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Clinical implications of coagulotoxic variations in Mamushi (Viperidae: *Gloydius*) snake venoms

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A B S T R A C T

Snake bite is currently one of the most neglected tropical diseases affecting much of the developing world. Asian pit vipers are responsible for a considerable amount of envenomations annually and bites can cause a multitude of clinical complications resulting from coagulopathic and neuropathic effects. While intense research has been undertaken for some species of Asian pit viper, functional coagulopathic effects have been neglected for others. We investigated their effects upon the human clotting cascade using venoms of four species of *Gloydius* and *Ovophis okinavensis*, a species closely to *Gloydius*. All species of included within this investigation displayed varying fibrinogenolytic effects, resulting in a net anticoagulant outcome. *Gloydius saxatilis* and *Gloydius ussuriensis* displayed the most variable effects from differing localities, sampled from Russia and Korea. As this *Gloydius* investigation includes some geographical variation, notable results indicate key variations of these species that point to possible limitations in antivenom cross-reactivities, which may have implications for the clinical care of victims envenomed by these snakes.

1. Introduction

Snake bite is currently a globally neglected tropical disease with many of the poorer regions of the world mostly effected as they live in extremely close proximity to highly venomous snakes (Fry, 2018; Gutiérrez et al., 2017; Kasturiratne et al., 2008). Snake venoms are complex cocktails of toxins that exert effects on any part of the body reachable by the bloodstream (Casewell et al., 2013). These mixtures of toxins have evolved and diversified over millions of years primarily to facilitate predation (and defence in some cases (Panagides et al., 2017)), and the toxin combinations differ between snake families, genus and species with many varying clinical effects (Fry et al., 2008). Clinical effects range from wide spread neurotoxicity, myotoxicity, necrosis and coagulotoxicity through to debilitating permanent damages if bite victims survive (Casewell et al., 2013; Fry, 2018; Fry et al., 2008; Slagboom et al., 2017).

Coagulotoxic venom components interfere with normal haemostasis and are capable of eliciting an immediate procoagulant or anticoagulant (*i.e.* haemorrhagic) response upon injection into the bloodstream. Procoagulant venoms are known to activate Factor X or prothrombin, resulting in the generation of endogenous thrombin and subsequent stable fibrin clots (Joseph and Kini, 2002; Kini, 2005, 2006; Kornalik and Blombäck, 1975; Lister et al., 2017; Oulion et al., 2018; Rogalski et al., 2017; Rosing and Tans, 1991, 1992, 2010; Sousa et al., 2018; Tans and Rosing, 2002; Yamada et al., 1996; Zdenek et al., 2019). Anticoagulant effects can be accomplished through the inhibition of clotting enzymes, such as Factors Xa, XIa, IXa or thrombin, or through fibrinogenolytic effects (Youngman et al., 2018). Fibrinogen cleavage occurs either in a non-clotting, destructive manner, or results in the formation of aberrant fibrin clots that are short lived and rapidly degraded (Coimbra et al., 2018; Debono et al., 2018). Both types of fibrinogen cleavage have a net anticoagulant effect through a reduction in the amount of normal, intact fibrinogen available for cleavage by endogenous thrombin to form stable fibrin clots.

Asian pit vipers are characterised by haemorrhagic venoms. *Gloydius* is a wide-spread Asian pit viper genus, spanning Japan, China, Mongolia and surrounding borders, Siberian Russia and Korea, occupying many distinct and variable climatic locations (Vogel, 2006). There has been little comparative research performed on species of this genus despite thousands of Mamushi (*G. blomhoffi*) bites (and related species) each year across Japan and surrounding areas (Hifumi et al., 2015, 2011). Bite case reports describe classic haemorrhagic pit viper symptoms including wide spread bleeding which can be fatal (Okamoto et al., 2017; Toh Yoon et al., 2017). The mechanisms behind these widespread symptoms has been subjected to minimal investigation, with majority of the results illustrating fibrinogenolytic effects upon coagulation (Cho et al., 2001; Choi and Lee, 2013; Huang et al., 2011; Zhang et al., 2007). Most studies have focussed not on functional

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aspects but rather concentrated on proteomic isolation and purification from single species (Cho et al., 2001; Choi and Lee, 2013; Fujisawa et al., 2009; Huang et al., 2011; Sun et al., 2006; Yang et al., 2015; Zhang et al., 2007).

It has been established in the literature that venom composition between closely related species, or within different populations of a single species, may vary considerably due to differential pressures regarding prey availability and prey escape potential. This variation has direct implications for the relative clinical usefulness of antivenoms against species not included in the immunizing mixture, or varying populations of the species used (Debono et al., 2017; Dobson et al., 2018; Fry et al., 2003; Lister et al., 2017; Rogalski et al., 2017; Sousa et al., 2018; Sunagar et al., 2014; Tan et al., 2016; Tan et al., 2017; Yang et al., 2016; Zdenek et al., 2019). Many current antivenomic strategies are mostly limited to the immunoreactivity of the antivenom towards the venom components, these do not ascertain whether the antivenom is effective in neutralising venom pathological functions (Jones et al., 2019; Williams et al., 2011; Xu et al., 2017). Out of the ten commonly described *Gloydius* species, antivenom exists only for four (*G. blomhoffii*, *G. brevicaudus*, *G. halys* and *G. ussuriensis*) (Supplementary Table 1). With antivenom available for only a select few species, this creates potential life-threatening challenges for bite victims of other *Gloydius* species. In addition, localities for these species from which antivenom is made is restricted, resulting in unknown medical challenges if a bite victim is bitten by a species from an alternative geographical location.

In this study we investigated the effects on coagulation of the venoms from multiple species of *Gloydius*, and populations within some species, in addition to *Ovophis okinavensis*, which sits basal to *Gloydius* rather than being closely related to other species within the *Ovophis* genus, and therefore may be considered as part of an expanded *Gloydius* genus (Alencar et al., 2018; Malhotra and Thorpe, 2004). We aimed to highlight coagulotoxic differences between the venoms and how this relates to potential clinical effects of the envenomed patient. This investigation will provide important insight into the key variations of these species which is fundamental for clinical care as well as snake bite management across varying localities within Japan and surrounding countries.

2. Material and methods

2.1. Venoms

Lyophilized venom from a total of nine venom samples from four adult *Gloydius* species (pooled or sourced from single specimen) and one sample from an adult *O. okinavensis* species were investigated for effects upon coagulation (Table 1) (Alencar et al., 2016; Malhotra and Thorpe, 2004). Venoms were resuspended in deionized H_20 and protein concentrations (mg/ml) determined using a ThermoFisher Scientific Nanodrop™ 2000c Spectrophotometer. Working stocks of 50% deionized water/50% glycerol (> 99%, Sigma-Aldrich) for all venoms were prepared at 1 mg/ml and stored at −20 °C to preserve enzymatic activity and reduce enzyme degradation. Where possible, both pooled and individual samples were tested for maximum variation. Where results were the same, representatives were used to minimise redundancy. All venom work was undertaken under University of Queensland Biosafety Approval #IBC134BSBS2015.

2.2. Coagulation analysis

Venoms were ascertained for their ability to clot or inhibit the clotting of recalcified plasma, or to form fibrin clots in fibrinogen solutions using methods previously validated by us (Debono et al., 2019, 2018, 2017). Healthy human plasma (citrate 3.2%, Lot#1690252, research agreement #16-04QLD-10) was obtained from the Australian Red Cross (44 Musk Street, Kelvin Grove, Queensland 4059). All human plasma work was undertaken under University of Queensland Biosafety Approval #IBC134BSBS2015 and Human Ethics Approval # 2016000256. Human fibrinogen (Lot#F3879, Sigma Aldrich, St. Louis, Missouri, United States) was reconstituted to a concentration of 1 mg/ml in isotonic saline solution, flash frozen in liquid nitrogen and stored at −80 °C until use. Coagulopathic toxin effects were measured by a modified procoagulant protocol on a Stago STA-R Max coagulation robot using Stago Analyser software v0.00.04 (Stago, Asniéres sur Seine, France). Plasma clotting baseline parameters were determined by performing the standardised activated Partial Thromboplastin Time (aPTT) test (Stago Cat# T1203 TriniCLOT APTT HS). In order to determine clotting times effected by the addition of varying venom concentrations, a modified aPTT test was developed, in which 50 µL of venom (20–0.05 µg/ml) dilutions in STA Owren Koller Buffer (Stago Cat# 00360), 50 µL $CaCl_2$ (5 mM final, Stago Cat# 00367 STA $CaCl_2$ 0.025 M), and 50 µL phospholipid (solubilized in Owren Koller Buffer adapted from STA C.K Prest standard kit, Stago Cat# 00597) in a final volume of 175 µL in Owren Koller Buffer was incubated for 120 s at 37 °C before adding 75 µL of human plasma. Relative clotting was then monitored for 999 s or until plasma clotted (whichever was sooner). Calcium or phospholipid dependency was assessed by replacing calcium or phospholipid for Owren Koller Buffer, respectively.

