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Trimeresurus albolabris snakebite treatment implications arising from ontogenetic venom comparisons of anticoagulant function, and antivenom efficacy

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ABSTRACT

Does the venom of *Trimeresurus albolabris* (white-lipped pit viper) differ between neonate and adults? This species is responsible for most snakebites within south and southeast Asia, yet it is unknown whether ontogenetic variation in venom composition occurs in this species, or how this might affect antivenom efficacy. Using a coagulation analyser robot, we examined the anticoagulant activity of *T. albolabris* venom from eight individuals across multiple age classes. We then compared the efficacy of Thai Red Cross Green Pit Viper Antivenom across these age classes. Venoms from all age classes were equally potent in their pseudo-procoagulant, fibrinogenolytic activity, in that fibrinogen was cleaved to form weak, unstable fibrin clots that rapidly broke down, thus resulting in a net anticoagulant state. Similarly, this coagulotoxic activity was well neutralised by antivenom across all venoms. Given that coagulotoxicity is the primary serious pathology in *T. albolabris* envenomations, we conclude that Thai Red Cross Green Tree Pit Viper Antivenom is a valid treatment for envenomations by this species, regardless of age or sex of the offending snake. These results are relevant for clinical treatment of envenomations by *T. albolabris*.

1. Introduction

Snakebite is a global public health issue (Chippaux, 1998; Fry, 2018; Kasturiratne et al., 2008) and is often associated with disruption of blood coagulation pathways (Kini and Koh, 2016; White, 2005). In human snakebite victims, the venom can disrupt the coagulation cascade in either a procoagulant or anticoagulant manner. Procoagulant venoms exhibit their effects by either activating thrombin directly, through converting prothrombin to thrombin, or indirectly by activating upstream zymogens such as Factor X (Lister et al., 2017; Oulion et al., 2018; Rogalski et al., 2017; Zdenek et al., 2019a, b). Anticoagulant venoms produce their effect in one of two ways: anticoagulation through inhibition of coagulation factors, or depletion of fibrinogen levels by either cleaving in a destructive manner or cleaving in a pseudo-procoagulant manner such that weak, unstable, short-lived fibrin clots are formed that rapidly degrade (Bittenbinder et al., 2019, 2018; Debono et al., 2019a, b; Debono et al., 2019d; Youngman et al., 2019a, b).

Snake venoms are known to vary greatly between species, across geographical ranges within a species, between individuals of the same species, by sex, and by age (Amorim et al., 2018; Casewell et al., 2014; Cipriani et al., 2017; Gibbs et al., 2011; Jackson et al., 2016; Sousa et al., 2018; Tan et al., 2015; Zdenek et al., 2019b; Zelanis et al., 2016, 2010). The driving force behind intraspecies venom variation is thought to be prey specificity (Barlow et al., 2009; Daltry et al., 1996; Jorge da Silva and Aird, 2001), whereby diet affects venom composition so as to effectively immobilise specific prey types. Prey specialisation for different age groups can result in significant venom biochemistry changes during the life of a snake, such as in *Pseudonaja* (brown snake) species, whereby juveniles specialised upon lizard prey and had neurotoxic venoms, while adults specialised upon mammalian prey and had stroke-inducing procoagulant venoms (Cipriani et al., 2017; Jackson et al., 2016). Other studies have revealed no ontogenetic shift in snake venom, which suggests a consistent diet across life stages. For example, *Oxyuranus* (taipan) species specialise upon mammalian prey and have stroke-inducing, procoagulant venoms at all age classes (Jackson et al., 2016; Tan et al., 1992, 1993a).

Ontogenetic venom variation can have immediate implications for

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the treatment of the envenomed patients, as differences in the underlying biochemistry may radically influence the relative efficacy of antivenom treatment. Therefore, an imperative exists to identify parameters which influence venom variation within venomous snakes, particularly medically significant species.

The white-lipped pit viper (*Trimeresurus albolabris,*family: Viperidae) is an arboreal pit viper, inhabiting south and south-east Asia, recorded to feed mainly on small mammals and frogs (Devan-Song et al., 2017; Orlov et al., 2002). Being so widespread and abundant, *T. albolabris* is one of the most common causes of snakebites in its region (Cockram et al., 1990; Viravan et al., 1992), causing local swelling, necrosis, prolonged coagulopathy, defibrination (fibrinogen consumption), thrombocytopenia (low blood platelet counts), leucocytosis (abnormally high white blood cell counts), and spontaneous systemic bleeding in bite victims (Hutton et al., 1990; Pandey et al., 2019; Sarmin et al., 2013). Research has shown that the Thai Red Cross Green Tree Pit Viper antivenom is effective in neutralising the venom of adult specimens (Debono et al., 2019c). However, to date no studies have investigated the possibility of ontogenetic venom variation in this species. This study therefore investigated for the first time whether ontogenetic or sexual variation in venom potency occurs in *T. albolabris* venom, and whether this influenced antivenom efficacy.

2. Materials and methods

2.1. Venom stock preparation

All venom samples were provided in lyophilised form from the University of Queensland's Venom Evolution Lab's collection and stored in a −80 °C freezer. Eight venom samples of *T. albolabris* within life stages: adult female #1, adult female #2, adult male, sub-adult male, juvenile females (pooled n = 4), juvenile males (pooled n = 4), neonate females (pooled n = 4), and neonate males (pooled n = 5), were analysed in the study. The concentration of venom in each sample was calculated using a Thermo Fisher Scientific™ NanoDrop 2000 UV–Vis Spectrophotometer (Thermofisher, Sydney, NSW, Australia). Based on Nanodrop calculations the required volume of venom, deionised water and glycerol were added to 1.5 ml aliquots to make 1 mg/ml working stocks of venom. Glycerol was added to preserve enzymatic activity, and these stocks were stored in a −20 °C freezer.

