, pp. 291–299.
Tempone, A.G., Andrade Jr, H.F., Spencer, P.J., Lourenco, C.O., Rogero, J.R., Nascimento, N., 2001. *Bothrops moojeni* venom kills *Leishmania* spp. with hydrogen peroxide generated by its L-amino acid oxidase. Biochem. Biophys. Res. Commun. 280 (3), 620–624.
Toledo, L.F., Ribeiro, R.S., Haddad, C.F.B., 2007. Anurans as prey: an exploratory analysis and size relationships between predators and their prey. J. Zool. 271, 170–177. http://dx.doi.org/10.1111/j.1469-7998.2006.00195.x.
Underwood, G., 1967. A Contribution to the Classification of Snakes. England, Staples Printers LTD, pp. 135.
Vaiyapuri, S., Thiyagarajan, N., Hutchinson, E.G., Gibbins, J.M., 2012. Sequence and phylogenetic analysis of viper venom serine proteases. Bioinformation 8 (16), 763–772.
Vaiyapuri, S., Sunagar, K., Gibbins, J.M., Jackson, T.N.W., Reeks, T., Fry, B.G., 2015. Kallikrein Enzymes. In: Fry, B.G. (Ed.), Venomous Reptiles and Their Toxins: Evolutionary, Pathophysiological and Biodiscovery Implications. Oxford University Press, New York, pp. 267–281.
Wagstaff, S.C., Harrison, R.A., 2006. Venom gland EST analysis of the saw-scaled viper, *Echis ocellatus*, reveals novel alpha(9)beta(1) integrin-binding motifs in venom metalloproteinases and a new group of putative toxins, renin-like aspartic proteases. Gene 377, 21–32.
Wagstaff, S.C., Sanz, L., Juarez, P., Harrison, R.A., Calvete, J.J., 2009. Combined snake venomics and venom gland transcriptomic analysis of the ocellated carpet viper, *Echis ocellatus*. J. Proteome 71 (6), 609–623.
Warrell, D.A., Ormerod, L.D., Davidson, A.M., 1976. Bites by the night adder and burrowing vipers in Nigeria. Am. J. Trop. Med. Hyg. 25 (3), 517–524.
World Health Organization, 2010. Guidelines for the Prevention and Clinical Management of Snakebite in Africa. WHO, Geneva Document. http://apps.who.int/medicinedocs/documents/s17810en/s17810en.pdf (page 52. Accessed on 12/05/18).
Wüster, W., Peppin, L., Pook, C.E., Walker, D.E., 2008. A Nesting of Vipers: Phylogeny and Historical Biogeography of the Viperidae (Squamata: Serpentes). Molecular Phylogenetics and Evolution. 49. Elsevier Inc., pp. 445–459. (2). https://doi.org/10.1016/j.ympev.2008.08.019.
Yang, D.C., Deuis, J.R., Dashevsky, D., Dobson, J., Jackson, T.N.W., Brust, A., Xie, B., Koludarov, I., Debono, J., Hendrikx, I., Hodgson, W.C., Josh, P., Nouwens, A., Baillie, G.J., Bruxner, T.J.C., Alewood, P.F., Kok, K., Lim, P., Frank, N., Vetter, I., Fry, B.G., 2016. The Snake With the Scorpion's Sting: Novel Three-finger Toxin Sodium Channel Activators From the Venom of the Long-glanded Blue Coral Snake. pp. 1–21. http://dx.doi.org/10.3390/toxins8100303.