Inhibition of venom serine protease activity was determined using 4-benzenesulfonyl fluoride hydrochloride (AEBSF), a known serine protease inhibitor (Kuniyoshi et al., 2017; Torres-Bonilla et al., 2018; Xin et al., 2009; Yamashita et al., 2014) in the modified aPTT assay. For this purpose, 25 µL of (diluted) venom, 50 µL $CaCl_2$ (5 mM final), 50 µL phospholipid, and 50 µL AEBSF (2 mM final) were incubated for 2 min at 37 °C before adding 75 µL of human fibrinogen (1.2 mg/ml final).

Additional procoagulation (Factor X and prothrombin activation) or anticoagulation (inhibition of FXa, prothrombinase complex formation, thrombin, or fibrinogen cleavage, Supplementary Table 2) assays were also conducted, using methods previously validated by us (Debono et al., 2019, 2018; Youngman et al., 2018). To identify the possible target in the clotting cascade which the venom was acting upon, inhibition assays were performed in which either plasma or individual

Table 1
Species list of samples from the genus *Gloydius* and *Ovophis*. Pooled samples are of an unknown number of species and unknown specific localities.

Species	Sample composition	Sex (adult)	Origin
Gloydius brevicaudus	Individual	Female	Hapcheon-gun, Gyeongsangnam-do, Korea
Gloydius saxatilis	Pooled	NA	Russia
Gloydius saxatilis	Individual	Male	Yangsan-si, Gyeongsangnam-do, Korea
Gloydius saxatilis	Individual	Male	Yangsan-si, Gyeongsangnam-do, Korea
Gloydius tsushimaensis	Pooled	NA	Tsushuma Island, Japan
Gloydius ussuriensis	Pooled	NA	Russia
Gloydius ussuriensis	Individual	Male	Wonju-si, Gangwon-do, Korea
Gloydius ussuriensis	Individual	Female	Wonju-si, Gangwon-do, Korea
Gloydius ussuriensis	Individual	Female	Wonju-si, Gangwon-do, Korea
Ovophis okinavensis	Pooled	NA	Ryukyu Islands, Japan

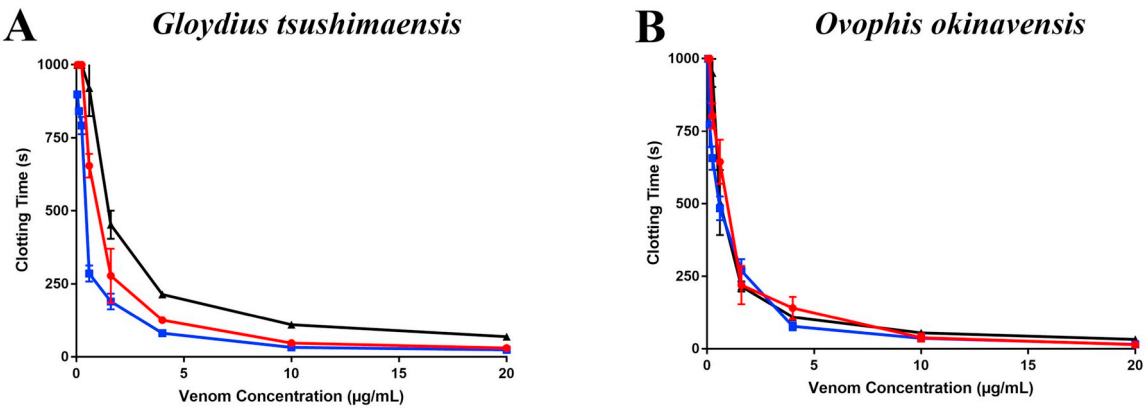

Fig. 1. Concentration-response curves the ability of the venoms to clot human plasma in the presence of both calcium and phospholipid (red line), in the absence of phospholipids (blue line), or in the absence of calcium (black line) for A) *G. tsushimaensis* and B) *O. okinavensis*. Data points are $N = 3$ with standard deviations.

clotting factors were incubated with the sample venom, as shown in (Youngman et al., 2018). Data was analysed using GraphPad PRISM 7.0 (GraphPad Prism Inc., La Jolla, CA, USA).

2.3. Fibrinogenolysis analysis

Fibrinogenolytic activity was assessed as previously described (Debono et al., 2019, 2018). In brief, fibrinogen (1 mg/ml final) was incubated with venom (10 µg/ml final) for 60 min at 37 °C, and fibrinogen cleavage was assessed at several time points (1, 5, 10, 15, 20, 30, 45 and 60 min) using SDS-PAGE analysis under reducing conditions followed by staining with Coomassie Brilliant Blue R-250. The visualized protein fragments were quantified using ImageJ software (V1.51r, Java 1.6.0_24, National Institutes of Health, Bethesda, Maryland, USA) and analysed using GraphPad PRISM 7.0 (GraphPad Prism Inc., La Jolla, CA, USA).

2.4. Thromboelastography

Venoms were investigated for clot strength ability on fibrinogen and plasma employing thromboelastography using a Thrombelastogram® 5000 Haemostasis analyser (Haemonetics®, Haemonetics Australia Pty Ltd., North Rdye, Sydney 2113, Australia) as described (Debono et al., 2019, 2018). Human fibrinogen was reconstituted in enzyme buffer (150 mM NaCl and 50 mM Tri-HCl (pH 7.3)). Briefly, 7 µl venom working stock (1 mg/ml outlined in (Debono et al., 2017)) or 7 µl thrombin as a positive control (stable thrombin from Stago Liquid Fib kit, Stago Cat#115081 Liquid Fib), 72 µl $CaCl_2$ (25 mM stock solution Stago Cat# 00367 STA), 72 µl phospholipid (solubilized in Owren Koller Buffer adapted from STA C.K Prest standard kit, Stago Cat# 00597), and 20 µl Owren Koller Buffer (Stago Cat# 00360) were combined with 189 µl fibrinogen or human plasma and run immediately for 30 min to allow for ample time for clotting formation. An additional positive control of 7 µl Factor Xa (Liquid Anti-Xa FXa Cat#253047, Stago) was also incorporated for plasma only. When no clot was formed from the effects of anticoagulant venoms, an additional 7 µl thrombin was added to the pin and cup to generate a clot and to determine the effects of fibrinogen degradation.

2.5. Fibrinolysis

The ability for venoms of the *Gloydius* and *Ovophis* genus to actively lyse fibrin clots was investigated following methods described previously (Debono et al., 2019, 2018). Varying concentrations of crude venom (1–0.1 µg/µl) were tested either with or without the addition of tissue plasminogen activator (tPA, Sekisui Diagnostics, Lexington, MA, USA). Briefly, tissue factor (TF) (Innovin, Siemens, USA) and phospholipid vesicles (PCPS, 75% phosphatidylcholine and 25% phosphatidylserine, Avanti Polar Lipids, Alabama, USA) were incubated at 37 °C for 1 h in HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 0.1% BSA (Bovine Serum Albumin A7030, Sigma Aldrich, St Louis, MD, USA), pH 7.4). To the TF/PCPS mixture (1.8 pM/3 µM final), CaCl2 (17 mM final), tPA (37.5 U/ml final, diluted in 20 mM HEPES, 150 mM NaCl, 0.1% PEG-8000, pH 7.5), venom (50% v/v), and plasma (50% v/v, prewarmed at 37 °C) were added. The fibrin clot formation and the subsequent lysis were monitored by measuring the absorbance at 405 nm for every 30 s. during 3 h at 37 °C in a SpectraMax M2e microplate reader. The onset of clot formation was defined as the time point at which the turbidity increased (delta absorbance > 0.04); the clotting time was the time from the start of the assay to the onset of clot formation. The clot lysis time was the interval between the clear to turbid transition (defined as the midpoint between the onset of clot formation and the maximum turbidity) and the turbid to clear transition; the latter was determined by a sigmoidal fit of the turbidity plots using GraphPad Prism 7.