2.2. Fibrinogen coagulation assays

A Stago STA-R Max haemostasis analyser (Stago, Asnières sur Seine, France) was employed to perform fibrinogen coagulation assays. To investigate the effect of venom on human fibrinogen clotting time, fibrinogen (4 mg/ml, Sigma Aldrich, St. Louis, Missouri, United States) was prepared by diluting 100 mg of fibrinogen with Owren Koller (OK) buffer (Stago Cat #00360) to a volume of 250 µl, pipetting 1 ml of solution into 1.5 ml tubes, and immediately flash freezing. Fibrinogen was stored in a −80 °C freezer and thawed for 5 min in a 37 °C water-bath before use in assays. In each analysis, 40 µl of venom was diluted with 360 µl of diluent (OK buffer) before being placed into the Stago STA-R Max haemostasis analyser. The clotting time of venom was measured in triplicate at eight different venom concentrations (20 µg/ml, 10 µg/ml, 4 µg/ml, 1.6 µg/ml, 0.66 µg/ml, 0.25 µg/ml, 0.125 µg/ml, and 0.05 µg/ml). For the 1:1 dilution (20 µg/ml) the machine added a starting volume of 50 µl of the venom sample, 50 µl of phospholipid (solubilised in OK buffer, Stago Cat# 00597), 50 µl of CaCl (25 mM stock solution Stago Cat# 00367), and 25 µl (OK buffer), and incubated in a cuvette for 120 s before the addition of 75 µl of fibrinogen. Volume of venom was automatically diluted with diluent (OK buffer) for subsequent dilutions. Clotting time was measured up until the machine max of 999 s, and each venom sample tested in triplicate. Eight-point dilution curves were graphed in GraphPad PRISM (GraphPad Prism Inc., La Jolla, CA, USA)

2.3. Antivenom assays

Antivenom assays were performed on a Stago STA-R Max haemostasis analyser (Stago, Asnières sur Seine, France). Fibrinogen was prepared as above. Monovalent Green Pit Viper Antivenom (*Trimeresurus albolabris*) (Lot #TA00119, Expiry date: 15th Jan 2024, The Thai Red Cross Society Bangkok, Thailand) was used. One vial of dry antivenom was reconstituted in 10 ml provided saline solution (using manufacturers guide) and centrifuged at 12,000 rpm on an Allegra™ X-22R Centrifuge (Lot#982501, Beckman Coulter, Brea, CA, USA) for 10 min at 4 °C, upon which the supernatant was removed, filtered (0.45 µm Econofltr PES, Agilent Technologies, Beijing, China), aliquoted, and stored at 4 °C. In our previous work (Debono et al., 2019c) 5% working stock (0.5 % final reaction concentration) antivenom was extremely effective against *T. albolabris* venom, rapidly neutralising even the higher concentrations of venom. Thus in order to better discriminate between the *T. albolabris* venoms in this study, a 2% working stock (0.2 % final reaction concentration) antivenom was used. To test antivenom efficacy the same fibrinogen coagulation procedure was used (as above) except with the antivenom replacing the 25 µl OK buffer. Briefly, 25 µl of 2% working stock antivenom, 50 µl of venom (20 – 0.05 µg/ml dilutions), 50 µl $CaCl_2$ (5 mM final), and 50 µl phospholipid in a final volume of 175 µl in Owren Koller Buffer was incubated for 120 s at 37 °C, after which 75 µl of human fibrinogen (1.2 mg/ml final reaction concentration) was added. Relative clotting was then monitored for 999 s or until plasma clotted (whichever was sooner). Note that antivenom does not clot fibrinogen and that a control was performed to rule out any additional effects antivenom has on fibrinogen, in which antivenom was substituted into the above outlined protocol in replacement of a venom sample. Eight-point dilution curves were graphed in GraphPad PRISM (GraphPad Prism Inc., La Jolla, CA, USA), and overlaid with fibrinogen coagulation assays.

2.4. Thromboelastography

To measure plasma and fibrinogen clot strength a Thrombelastogram® 5000 Haemostasis analyser (Haemonetics®, Haemonetics Australia Pty Ltd., North Rdye, Sydney 2113, Australia) was employed. Tests were performed on human fibrinogen and human plasma. Fibrinogen was prepared as above. Human plasma (3.2 % citrated, Batch #4133302 + 6187639) was obtained from the Australian Red Cross (research approval #16-04QLD-10; 44 Musk Street, Kelvin Grove, Queensland 4059). Natural cups and pins were used. A standard procedure was performed in which the cup was pipetted with 72 µl CaCl, 72 µl phospholipid, 20 µl OK buffer, and either 7 µl of venom, 7 µl 50 % deionised water/ 50 % glycerol for the spontaneous clot control, 7ul of thrombin (Stago Cat#115081 Liquid Fib) for the thrombin control, or 7 µl Factor Xa (Stago Cat#253047 Liquid Anti-Xa) for the Factor Xa control. After defrosting for 5 min in a 37 °C water-bath 189 µl of human fibrinogen or human plasma (depending on the test) was pipetted into the cup. The total volume in each cup was 360 µl and each test ran for 30 min. All controls (spontaneous clot control, thrombin control, and FXa control) were performed when venoms were tested on human plasma, while only the thrombin control was used for fibrinogen testing. Traces were exported from the Thrombelastogram and Adobe Photoshop used to produce figures.

2.5. Statistical analyses

Most statistical analyses were performed in GraphPad PRISM 8.1.1 (GraphPad Prism Inc., La Jolla, CA, USA) and the significance level set at 0.05. For venom fibrinogen clotting time and clotting time with antivenom an area under the curve value (AUC) was derived from each venom curve, and an X-fold shift calculated to test antivenom efficacy. The X-fold shift was calculated by dividing the venom + antivenom AUC over the venom AUC and subtracting the total by 1. A value of 0

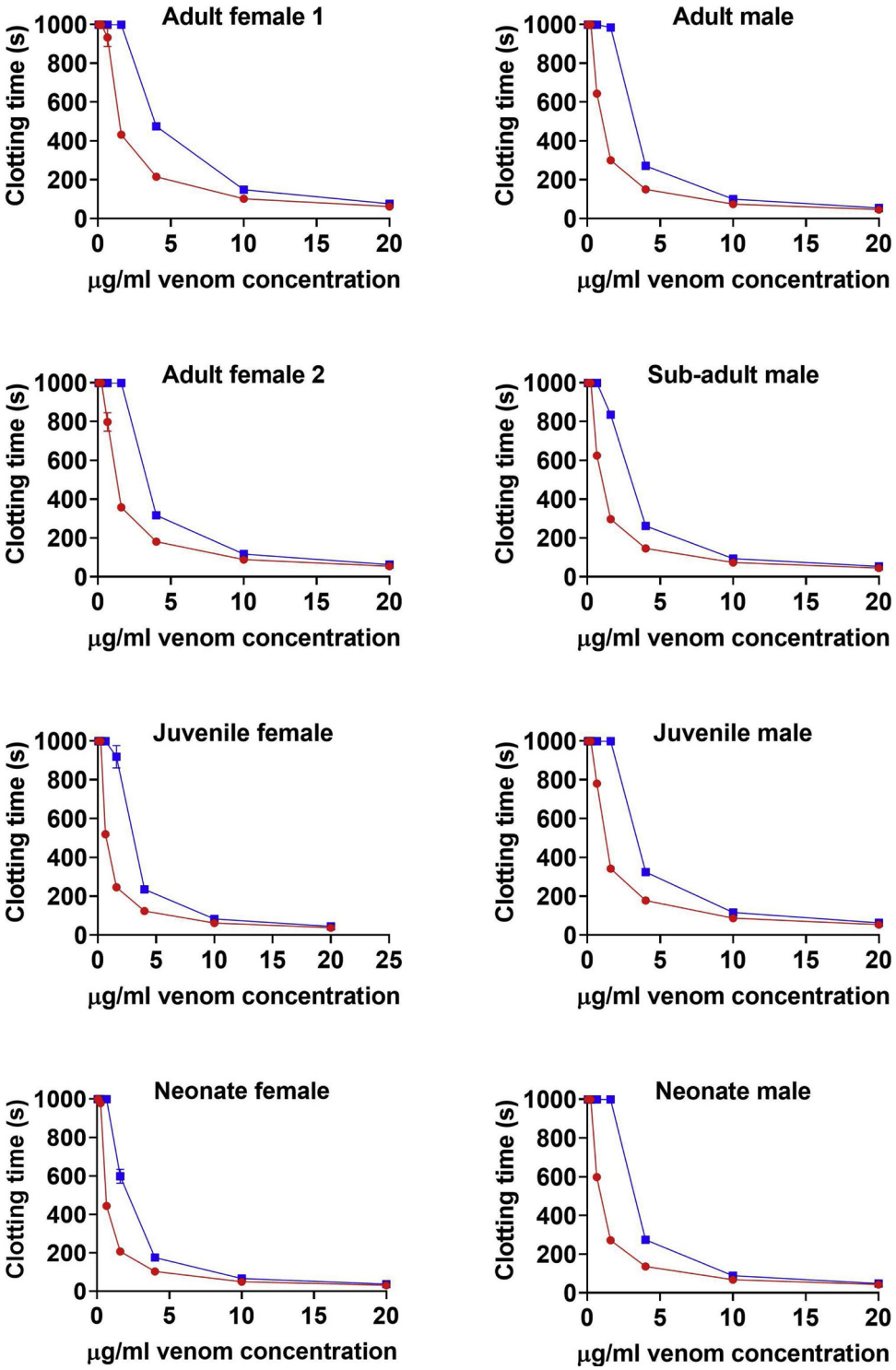

Fig. 1. Fibrinogen clotting time of *Trimeresuru salbolabris* venom (red line) and *T. albolabris* venom with antivenom (blue line) over eight different venom concentrations (20 µg/ml, 10 µg/ml, 4 µg/ml, 1.6 µg/ml, 0.66 µg/ml, 0.25 µg/ml, 0.125 µg/ml, and 0.05 µg/ml). All venoms tested in triplicate (N = 3) and shown with dots and standard error. Most standard errors are too small to see due to the very high quality of the data.

indicates no neutralisation of the venom by the antivenom (no shift in clotting time curves), while a value above 0 indicates venom neutralisation (shift in clotting time curves). The lowest and highest x-fold shift values were tested for significance with an unpaired T-test. For Thromboelastography tests a Ordinary one-way ANOVA, with Dunnett's multiple comparisons tests, was used to test for significant differences in clot strength (maximum amplitude value, mm) and initation (split point value, min) between venom and the controls. All datasets were checked for normality using quantile-quantile plots and homogeneity of variance using Brown-Forsythe test. AUC and maximum amplitude values (MA) are shown as the mean +/- standard deviation.