3. Results

3.1. Procoagulation and anticoagulation studies

At the initial 20 µg/ml venom concentration, only *G. tsushimaensis* (35 ± 3.7 s,) and *O. okinavensis* (54.6 ± 1 s) displayed the ability to clot plasma quicker than the negative control (spontaneous clotting of recalcified plasma) time of 350 ± 50 s (Fig. 1). All other venom species were equal or above the negative control (data not shown). An 8-point dilution series was undertaken with the two clotting venoms (*G. tsushimaensis* and *O. okinavensis*) under three experimental conditions: with both cofactors (calcium and phospholipid), in the absence of phospholipids, and in the absence of calcium (Fig. 1). Phospholipid was not a significant variable for either venom, shifting the clotting curve only 0.06 ± 0.01% for *G. tsushimaensis* and 0.004 ± 0.001 for *O. okinavensis*. However, for the calcium-dependence there was an area under the curve shift of 90 ± 4% for *G. tsushimaensis* ($p = 0.0075$ and therefore significant) and a shift of 65 ± 1% for *O. okinavensis* ($p = 0.0004$ and therefore significant) (Fig. 1). An 8-point dilution series was also undertaken for *G. tsushimaensis* and *O. okinavensis* with fibrinogen under the same three experimental conditions (Fig. 2). *Gloydius tsushimaensis* had an area under the curve shift of 52 ± 3% for phospholipid dependence ($p = 0.07$, and therefore only marginally insignificant), while *O. okinavensis* displayed a lower phospholipid dependence with a curve shift of 20 ± 5% ($p = 0.25$ and therefore insignificant). There was a significant dependency evident for calcium for *G. tsushimaensis*, with the area of the curve shifting 152 ± 5% (p = 0.007), while *O. okinavensis* displayed only lower calcium

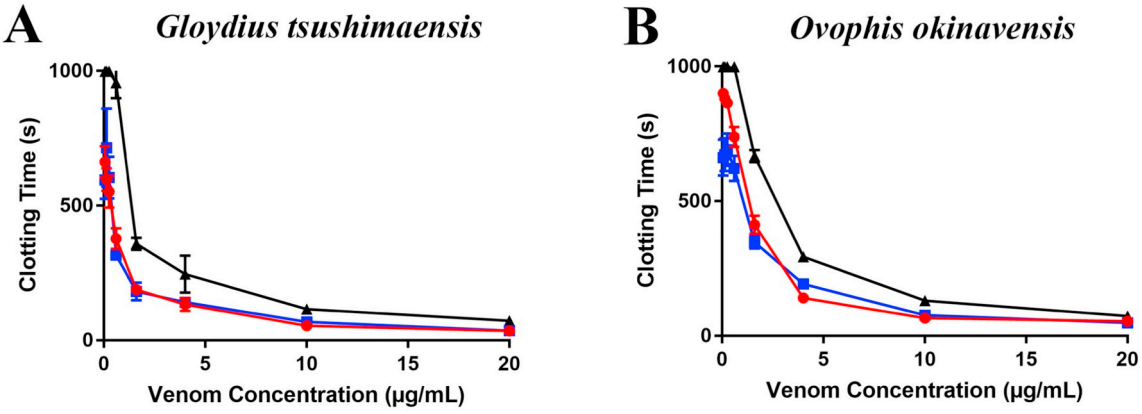

Fig. 2. Concentration-response curves the ability of the venoms to clot human fibrinogen in the presence of both calcium and phospholipid (red line), in the absence of phospholipids (blue line), or in the absence of calcium (black line) for A) *G. tsushimaensis* and B) *O. okinavensis*. Data points are $N = 3$ with standard deviations.

dependency with an area under the curve shift of 21 ± 2% ($p = -0.25$ and therefore insignificant) (Fig. 2).

As the clotting of plasma could be due to procoagulant functions through the generation of endogenous thrombin (producing strong fibrin clots), or pseudo-procoagulant functions by directly acting upon fibrinogen (producing weak clots), additional tests were conducted to ascertain the ability to activate Factor X or prothrombin, and also to determine the relative strength of fibrin clots in the plasma tests. None of the venoms, even the ones which produced clots in the above analyses, were able to activate FX or prothrombin, thus indicating pseudo-procoagulant actions directly upon fibrinogen.

Our study aimed to characterise the function and underlying haemorrhagic mechanisms of the *Gloydius* genus. Addition of the remaining venoms to recalcified human plasma extended the clotting time which exceeded the machine maximum reading time of 999 s. Investigations to determine the site of action proceeded in a stepwise manner in order to ascertain specific sites of action (Supplementary Table 2): i) incubation with FXa followed by addition of plasma to determine the ability to directly inhibit FXa; ii) incubation with plasma followed by addition of FXa to determine effects on prothrombinase complex formation (with the site of action determined by comparison with iii and iv, whereby if a strong effect was noted here but corresponding strong effects were not noted in iii or iv, then this indicated inhibition of the formation of the prothrombinase complex, as previously validated by us (Youngman et al., 2018); iii) incubation with thrombin followed by addition of fibrinogen to determine direct inhibition of thrombin; and iv) incubation with fibrinogen followed by addition of thrombin to determine the ability to degrade fibrinogen. Results were as follows (Fig. 3): i) intra-specific and inter-specific variation in FXa inhibition was notable, with the Russian *G. saxatilis* population being potent in this regard, but the Korean *G. saxatilis* were low in activity, with the other species showing activities levels approximately half of that of the Russian *G. saxatilis* (*G. brevicaudus*, *G. tsushimaensis*, and *G. ussuriensis*) or inactive in this assay (*O. okinavensis*) (Fig. 3A); ii) incubation with plasma followed by addition of FXa did not produce a relative strong effect for any of the venoms (Fig. 3B); iii) incubation with thrombin also revealed negligible effects in this inhibitory function (Fig. 3C); iv) incubation with fibrinogen revealed a very strong non-clotting, destructive fibrinogenolytic effect for *G. brevicaudus*, a lower but still strong effect for on *G. ussuriensis* male venom sample, but only moderate to low for all other samples (Fig. 3D).

3.2. Fibrinogenolytic activity

In order to determine the specific fibrinogen chains cleaved by the venoms, additional assays were undertaken to determine the time-dependent effects upon fibrinogen each chain (Figs. 4 and 5). A wide variation in pattern emerged, with many of the venoms displaying the ability to cleave the alpha chain (with the exception of *G. saxatilis* [Korea, male]). In most other samples, the alpha chain was initially quite rapidly degraded, quickly followed by the beta chain, and in some cases the gamma chain (*O. okinavensis*) displaying full cleavage of all three chains, with partial degradation of the gamma chain noted for *G. ussuriensis* and *G. tsushimaensis*. *Ovophis okinavensis* degraded all three chains the quickest, congruent with its ability to clot plasma. There was a geographical variation pattern emerging between Russian localities and Korean localities, with diversity in chain degradation shown among *G. ussuriensis* samples and *G. saxatilis* samples, with *G. saxatilis* being the most notable. For species with multiple samples (*G. saxatilis* Korea males and *G. ussuriensis* Korea male/females), representatives were chosen to be displayed as there was no differences between fibrinolytic actions and chain degradation within this assay (data not shown).

3.3. Thromboelastography

Clot strength on plasma was investigated in the presence of Ca^{2+} and phospholipid using thromboelastography (Fig. 6). There was a wide variation in ability to clot plasma, however these results coincided with additional above analysis on plasma. Plasma clotted faster than that of the negative control (SP = 8.5 ± 0.8, R = 9.9 ± 0.7, A = 15.4 ± 0.7, MRTGG 1.35 ± 0.09, TMRTG 11.8 ± 0.9 and TGG 90.7 ± 5.4), for *G. ussuriensis* (Russia) which formed a very small, weak clots (SP = 2.5 ± 0.3, R = 18.5 ± 2.1, A = 2.9 ± 0.8, MRTGG = 0.17 ± 0.01, TMRTG = 2.86 ± 0.34 and TGG = 14.2 ± 4.2); *G. tsushimaensis* which formed a very small, weak clot (SP = 1.9 ± 0.1, R = NA, A = 1.4 ± 0.05, MRTGG 0.27 ± 0.02, TMRTG 2.4 ± 0.2 and TGG 6.8 ± 0.3); *O. okinavensis* produced a more robust clot, albeit this being smaller than that of the controls (SP = 2.1 ± 0.1, R = 9.4 ± 2.5, A = 10.4 ± 0.5, MRTGG = 0.4 ± 0.08, TMRTG = 10.7 ± 4.5 and TGG = 57.3 ± 3.1). In contrast, *G. saxatilis* (Russia) delayed the formation of the clot, consistent with the FXa inhibition noted in Fig. 3A, and decreased the clot strength consistent with destructive, non-clotting cleavage of fibrinogen. *Gloydius brevicaudus*, *G. saxatilis* (Korea, male), and *G. ussuriensis* all prevented plasma spontaneous clotting, indicative of destructive, non-clotting cleavage of fibrinogen.