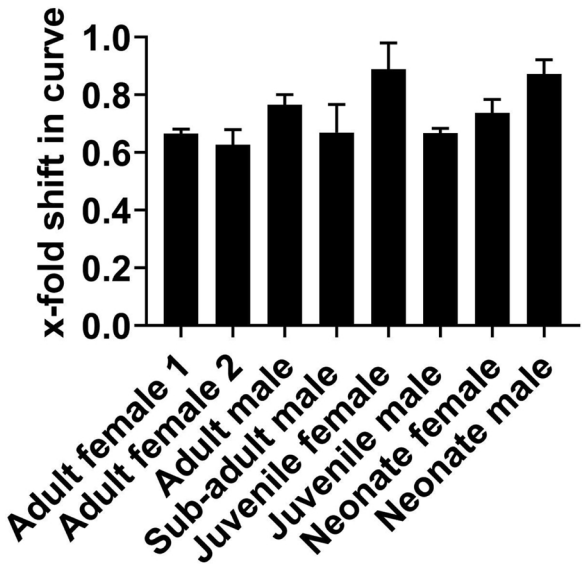

Fig. 2. X-fold shift in fibrinogen clotting time curves showing antivenom neutralisation across *Trimeresurus albolabris* life histories. X-fold shift was calculated by dividing the AUC for each venom with antivenom added fibrinogen clotting time curves over the AUC for venom fibrinogen clotting time curves and subtracting the total by 1. A value of 0 is no shift (antivenom does not neutralise venom), while a value above 0 indicates neutralisation.

3. Results

3.1. Fibrinogen coagulation assays and antivenom testing

All venoms exhibited a procoagulant effect, clotting fibrinogen in concentration dependent manners (Fig. 1). Ranking of area under the curve (AUC) values (with the more potent the venom, the smaller the value) were: adult female #1 3789 +/- 57.16, adult female #2 3280 +/- 45.64, juvenile males (pooled) 3210 +/- 15.71, adult male 2798 +/- 18.73, sub-adult male 2753 +/- 20.35, neonate males (pooled) 2592 +/- 30.25, juvenile females (pooled) 2375 +/- 21.92, and neonate females (pooled) 2032 +/- 17.99. Antivenom shifted the clotting curve upwards, with ranking of x-fold changes (the more effective the antivenom, the larger the number) being (Fig. 2): adult female #2 0.627 +/- 0.053, adult female #1 0.666 +/- 0.015, juvenile males (pooled) 0.667 +/- 0.017, sub-adult male 0.669 +/- 0.098, neonate females (pooled) 0.738 +/- 0.047, adult male 0.765 +/- 0.036, neonate males (pooled) 0.872 +/- 0.05, and juvenile females (pooled) 0.889 +/- 0.092. While all venoms were well neutralised by the antivenom, slight individual variation in antivenom neutralisation was observed, with juvenile females venom the best neutralised (x-fold shift = 0.889 +/ 0.092) and adult female #2 venom the least neutralised (x-fold shift = 0.627 +/- 0.053) (Fig. 2). This difference was significant (T-test $df = 4$, $t = 4.287$, $p = 0.0128$).

3.2. Thromboelastography

Thromboelastography on human plasma (Fig. 3) showed the ability of the venoms to intitiate clotting in plasma significantly faster than the spontaneous clotting control ($p = < 0.0001$), but producing the weak, unstable clots consistent with pseudo-procoagulant actions upon fibrinogen, with there being no sexual or ontogenetic pattern to the clot strength (MA) variation: juvenile males (pooled) 2.4 +/- 0.1, juvenile females (pooled) 2.5 +/- 0.1, adult female #2 2.7 +/- 0.1, adult male 2.7 +/- 0.2, sub-adult male 2.9 +/- 0.2, adult female #1 3.1 +/- 0.8, neonate males (pooled) 3.8 +/- 0.7, and neonate females (pooled) 4.3 +/- 0.2. Venoms formed significantly weaker plasma clots compared to the spontaneous clotting control ($p = < 0.0001$), thrombin control ($p = < 0.0001$), and FXa control ($p = < 0.0001$). Consistent with the actions upon plasma, all *T. albolabris* venoms exhibited weaker fibrinogen clots than the thrombin control ($p = < 0.0001$), thus indicating a pseudo-procoagulant effect forming weak, transient fibrin clots (Fig. 4). There was no pattern of ontogenetic venom variation, with all producing very similar clot strength (MA) values: adult male 10.4 +/- 0.2, adult female #1 10.5 +/- 0.3, neonate males (pooled) 10.7 +/- 0.3, sub-adult male 11.2 +/- 0.6, juvenile males (pooled) 11.6 +/- 0.5, adult female #2 11.8 +/- 0.5, neonate females (pooled) 11.8 +/- 0.6, and juvenile females (pooled) 12.0 +/- 0.5.

4. Discussion

Our study aimed to investigate the effects of *T. albolabris* venom on human fibrinogen and plasma, if ontogenetic or sexual differences in venom function occurred, and if the efficacy of antivenom was the same for all venoms. Venom from all life stages of *T. albolabris* clotted fibrinogen and human plasma in a pseudo-procoagulant manner, forming significantly weaker fibrin clots than the controls (Figure 3 and 4). The results of both these analyses are consistent with *T. albolabris* venoms directly cleaving fibrinogen at atypical cleavage sites to produce unstable fibrin clots, leading to fibrinogen depletion, thereby resulting in a net anticoagulant state. These results are consistent with the coagulation abnormalities seen in envenomated patients (Hutton et al., 1990; Pandey et al., 2019; Sarmin et al., 2013).

Our study found no evidence for differences in venom function across different life stages of the species or consistent variation between sexes. Venom function did vary between samples; however, this was attributed to individual venom variation rather than ontogenetic variation. On fibrinogen female *T. albolabris* neonates and juveniles did have faster acting venom (AUC = 2032 +/- 17.99 and 2375 +/- 21.92, respectively) compared to female adult #1 (AUC = 3789 +/- 57.16) and female adult #2 (AUC = 3280 +/- 45.64) venoms. The same trend was not observed in male venoms. Neonate male and female venoms also had slightly stronger clots on human plasma (MA = 3.8 +/- 0.7 and 4.3 +/- 0.2, respectively) compared to adult male (MA = 2.7 +/- 0.2), adult female #1 (MA = 3.1 +/- 0.8) and adult female #2 venoms (MA = 2.7 +/- 0.1). These differences; however, are minimal when compared against other studies where extremely pronounced ontogenetic shifts were observed for other types of snakes such as *Pseudonaja* species (Cipriani et al., 2017; Jackson et al., 2016).