As the actions of *G. ussuriensis* (Russia), *G. tsushimaensis*, and *O. okinavensis* indicated pseudo-procoagulant effects whereby fibrinogen was directly cleaved by the venom, to produce weak fibrin clots, additional thromboelastography studies were undertaken. In addition, these additional studies would determine if *G. brevicaudus*, *G. saxatilis* (Korea, male), and *G. ussuriensis* prevented plasma spontaneous clotting

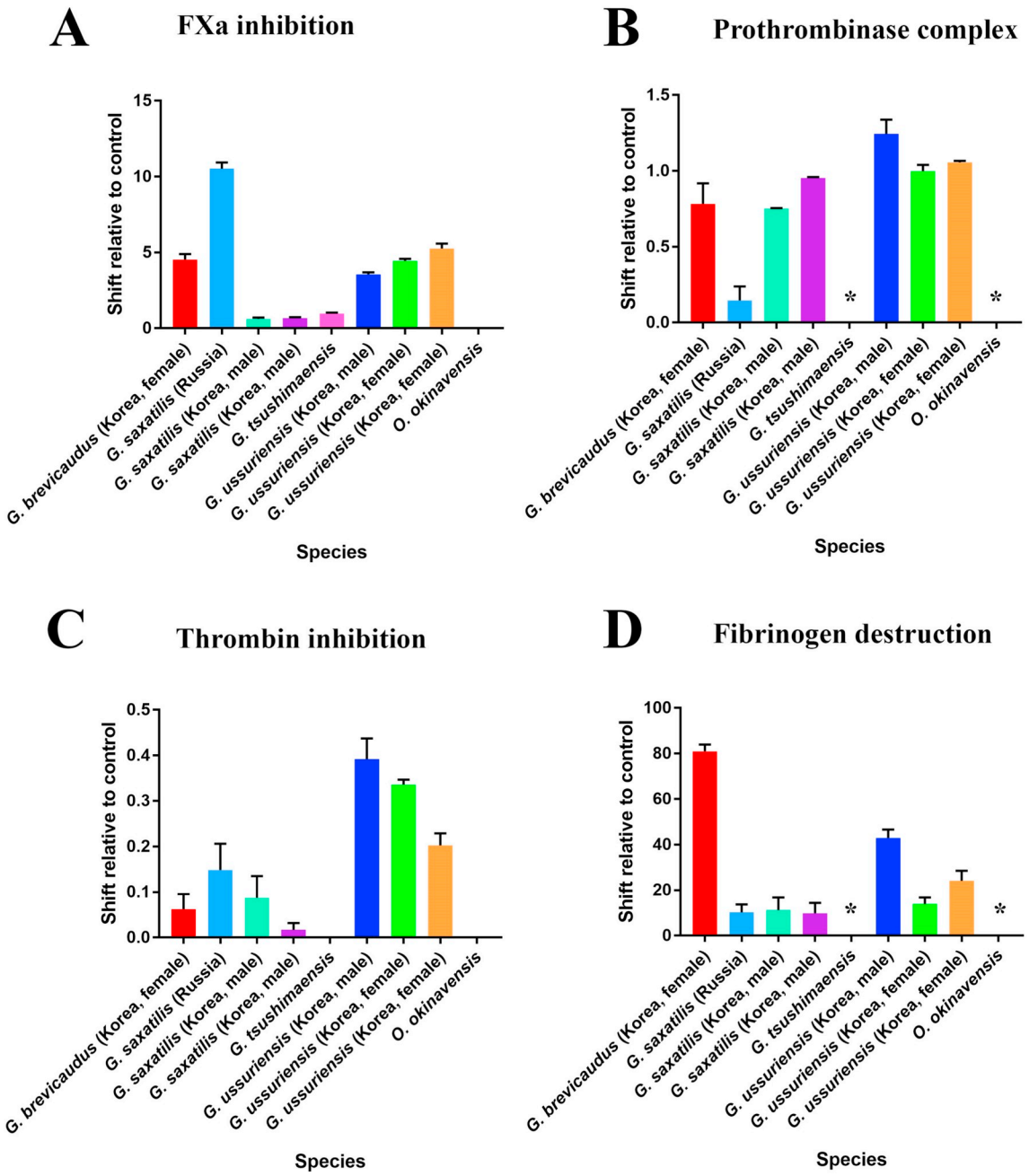

Fig. 3. Clotting factor inhibition showing the relative inhibitory effects of *Gloydius* and *Ovophis* venom on preventing the activation of: A) FXa, B) prothrombin to thrombin (Prothombinase complex), C) thrombin inhibition and D) destruction of fibrinogen. For A) and C) venom was incubated with FXa or thrombin for 2 min before adding plasma or fibrinogen, with clot time then immediately measured. For B) and D) venom was incubated with human plasma or fibrinogen for 2 min before adding Factor Xa or thrombin with clot time then immediately measured. Data points are N = 3 displayed as a shift away from the control with standard deviations (no shift from control values would be a zero value). Star icons represent values that could not be recorded due to premature clotting of plasma or fibringoen prior to addition of thrombin or FXa, thus no inhibition.

through the destructive, non-clotting cleavage of fibrinogen. In order to determine the strength of the clots formed by venom-dependent fibrinogen cleavage, we investigated the ability for the venoms to clot fibrinogen in the presence of Ca^{2+} and phospholipid using thromboelastography (Fig. 7). Results revealed wide variation between the venoms. Consistent with the effects noted upon plasma, *G. tsushimaensis*, *G. ussuriensis* (Russian pooled sample), and *O. okinavensis* all clotted fibrinogen within the initial 30 min analysis in a pseudo-procoagulant manner with a reduction in clot strength relative to the thrombin control. As the remainder of the samples did not display any ability to directly clot fibrinogen within the allocated 30 min, thrombin was added after 30 min to attempt to generate a clot and to determine if the venoms were destroying fibrinogen directly to impede a clot being formed (Fig. 7). All fibrinogen samples which were not clotted by the venoms directly, displayed high level of destructive cleavage of fibrinogen after the addition of thrombin, whereby thrombin produced only weak clots for some venoms (*G. brevicaudus*, and *G. ussuriensis* female), consistent with substantial degradation of the fibrinogen by the venoms, yet was unable to produce clots at all for other venoms (*G. ussuriensis* male, and *G. saxatilis*) consistent with complete destruction of fibrinogen by the venoms. Diversification in fibrinogenolytic activity was shown between *G. ussuriensis* samples from Korea *versus* Russia. For species with multiple samples (*G. saxatilis* Korea males and *G. ussuriensis* Korea females), representatives were chosen to be displayed as

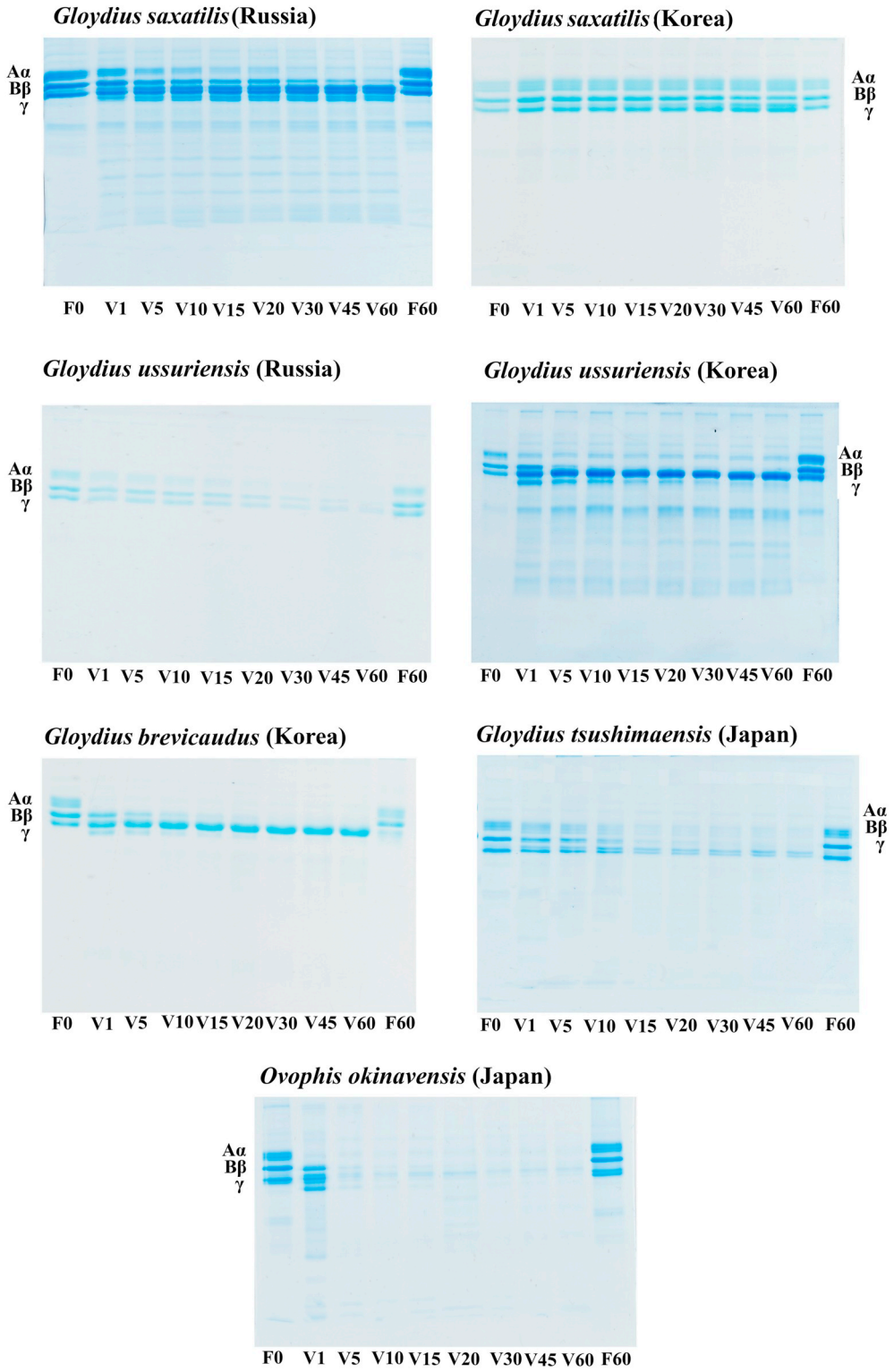

Fig. 4. 1D SDS PAGE time dependent fibrinogen chain degradation (α, β or γ) by venom at 0.1 μg/μl concentration at 37 °C over 60 min. F = fibrinogen at 0 min or 60 min incubation controls, V = venom at 1, 5… 60 min incubation.

there was no differences in clot time, clot strength or clot output among them within these assays (data not shown).

3.4. Fibrinolysis assessment

The ability for the venoms to actively lyse plasma clots was investigated with the presence and absence of tPA (tissue plasminogen activator) (Fig. 8). Although plasma clots were unable to be lysed by any of the venoms, some species increased the ability for tPA to lyse clots. In the presence of tPA, some species (*G. brevicaudus*, *G. tsushimaensis* pooled) were able to decrease clot lyses time (CLT) as compared to the control (Fig. 8). This could be due to fibrinogen structure of the clot in the presence of venom whereby tPA is better able to lyse a particular structure. Albeit, coupled with thromboelastography clot

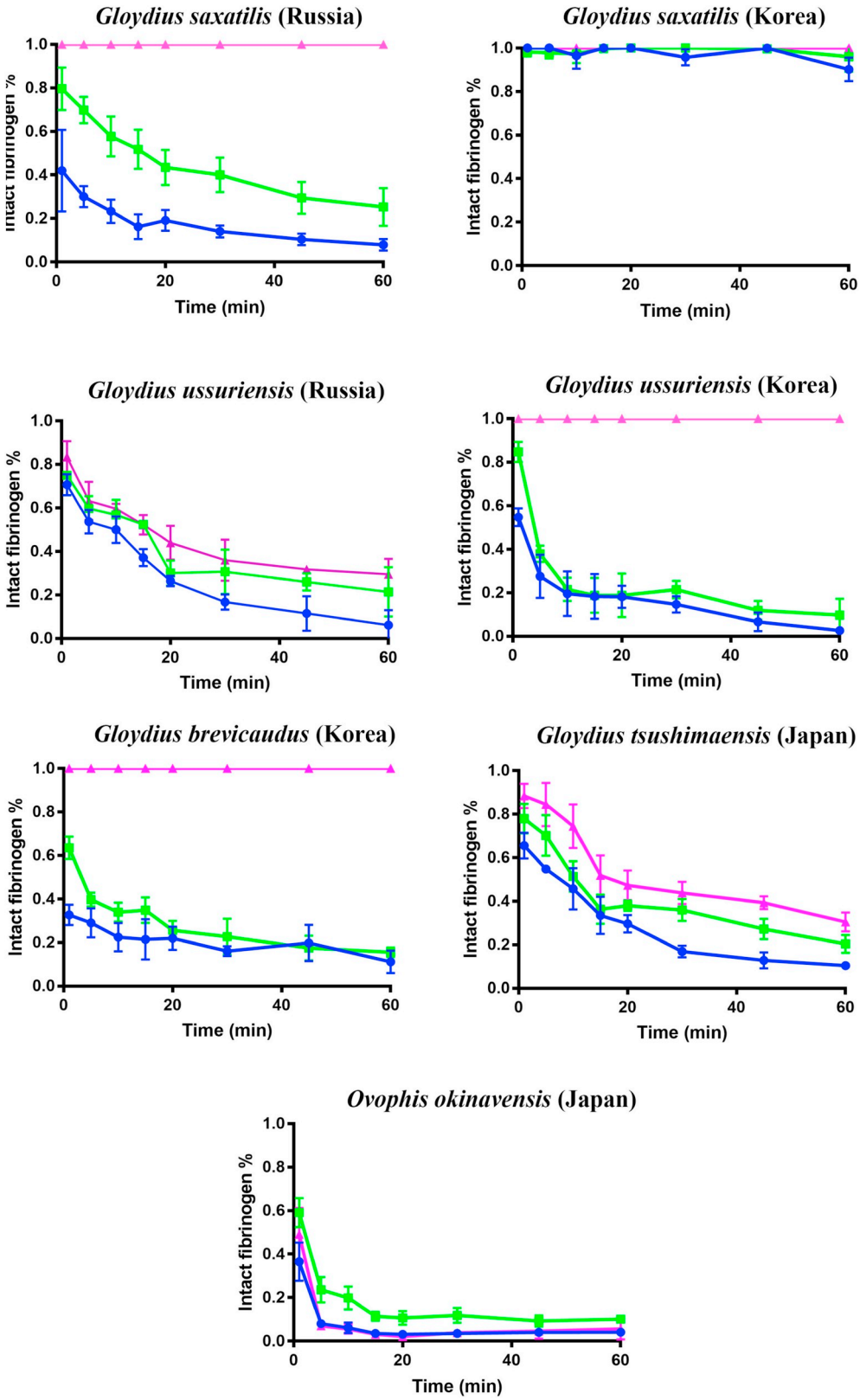

Fig. 5. Relative cleavage of alpha (blue), beta (green) or gamma (pink) chains of fibrinogen. X-axis is time (min), y-axis is percentage of intact chain remaining. Error bars indicate standard deviation and N = 3 means.

strength outputs, the ability for these specific venoms to reduce clot strength was demonstrated by all. Due to venom availability at the time of assay completion, certain venoms were unable to be included in this assay.

3.5. Snake venom serine protease contribution

As *Gloydius tsushimaensis* and *Ovophis okinavensis* both displayed clotting ability in the above analysis, both species were subjected to

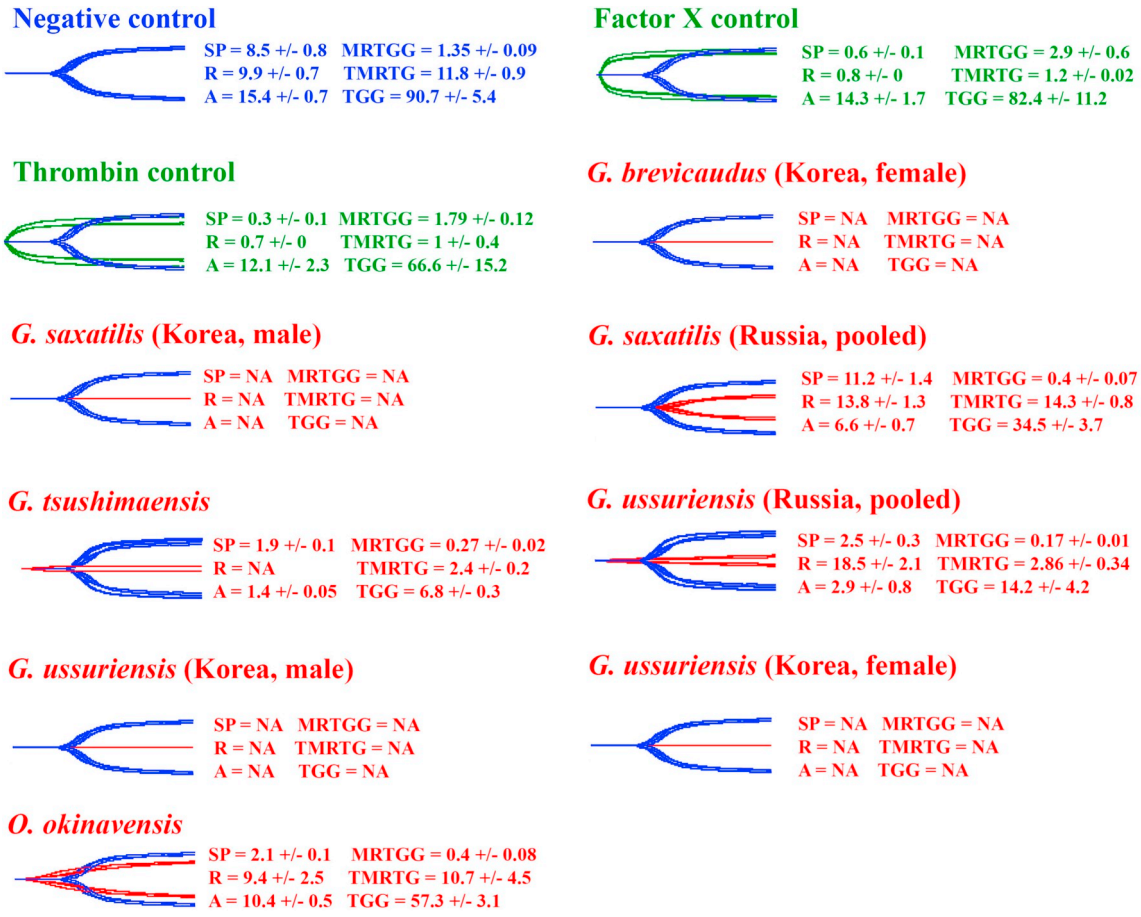

Fig. 6. Overlaid thromboelastography traces showing effects of venoms ability to clot plasma relative to spontaneous clot control where species cleave plasma in a clotting manner to form weak clots. Blue traces = spontaneous clot controls, green traces = thrombin induced clot or Factor Xa induced clot, red traces = samples. SP = split point, time taken until clot begins to form (min). R = time to initial clot formation where formation is 2 mm + (min). A = amplitude of detectable clot (mm). MRTGG = maximum rate of thrombus generation (dsc, dynes/cm^2/s). TMRTG = time to maximum rate of thrombus generation (min). TGG = total thrombus generation (dynes/cm^2). Overlaid traces are N = 3 for each set of control or experimental conditions. Values are N = 3 means and standard deviation.

additional inhibition studies. Fibrinogen clotting by *Ovophis okinavensis* venom was completely inhibited by 2 mM AEBSF (venom without inhibitor = 23.5 ± 1.3, while venom + inhibitor = 999.99 ± 0 s), while that of the *G. tsushimaensis* venom was only partially inhibited (venom without inhibitor = 59.0 ± 1.6, while venom + inhibitor 207.8 ± 27.6 s) under these conditions (Fig. 9), as clotting times for fibrinogen did not reach the maximum output of 999.99 s for *G. tsushimaensis*. This indicates that the venom of *O. okinavensis* is heavily dominated by SVSP proteins actively responsible for fibrinogen-specific coagulation effects, as opposed to *G. tsushimaensis*, which may have a wider composition of coagulatoxic proteins including contribution by metalloproteases.