The minimal venom variation observed in this present study is likely due to individual variation rather than age or sex, specifically between adult and sub-adult venoms which were individual snake venoms, rather than pooled venoms. Individual venom variation has been observed in numerous snake species (Currier et al., 2010; Smiley-Walters et al., 2019), and pooled samples can help eliminate this possible variation (Borja et al., 2013; Galizio et al., 2018). Both adult female samples differed in their clotting time and clot strength (Figs. 1, 3 and 4) indicating slight individual venom variation, thus future research needs to be done with a larger set of pooled venom samples to test for ontogenetic differences. Even if present, our study shows these differences are minute compared to other studies (Cipriani et al., 2017; Jackson et al., 2016). Other research backs up our finding suggesting some snakes simply do not have ontogenetic (Tan et al., 1992, 1993a, b) or sexual dimorphic (Tan et al., 2017) venom shifts, possibly correlated with minimal selection pressures due to similar diets or conserved toxin types.

Clinically, a bite from any *T. albolabris* individual, regardless of sex or age, may produce the same potent anticoagulant action upon clotting due to fibrinogen depletion resulting from pseudo-procoagulant mechanisms that produce weak, unstable, transient fibrin clots (Debono et al., 2019c). Despite some slight intraspecific variation in antivenom neutralisation (Fig. 1) all the venoms were effectively neutralised by the Thai Red Cross Green Pit Viper antivenom (Figs. 1 and 2). The Thai Red Cross Green Pit Viper antivenom has already been proven effective

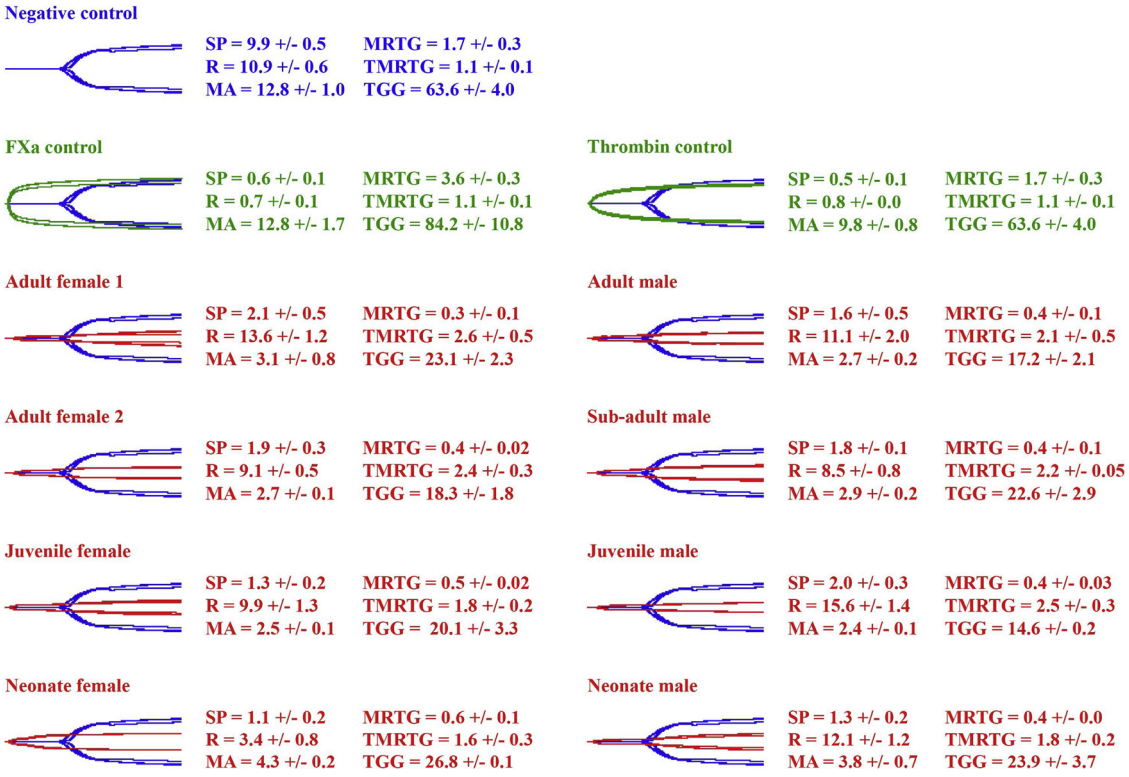

Fig. 3. Thromboelastography using human plasma. Blue traces = spontaneous clot control (negative control), green traces = thrombin and FXa control, red traces = venom samples. All venom traces overlaid with the spontaneous clot control. Parameter SP = the split point (time till clot formation begins) (min), R = time until detectable clot (2 mm +) is formed (min), MA = maximum amplitude of clot (mm), MRTG = maximum rate of thrombus generation (dynes/cm^2/s), TMRTG = time to maximum rate of thrombus generation (min), TGG = total thrombus generated (dynes/cm^2). Each trace is N = 3 and values are N = 3 means +/- standard deviation.