4. Discussion

Our study aimed to investigate the differential coagulotoxic mechanisms within *Gloydius* venoms across majority of the genus, with the addition of *O. okinavensis*. *Ovophis okinavensis* was added as an additional species as it sits basal to *Gloydius* and is phylogenetically distinct from all other species currently placed in the *Ovophis* genus (Alencar et al., 2018; Malhotra and Thorpe, 2004) and therefore may be included in an expanded consideration of the *Gloydius* genus along with '*Trimeresurus*' *gracilis*. Extensive functional and coagulotoxic variation was evident among all samples tested across a myriad of assays, depicting variances across the genus and within a single species of differing geographical locations, with venoms falling into two broad anticoagulant functional categories in regards to their actions upon fibrinogen: directly anticoagulant through the destructive cleavage of fibrinogen (*G. brevicaudus*, *G. saxatilis*, *G. ussuriensis* [Korea population]); and pseudo-procoagulant whereby fibrinogen levels are depleted by cleavage to form weak, unstable, short-lived fibrin clots (*G. tsushimaensis*, *G. ussuriensis* [Russia population], and *O. okinavensis*). Inhibition of clotting enzymes was not shown to be a major feature, with the exception of the potent FXa inhibition effect noted for the Russian population of *G. saxatilis*, and more moderate yet still considerable FXa inhibition by *G. brevicaudus* and *G. ussuriensis*. Inhibition of FXa would have a synergistic anticoagulant activity with the depletion of fibrinogen levels. Coagulatoxic effects from *O. okinavensis* can be directly attributed to its venom composition being dominated by SVSPs (Fig. 9) (Aird et al., 2013). Comparatively, *G. tsushimaensis* pseudo-procoagulant effects are due to a more complex venom composition, as fibrinogen clotting was only partially inhibited by the known serine protease inhibitor AEBSF (Kuniyoshi et al., 2017; Torres-Bonilla et al., 2018; Xin et al., 2009; Yamashita et al., 2014). This variation between two pseudo-procoagulant venoms with seemingly similar venom actions *in vitro* can have vast implications clinically as the variation in underlying venom biochemistry is indicative of differential toxins exerting the pathophysiological actions, which may therefore result in variations in antivenom efficacy. Future work is needed however to elucidate such variations in antivenom efficacy in order to fully ascertain such clinical implications.

Variation in action upon fibrinogen was shared among the other

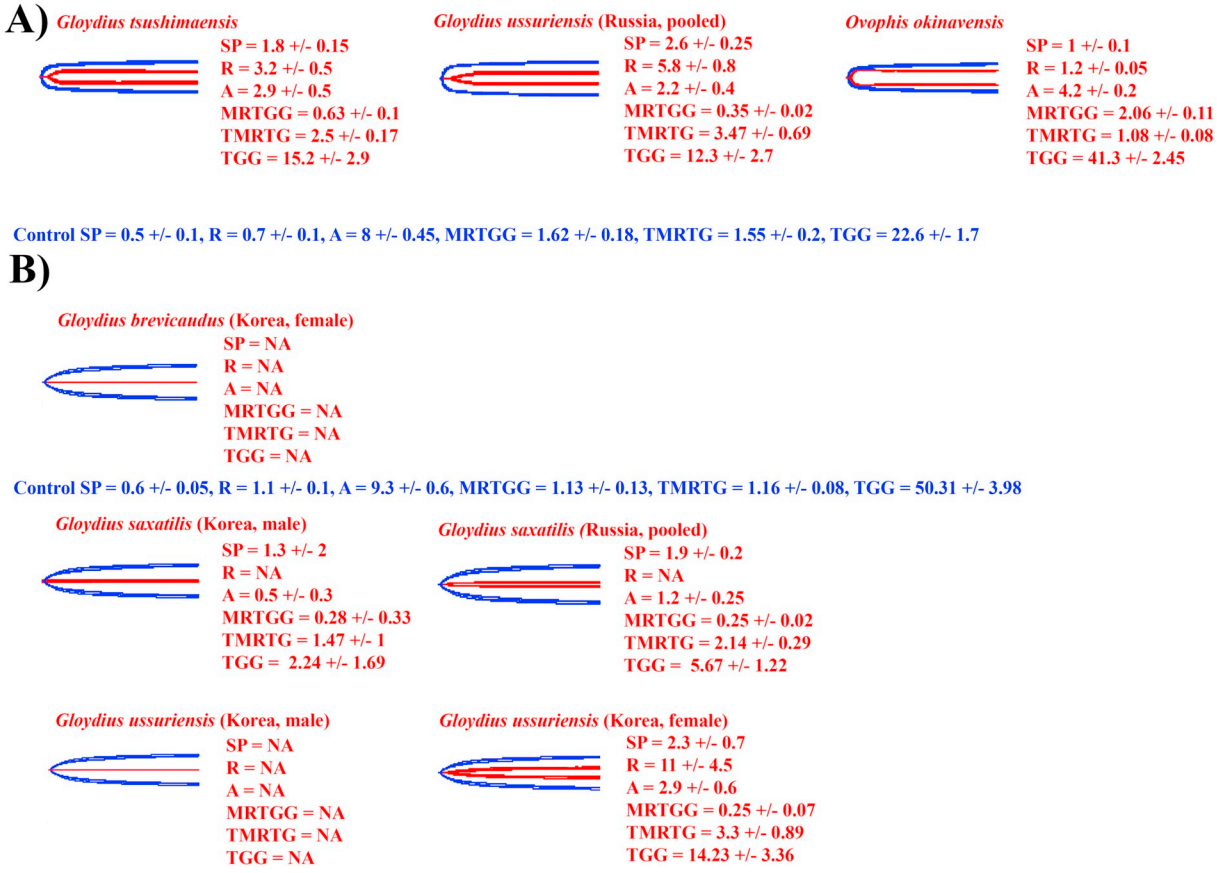

Fig. 7. Overlaid thromboelastography traces showing tests for A) ability to clot fibrinogen relative to thrombin control; or B) test for the ability to degrade fibrinogen for species which did not clot in (A) whereby thrombin was added at the end of the 30 min runs to test for intact fibrinogen. Blue traces = thrombin controls, red traces = samples. SP = split point, time taken until clot begins to form (min). R = time to initial clot formation where formation is 2 mm + (min). A = amplitude of detectable clot (mm). MRTGG = maximum rate of thrombus generation (dynes/cm^2/s). TMRTG = time to maximum rate of thrombus generation (min). TGG = total thrombus generation (dynes/cm^2). Overlaid traces are N = 3 for each set of control or experimental conditions. Values are N = 3 means and standard deviation.

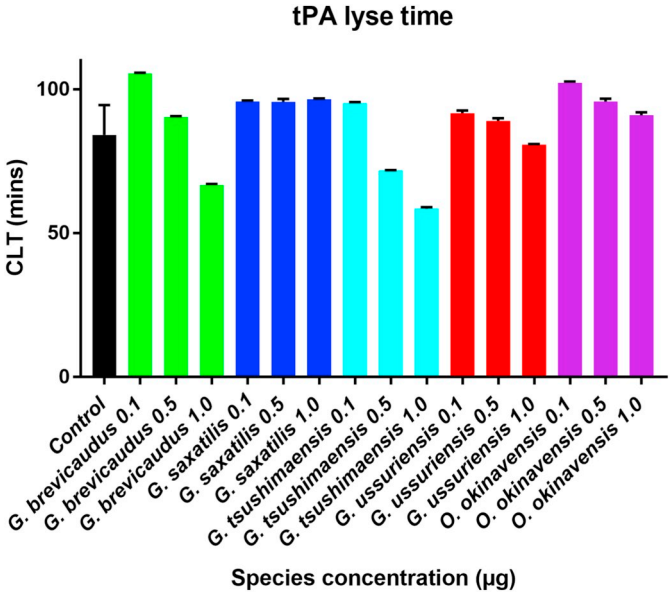

Fig. 8. Clot Lysis Time (CLT) for each species within the basal clade in the presence of tPA at 0.1 µg, 0.5 µg and 1 µg venom concentrations. The CLT of normal human plasma in the absence of venom is indicated in black. Columns are averages of triplicates and error bars given for each.

species included within this study. Ecological niche partitioning and a shift in diet would contribute to venom evolution of the species across varying localities and habitats, with consequential impacts upon relative clinical effects (Casewell et al., 2013; Fry et al., 2008). Such wide variation depicted within a single genus has also been shown within many other groups of closely related snakes, each inhabiting slightly varied ecological niches (Debono et al., 2019, 2018, 2017; Dobson et al., 2018; Rogalski et al., 2017) as well as variation between localities of the same species (Sousa et al., 2018). While some ontogenetic shift has been shown within one species of *Gloydius* (Gao et al., 2013), due to lack of sample availability the impact upon relative coagulotoxicity was not investigated in the present study and should be the subject of future work.

Variation in venom, dependent on its geographical location, can create many new challenges when seeking medical attention post envenomation. With antivenoms being reared among one or two species of a single locality, envenomation from another locality is not always effectively neutralised, as shown, for example, for the *Bothrops* genus of pit vipers of South America (Sousa et al., 2018), *Crotalus* (Dobson et al., 2018), and *Echis* genus of true vipers in Africa (Rogalski et al., 2017). Such complications must be taken into consideration by clinicians when treating an envenomated patient, while differences in venom composition lead to differences in relative venom efficacy. Future work should investigate the variations in antivenom efficacy for the available antivenoms against these species (and others within this genus) in order to map out problematic species or localities.

The variations exhibited by these species in this study highlight the

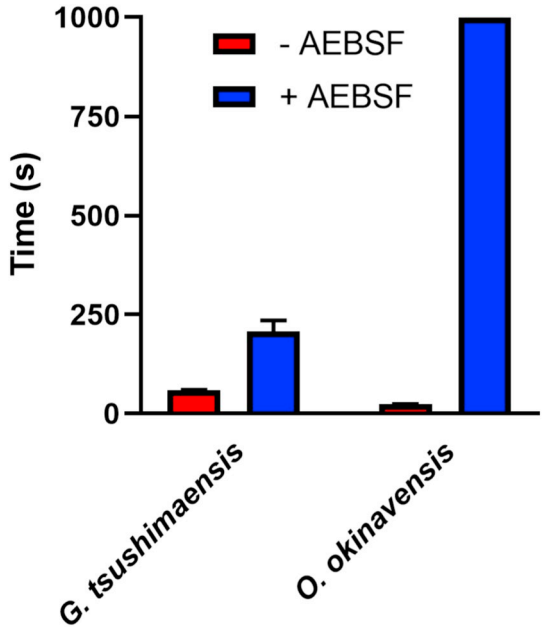

Fig. 9. Snake venom serine protease inhibition. Venom-induced fibrin clot formation of fibrinogen was analysed for *G. tsushimaensis* or *O. okinavensis* venom in the presence or absence of the serine protease inhibitor AEBSF (2 mM). The values represent the average ± S.D. of three identical experiments. 999 = machine maximum reading time.

importance of a combined evolutionary and clinical approach to snake venom research. While clinical biology can demonstrate physiological effects, evolutionary biology can communicate variations among a genus and infer possible explanations as seen with *G. tsushimaensis* and *O. okinavensis*, two species with similar *in vitro* effects occupying two extremely isolated habitats. Although this study included only a select few species and of geographical locations, the results are indicative of a large clinical and biodiscovery potential for future medical implications and bite case management. The substantial differences in fundamental venom biochemistry of these species indicate potential limitations in intra-specific and inter-specific cross-reactivity for antivenoms which include *Gloydius* venoms in the immunizing mixtures. Future research should investigate such limitations in these life-saving medications.

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References

Aird, S.D., Watanabe, Y., Villar-Briones, A., Roy, M.C., Terada, K., Mikheyev, A.S., 2013. Quantitative high-throughput profiling of snake venom gland transcriptomes and proteomes (*Ovophis okinavensis* and *Protobothrops flavoviridis*). BMC Genomics 14, 790.

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

Alencar, L.R., Martins, M., Greene, H.W., 2018. Evolutionary history of vipers. eLS 1–10.

Casewell, N.R., Wüster, W., Vonk, F.J., Harrison, R.A., Fry, B.G., 2013. Complex cocktails: the evolutionary novelty of venoms. Trends Ecol. Evol. 28, 219–229.

Cho, S.Y., Hahn, B.-S., Yang, K.Y., Kim, Y.S., 2001. Purification and characterization of calobin II, a second type of thrombin-like enzyme from *Agkistrodon caliginosus* (Korean viper). Toxicon 39, 499–506.

Choi, S.-H., Lee, S.-B., 2013. Isolation from *Gloydius blomhoffii siniticus* venom of a fibrin (ogen) olytic enzyme consisting of two heterogenous polypeptides. J. Pharmacopunct. 16, 46.

Coimbra, F.C., Dobson, J., Zdenek, C.N., Op den Brouw, B., Hamilton, B., Debono, J., Masci, P., Frank, N., Ge, L., Kwok, H.F., Fry, B.G., 2018. Does size matter? Venom proteomic and functional comparison between night adder species (Viperidae: *Causus*) with short and long venom glands. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 211, 7–14.

Debono, J., Dobson, J., Casewell, N.R., Romilio, A., Li, B., Kurniawan, N., Mardon, K., Weisbecker, V., Nouwens, A., Kwok, H.F., Fry, B.G., 2017. Coagulating Colubrids: evolutionary, pathophysiological and biodiscovery implications of venom variations between boomslang (*Dispholidus typus*) and twig snake (*Thelotornis mossambicanus*). Toxins 9, 171.

Debono, J., Bos, M.H., Nouwens, A., Ge, L., Frank, N., Kwok, H.F., Fry, B., 2018. Habu coagulotoxicity: clinical implications of the functional diversification of *Protobothrops* snake venoms upon blood clotting factors. Toxicol. in Vitro 55, 62–74.

Debono, J., Bos, M.H., Coimbra, F., Ge, L., Frank, N., Kwok, H.F., Fry, B., 2019. Basal but divergent: clinical implications of differential coagulotoxicity in a clade of Asian vipers. Toxicol. in Vitro 58, 195–206.

Dobson, J., Yang, D.C., Op den Brouw, B., Cochran, C., Huynh, T., Kurrupu, S., Sánchez, E.E., Massey, D.J., Baumann, K., Jackson, T.N., Nouwens, A., Josh, P., Neri-Castro, E., Alagón, A., Hodgson, W.C., Fry, B.G., 2018. 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., Part C: Toxicol. Pharmacol. 205, 62–69.

Fry, B.G., 2018. Snakebite: when the human touch becomes a bad touch. Toxins 10, 170.

Fry, B.G., Winkel, K.D., Wickramaratna, J.C., Hodgson, W.C., Wüster, W., 2003. Effectiveness of snake antivenom: species and regional venom variation and its clinical impact. J. Toxicol., Toxin Rev. 22, 23–34.

Fry, B.G., Scheib, H., van der Weerd, L., Young, B., McNaughtan, J., Ramjan, S.R., 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, 215–246.

Fujisawa, D., Yamazaki, Y., Morita, T., 2009. Re-evaluation of M-LAO, l-amino acid oxidase, from the venom of *Gloydius blomhoffi* as an anticoagulant protein. J. Biochem. 146, 43–49.

Gao, J.-F., Qu, Y.-F., Zhang, X.-Q., He, Y., Ji, X., 2013. Neonate-to-adult transition of snake venomics in the short-tailed pit viper, *Gloydius brevicaudus*. J. Proteome 84, 148–157.

Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017. Snakebite envenoming. Nat. Rev. Dis. Primers. 3, 17063.

Hifumi, T., Yamamoto, A., Morokuma, K., Ogasawara, T., Kiriu, N., Hasegawa, E., Inoue, J., Kato, H., Koido, Y., Takahashi, M., 2011. Surveillance of the clinical use of mamushi (*Gloydius blomhoffii*) antivenom in tertiary care centers in Japan. Jpn. J. Infect. Dis. 64, 373–376.

Hifumi, T., Sakai, A., Kondo, Y., Yamamoto, A., Morine, N., Ato, M., Shibayama, K., Umezawa, K., Kiriu, N., Kato, H., 2015. Venomous snake bites: clinical diagnosis and treatment. J. Intensive Care 3, 16.

Huang, K., Zhao, W., Gao, Y., Wei, W., Teng, M., Niu, L., 2011. Structure of saxthrombin, a thrombin-like enzyme from *Gloydius saxatilis*. Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun. 67, 862–865.

Jones, B.K., Saviola, A., Reilly, S.B., Stubbs, A.L., Arida, E., Iskandar, D.T., McGuire, J.A., Yates, J.R., Mackessy, S.P., 2019. Venom composition in a phenotypically variable pit viper (*Trimeresurus insularis*) across the Lesser Sunda Archipelago. J. Proteome Res. 18 (5), 2206–2220.