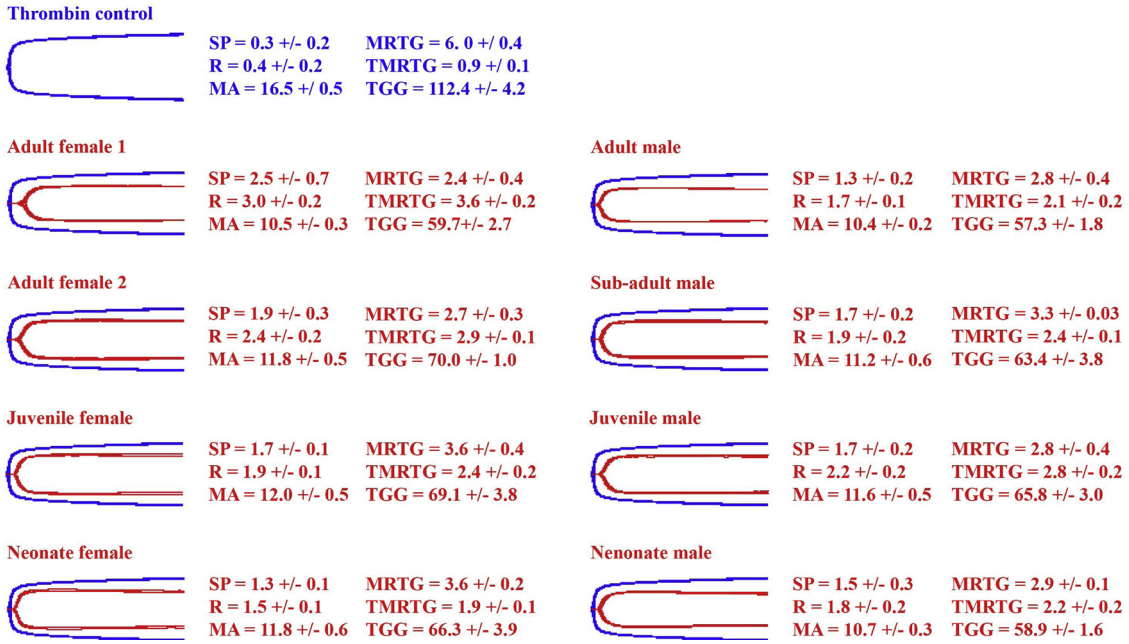

Fig. 4. Thromboelastography using human fibrinogen. Blue traces = thrombin control, red traces = venom samples. All venom traces overlaid with the thrombin control trace. Parameter SP = the split point (time till clot formation begins) (min), R = time until detectable clot (2 mm +) is formed (min), MA = maximum amplitude of clot (mm), MRTG = maximum rate of thrombus generation (dynes/cm^2/s), TMRTG = time to maximum rate of thrombus generation (min), TGG = total thrombus generated (dynes/cm^2). Each trace is N = 3 and values are N = 3 means +/- standard deviation.

against adult *T. albolabris* venom (Debono et al., 2019c); however, the present study is the first to validate antivenom efficacy for different life stages and sexes of *T. albolabris*. This result is important in indicating that for this snake species the same medical treatment (antivenom administration) should be performed regardless of sex or age differences.

Overall, this study indicates that *T. albolabris* exhibits a conserved potency of pseudo-procoagulant venom throughout its life history. Unlike some snakes, such as the *Pseudonaja* genus whereby diet shifts across age classes influences venom composition (Cipriani et al., 2017; Jackson et al., 2016), *T. albolabris* individuals likely have a similar diet from neonates to adults. Furthermore, with the available antivenom proving effective for all tested specimens this research adds to important medical knowledge in treating snakebites from one of Southern Asia's most common culprits.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2020.03.009.

References

Amorim, F.G., Costa, T.R., Baiwir, D., De Pauw, E., Quinton, L., Sampaio, S.V., 2018. Proteopeptidomic, functional and immunoreactivity characterization of *Bothrops moojeni* snake venom: influence of snake gender on venom Composition. Toxins 10, 177. https://doi.org/10.3390/toxins10050177.

Barlow, A., Pook, C.E., Harrison, R.A., Wüster, W., 2009. Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. Proc. Biol. Sci. 276, 2443–2449. https://doi.org/10.1098/rspb.2009.0048.

Bittenbinder, M.A., Zdenek, C.N., Youngman, N.J., Dobson, J.S., Naude, A., Vonk, F.J., Fry, B.G., 2018. Coagulotoxic cobras: clinical implications of strong anticoagulant actions of African spitting Naja venoms that are not neutralised by antivenom but are by LY315920 (Varespladib). Toxins 10, 516. https://doi.org/10.3390/toxins10120516.

Bittenbinder, M.A., Dobson, J.S., Zdenek, C.N., op den Brouw, B., Naude, A., Vonk, F.J., Fry, B.G., 2019. Differential destructive (non-clotting) fibrinogenolytic activity in Afro-Asian elapid snake venoms and the links to defensive hooding behavior. Toxicol. In Vitro 60, 330–335. https://doi.org/10.1016/j.tiv.2019.05.026.

Borja, M., Lazcano, D., Martínez-Romero, G., Morlett, J., Sánchez, E., Cepeda-Nieto, A.C., Garza-García, Y., Zugasti-Cruz, A., 2013. Intra-specific variation in the protein composition and proteolytic activity of venom of *Crotalus lepidus morulus* from the northeast of Mexico. Copeia 2013, 707–716. https://doi.org/10.1643/OT-13-005.

Casewell, N.R., Wagstaff, S.C., Wüster, W., Cook, D.A.N., Bolton, F.M.S., King, S.I., Pla, D., Sanz, L., Calvete, J.J., Harrison, R.A., 2014. Medically important differences in snake venom composition are dictated by distinct postgenomic mechanisms. Proc. Natl. Acad. Sci. U.S.A. 111, 9205–9210. https://doi.org/10.1073/pnas.1405484111.

Chippaux, J.P., 1998. Snake-bites: appraisal of the global situation. Bull W H O 76, 515–524.

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. C Toxicol. Pharmacol. 197, 53–60. https://doi.org/10.1016/j.cbpc.2017.04.007.

Cockram, C.S., Chan, J.C., Chow, K.Y., 1990. Bites by the white-lipped pit viper (*Trimeresurus albolabris*) and other species in Hong Kong. A survey of 4 years' experience at the Prince of Wales Hospital. Am. J. Trop. Med. Hyg. 93, 79–86.