Joseph, J.S., Kini, R.M., 2002. Snake venom prothrombin activators homologous to blood coagulation factor Xa. Pathophysiol. Haemost. Thromb. 31, 234–240.

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. PLoS Med. 5, e218.

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

Kini, R.M., 2006. Anticoagulant proteins from snake venoms: structure, function and mechanism. Biochem. J. 397, 377–387.

Kornalik, F., Blombäck, B., 1975. Prothrombin activation induced by Ecarin-a prothrombin converting enzyme from *Echis carinatus* venom. Thromb. Res. 6, 53–63.

Kuniyoshi, A.K., Kodama, R.T., Moraes, L.H.F., Duzzi, B., Iwai, L.K., Lima, I.F., Cajado-Carvalho, D., Portaro, F.V., 2017. *In vitro* cleavage of bioactive peptides by peptidases from *Bothrops jararaca* venom and its neutralization by bothropic antivenom produced by Butantan Institute: major contribution of serine peptidases. Toxicon 137, 114–119.

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

Malhotra, A., Thorpe, R.S., 2004. A phylogeny of four mitochondrial gene regions suggests a revised taxonomy for Asian pitvipers (*Trimeresurus* and *Ovophis*). Mol. Phylogenet. Evol. 32, 83–100.

Okamoto, O., Nakashima, R., Yamamoto, S., Hashimoto, T., Takasaki, T., Tokuda, H., Sato, S., Gamachi, A., Hashimoto, H., Inagaki, N., 2017. A lethal case of mamushi (*Gloydius blomhoffii*) bite: severe bowel symptoms as a lethal sign. Acute Med. Surg. 4, 135–139.

Oulion, B., Dobson, J.S., Zdenek, C.N., Arbuckle, K., Lister, C., Coimbra, F.C., Op den Brouw, B., Debono, J., Rogalski, A., Violette, A., Fourmy, R., Frank, N., Fry, B.G., 2018. Factor X activating *Atractaspis* snake venoms and the relative coagulotoxicity neutralising efficacy of African antivenoms. Toxicol. Lett. 288, 119–128.

Panagides, N., Jackson, T., Ikonomopoulou, M., Arbuckle, K., Pretzler, R., Yang, D., Ali, S., Koludarov, I., Dobson, J., Sanker, B., Asselin, A., Santana, R.C., Hendrikx, I., Van der Ploeg, H., Tai-A-Pin, J., Van den Bergh, R., Kerkkamp, H.M.I., Vonk, F.J., Naude,

A., Strydom, M.A., Jacobsz, L., Dunstan, N., Jaeger, M., Hodgson, W.C., Miles, J., Fry, B.G., 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 9, 103.

Rogalski, A., Soerensen, C., Op den Brouw, B., Lister, C., Dashevsky, D., Arbuckle, K., Gloria, A., Zdenek, C.N., Casewell, N.R., Gutiérrez, J.M., 2017. Differential procoagulant effects of saw-scaled viper (Serpentes: Viperidae: *Echis*) snake venoms on human plasma and the narrow taxonomic ranges of antivenom efficacies. Toxicol. Lett. 280, 159–170.

Rosing, J., Tans, G., 1991. Inventory of exogenous prothrombin activators. Thromb. Haemost. 66, 627–630.

Rosing, J., Tans, G., 1992. Structural and functional properties of snake venom prothrombin activators. Toxicon 30, 1515–1527.

Rosing, J., Tans, G., 2010. Snake Venom Prothrombin Activators–The History, Toxins and Hemostasis. Springer, pp. 485–499.

Slagboom, J., Kool, J., Harrison, R.A., Casewell, N.R., 2017. Haemotoxic snake venoms: their functional activity, impact on snakebite victims and pharmaceutical promise. Br. J. Haematol. 177, 947–959.

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

Sun, M.-Z., Liu, S., Greenaway, F.T., 2006. Characterization of a fibrinolytic enzyme (ussurenase) from *Agkistrodon blomhoffii ussurensis* snake venom: insights into the effects of Ca2+ on function and structure. Biochim. Biophys. Acta, Proteins Proteomics 1764, 1340–1348.

Sunagar, K., Undheim, E.A., Scheib, H., Gren, E.C., Cochran, C., Person, C.E., Koludarov, I., Kelln, W., Hayes, W.K., King, G.F., Antunes, A., Fry, B.G., 2014. Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (*Crotalus oreganus helleri*): biodiscovery, clinical and evolutionary implications. J. Proteome 99, 68–83.

Tan, C.H., Liew, J.L., Tan, K.Y., Tan, N.H., 2016. Genus *Calliophis* of Asiatic coral snakes: a deficiency of venom cross-reactivity and neutralization against seven regional elapid antivenoms. Toxicon 121, 130–133.

Tan, C.H., Liew, J.L., Tan, N.H., Ismail, A.K., Maharani, T., Khomvilai, S., Sitprija, V., 2017. Cross reactivity and lethality neutralization of venoms of Indonesian *Trimeresurus* complex species by Thai Green Pit Viper Antivenom. Toxicon 140, 32–37.

Tans, G., Rosing, J., 2002. Snake venom activators of factor X: an overview. Pathophysiol. Haemost. Thromb. 31, 225–233.

Toh Yoon, E.W., Otani, Y., Kabuto, S., 2017. Severe Japanese Mamushi (*Gloydius blomhoffii*) bite. Clin. Case Rep. 5, 1548–1549.

Torres-Bonilla, K.A., Andrade-Silva, D., Serrano, S.M., Hyslop, S., 2018. Biochemical characterization of venom from *Pseudoboa neuwiedii* (Neuwied's false boa; *Xenodontinae*; *Pseudoboini*). Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 213, 27–38.

Vogel, G., 2006. Venomous Snakes of Asia. (Edition Chimaira).

Williams, D.J., Gutiérrez, J.-M., Calvete, J.J., Wüster, W., Ratanabanangkoon, K., Paiva, O., Brown, N.I., Casewell, N.R., Harrison, R.A., Rowley, P.D., 2011. Ending the drought: new strategies for improving the flow of affordable, effective antivenoms in Asia and Africa. J. Proteome 74, 1735–1767.

Xin, Y., Dong, D., Chen, D., Li, R., 2009. Structural and biological characterization of a novel acutobin-like enzyme isolated from the venom of the sharp-nosed pit viper (*Deinagkistrodon acutus*). Biotechnol. Appl. Biochem. 53, 123–131.

Xu, N., Zhao, H.-Y., Yin, Y., Shen, S.-S., Shan, L.-L., Chen, C.-X., Zhang, Y.-X., Gao, J.-F., Ji, X., 2017. Combined venomics, antivenomics and venom gland transcriptome analysis of the monocoled cobra (*Naja kaouthia*) from China. J. Proteome 159, 19–31.

Yamada, D., Sekiya, F., Morita, T., 1996. Isolation and characterization of carinactivase, a novel prothrombin activator in *Echis carinatus* venom with a unique catalytic mechanism. J. Biol. Chem. 271, 5200–5207.

Yamashita, K.M., Alves, A.F., Barbaro, K.C., Santoro, M.L., 2014. *Bothrops jararaca* venom metalloproteinases are essential for coagulopathy and increase plasma tissue factor levels during envenomation. PLoS Negl. Trop. Dis. 8, e2814.

Yang, Z.-M., Yang, Y.-E., Chen, Y., Cao, J., Zhang, C., Liu, L.-L., Wang, Z.-Z., Wang, X.-M., Wang, Y.-M., Tsai, I.-H., 2015. Transcriptome and proteome of the highly neurotoxic venom of Gloydius intermedius. Toxicon 107, 175–186.

Yang, D.C., Deuis, J.R., Dashevsky, D., Dobson, J., Jackson, T.N., 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., Lim, K.K.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 (*Calliophis bivirgatus*). Toxins 8, 303.

Youngman, N.J., Zdenek, C.N., Dobson, J.S., Bittenbinder, M.A., Gillett, A., Hamilton, B., Dunstan, N., Allen, L., Veary, A., Veary, E., 2018. Mud in the blood: novel potent anticoagulant coagulotoxicity in the venoms of the Australian elapid snake genus*Denisonia* (mud adders) and relative antivenom efficacy. Toxicol. Lett. 1;302, 1–6.

Zdenek, C.N., Hay, C., Arbuckle, K., Jackson, T.N., Bos, M.H., op den Brouw, B., Debono, J., Allen, L., Dunstan, N., Morley, T., Herrera, M., Gutiérrez, J.M., Williams, D.J., Fry, B.G., 2019. Coagulotoxic effects by brown snake (*Pseudonaja*) and taipan (*Oxyuranus*) venoms, and the efficacy of a new antivenom. Toxicol. in Vitro 58, 97–109.

Zhang, Q., Wang, J., Han, Y., Xie, Q., An, L., Bao, Y., 2007. Identification of a novel thrombin-like phospholipase A2 from Gloydius ussuriensis snake venom. Blood Coagul. Fibrinolysis 18, 723–729.