Currier, R.B., Harrison, R.A., Rowley, P.D., Laing, G.D., Wagstaff, S.C., 2010. Intra-specific variation in venom of the African Puff Adder (*Bitis arietans*): differential expression and activity of snake venom metalloproteinases (SVMPs). Toxicon 55, 864–873. https://doi.org/10.1016/j.toxicon.2009.12.009.

Daltry, J.C., Wüster, W., Thorpe, R.S., 1996. Diet and snake venom evolution. Nature 379, 537–540. https://doi.org/10.1038/379537a0.

Debono, J., Bos, M.H.A., Coimbra, F., Ge, L., Frank, N., Kwok, H.F., Fry, B.G., 2019a. Basal but divergent: clinical implications of differential coagulotoxicity in a clade of Asian vipers. Toxicol. In Vitro 58, 195–206. https://doi.org/10.1016/j.tiv.2019.03.038.

Debono, J., Bos, M.H.A., Do, M.S., Fry, B.G., 2019b. Clinical implications of coagulotoxic variations in Mamushi (Viperidae: gloydius) snake venoms. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 225, 108567. https://doi.org/10.1016/j.cbpc.2019.108567.

Debono, J., Bos, M.H.A., Frank, N., Fry, B., 2019c. Clinical implications of differential antivenom efficacy in neutralising coagulotoxicity produced by venoms from species within the arboreal viperid snake genus Trimeresurus. Toxicol. Lett. https://doi.org/10.1016/j.toxlet.2019.09.003.

Debono, J., Bos, M.H.A., Nouwens, A., Ge, L., Frank, N., Kwok, H.F., Fry, B.G., 2019d. Habu coagulotoxicity: clinical implications of the functional diversification of *Protobothrops* snake venoms upon blood clotting factors. Toxicol. In Vitro 55, 62–74. https://doi.org/10.1016/j.tiv.2018.11.008.

Devan-Song, A., Martelli, P., Karraker, N.E., 2017. Reproductive biology and natural history of the whitelipped pit viper (*Trimeresurus albolabris* Gray, 1842) in Hong Kong. Herpetol. Conserv. Biol. 12, 41–55.

Fry, B.G., 2018. Snakebite: when the human touch becomes a bad touch. Toxins 10, 170. https://doi.org/10.3390/toxins10040170.

Galizio, N.D.C., Serino-Silva, C., Stuginski, D.R., Abreu, P.A.E., Sant'Anna, S.S., Grego, K.F., Tashima, A.K., Tanaka-Azevedo, A.M., Morais-Zani, Kd., 2018. Compositional and functional investigation of individual and pooled venoms from long-term captive and recently wild-caught *Bothrops jararaca* snakes. J. Proteomics 186, 56–70. https://doi.org/10.1016/j.jprot.2018.07.007.

Gibbs, H.L., Sanz, L., Chiucchi, J.E., Farrell, T.M., Calvete, J.J., 2011. Proteomic analysis of ontogenetic and diet-related changes in venom composition of juvenile and adult Dusky Pigmy rattlesnakes (*Sistrurus miliarius barbouri*). J. Proteomics 74, 2169–2179. https://doi.org/10.1016/j.jprot.2011.06.013.

Hutton, R.A., Looareesuwan, S., Ho, M., Silamut, K., Chanthavanich, P., Karbwang, J., Supanaranond, W., Vejcho, S., Viravan, C., Phillips, R.E., Warrell, D.A., 1990. Arboreal green pit vipers (genus *Trimeresurus*) of south-east Asia: bites by *T. Albolabris* and *T. Macrops* in Thailand and a review of the literature. Trans. R. Soc. Trop. Med. Hyg. 84, 866–874. https://doi.org/10.1016/0035-9203(90)90111-Q.

Jackson, T.N.W., Koludarov, I., Ali, S.A., Dobson, J., Zdenek, C.N., Dashevsky, D., Op den Brouw, B., Masci, P.P., Nouwens, A., Josh, P., Goldenberg, J., Cipriani, V., Hay, C., Hendrikx, I., Dunstan, N., Allen, L., Fry, B.G., 2016. Rapid radiations and the race to redundancy: an investigation of the evolution of Australian elapid snake venoms. Toxins 8, 309. https://doi.org/10.3390/toxins8110309.

Jorge da Silva, N., Aird, S.D., 2001. Prey specificity, comparative lethality and compositional differences of coral snake venoms. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 128, 425–456. https://doi.org/10.1016/S1532-0456(00)00215-5.

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. https://doi.org/10.1371/journal.pmed.0050218.

Kini, R.M., Koh, C.Y., 2016. Metalloproteases affecting blood coagulation, fibrinolysis and platelet aggregation from snake venoms: definition and nomenclature of interaction sites. Toxins 8, 284. https://doi.org/10.3390/toxins8100284.

Lister, C., Arbuckle, K., Jackson, T.N.W., 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. C Toxicol. Pharmacol. 202, 39–54. https://doi.org/10.1016/j.cbpc.2017.07.005.

Orlov, N., Ananjeva, N., Khalikov, R., 2002. Natural History of Pitvipers in Eastern and Southeastern Asia. Eagle Mountain Publishing, Eagle Mountain, Utah.

Oulion, B., Dobson, J.S., Zdenek, C.N., Arbuckle, K., Lister, C., Coimbra, F.C.P., 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. https://doi.org/10.1016/j.toxlet.2018.02.020.

Pandey, D.P., Ghimire, A., Shrestha, B.R., 2019. Retrospective documentation of a confirmed white-lipped green pit viper (*Trimeresurus albolabris* Gray, 1842) bite in the south-central hills of Nepal. Wilderness Environ. Med. 30, 79–85. https://doi.org/10.1016/j.wem.2018.11.001.

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., Wüster, W., Ali, S.A., Masci, P., Rowley, P., Frank, N., Fry, B.G., 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. https://doi.org/10.1016/j.toxlet.2017.08.020.

Sarmin, S., Amin, M.R., Al-Mamun, H., Rahman, R., Faiz, M.A., 2013. Clinical aspects of green pit viper bites in Bangladesh: a study on 40 patients. Asia Pac. J. Med. Toxicol. 2, 96–100. https://doi.org/10.22038/apjmt.2013.1670.

Smiley-Walters, S.A., Farrell, T.M., Gibbs, H.L., 2019. High levels of functional divergence in toxicity towards prey among the venoms of individual pigmy rattlesnakes. Biol. Lett. 15, 20180876. https://doi.org/10.1098/rsbl.2018.0876.

Sousa, L.F., Zdenek, C.N., Dobson, J.S., Op den Brouw, B., Coimbra, F.C.P., Gillett, A., Del-Rei, T.H.M., Chalkidis, H.D.M., Sant'Anna, S., Teixeira-da-Rocha, M.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. https://doi.org/10.3390/toxins10100411.

Tan, N.-H., Armugam, A., Mirtschin, P.J., 1992. The biological properties of venoms from juvenile and adult taipan (*oxyuranus scutellatus*) snakes. Comp. Biochem. Physiol., B 103, 585–588. https://doi.org/10.1016/0305-0491(92)90375-2.

Tan, N.-H., Ponnudurai, G., Mirtschin, P.J., 1993a. A comparative study of the biological

properties of venoms from juvenile and adult inland taipan (*Oxyuranus microlepidotus*) snake venoms. Toxicon 31, 363–367. https://doi.org/10.1016/0041-0101(93)90155-C.

Tan, N.-H., Ponnudurai, G., Mirtschin, P.J., 1993b. A comparative study on the biological properties of venoms from juvenile and adult common tiger snake (*Notechis scutatus*) venoms. Comp. Biochem. Physiol., B 106, 651–654. https://doi.org/10.1016/0305-0491(93)90144-T.

Tan, K.Y., Tan, C.H., Fung, S.Y., Tan, N.H., 2015. Venomics, lethality and neutralization of *Naja kaouthia* (monocled cobra) venoms from three different geographical regions of Southeast Asia. J. Proteomics 120, 105–125. https://doi.org/10.1016/j.jprot.2015.02.012.

Tan, C.H., Tan, K.Y., Yap, M.K.K., Tan, N.H., 2017. Venomics of Tropidolaemus wagleri, the sexually dimorphic temple pit viper: unveiling a deeply conserved atypical toxin arsenal. Sci. Rep. 7, 43237. https://doi.org/10.1038/srep43237.

Viravan, C., Looareesuwan, S., Kosakarn, W., Wuthiekanun, V., McCarthy, C.J., Stimson, A.F., Bunnag, D., Harinasuta, T., Warrell, D.A., 1992. A national hospital-based survey of snakes responsible for bites in Thailand. Trans. R. Soc. Trop. Med. Hyg. 86, 100–106. https://doi.org/10.1016/0035-9203(92)90463-M.

White, J., 2005. Snake venoms and coagulopathy. Toxicon 45, 951–967. https://doi.org/10.1016/j.toxicon.2005.02.030.

Youngman, N.J., Debono, J., Dobson, J.S., Zdenek, C.N., Harris, R.J., Coimbra, F.C., Naude, A., Coster, K., Sundman, E., Braun, R., 2019a. Venomous landmines: clinical implications of extreme coagulotoxic diversification and differential neutralization by antivenom of venoms within the viperid snake genus *Bitis*. Toxins 11, 422. https://doi.org/10.3390/toxins11070422.

Youngman, N.J., Zdenek, C.N., Dobson, J.S., Bittenbinder, M.A., Gillett, A., Hamilton, B., Dunstan, N., Allen, L., Veary, A., Veary, E., Fry, B.G., 2019b. 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. 302, 1–6. https://doi.org/10.1016/j.toxlet.2018.11.015.

Zdenek, C.N., den Brouw, B.O., Dashevsky, D., Gloria, A., Youngman, N., Watson, E., Green, P., Hay, C., Dunstan, N., Allen, L., Fry, B.G., 2019a. Clinical implications of convergent procoagulant toxicity and differential antivenom efficacy in Australian elapid snake venoms. Toxicol. Lett. https://doi.org/10.1016/j.toxlet.2019.08.014.

Zdenek, C.N., Hay, C., Arbuckle, K., Jackson, T.N.W., Bos, M.H.A., op den Brouw, B., Debono, J., Allen, L., Dunstan, N., Morley, T., Herrera, M., Gutiérrez, J.M., Williams, D.J., Fry, B.G., 2019b. Coagulotoxic effects by brown snake *(Pseudonaja*) and taipan (*Oxyuranus*) venoms, and the efficacy of a new antivenom. Toxicol. In Vitro 58, 97–109. https://doi.org/10.1016/j.tiv.2019.03.031.

Zelanis, A., Tashima, A.K., Rocha, M.M.T., Furtado, M.F., Camargo, A.C.M., Ho, P.L., Serrano, S.M.T., 2010. Analysis of the ontogenetic variation in the venom proteome/peptidome of *Bothrops jararaca* reveals different strategies to deal with prey. J. Proteome Res. 9, 2278–2291. https://doi.org/10.1021/pr901027r.

Zelanis, A., Menezes, M.C., Kitano, E.S., Liberato, T., Tashima, A.K., Pinto, A.F.M., Sherman, N.E., Ho, P.L., Fox, J.W., Serrano, S.M.T., 2016. Proteomic identification of gender molecular markers in *Bothrops jararaca* venom. J. Proteomics 139, 26–37. https://doi.org/10.1016/j.jprot.2016.02.030.