



Swansea University
Prifysgol Abertawe



Cronfa - Swansea University Open Access Repository

This is an author produced version of a paper published in :
Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology

Cronfa URL for this paper:
<http://cronfa.swan.ac.uk/Record/cronfa33257>

Paper:

Cipriani, V., Debono, J., Goldenberg, J., Jackson, T., Arbuckle, K., Dobson, J., Koludarov, I., Li, B., Hay, C., Dunstan, N., Allen, L., Hendrikx, I., Kwok, H. & Fry, B. (2017). Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 197, 53-60.

<http://dx.doi.org/10.1016/j.cbpc.2017.04.007>

This article is brought to you by Swansea University. Any person downloading material is agreeing to abide by the terms of the repository licence. Authors are personally responsible for adhering to publisher restrictions or conditions. When uploading content they are required to comply with their publisher agreement and the SHERPA RoMEO database to judge whether or not it is copyright safe to add this version of the paper to this repository.
<http://www.swansea.ac.uk/iss/researchsupport/cronfa-support/>

Accepted Manuscript

Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*)

Vittoria Cipriani, Jordan Debono, Jonathan Goldenberg, Timothy N.W. Jackson, Kevin Arbuckle, James Dobson, Ivan Koludarov, Bin Li, Chris Hay, Nathan Dunstan, Luke Allen, Iwan Hendrikkx, Hang Fai Kwok, Bryan G. Fry

PII: S1532-0456(17)30092-3
DOI: doi:[10.1016/j.cbpc.2017.04.007](https://doi.org/10.1016/j.cbpc.2017.04.007)
Reference: CBC 8317

To appear in: *Comparative Biochemistry and Physiology Part C*

Received date: 25 February 2017
Revised date: 19 April 2017
Accepted date: 25 April 2017

Please cite this article as: Cipriani, Vittoria, Debono, Jordan, Goldenberg, Jonathan, Jackson, Timothy N.W., Arbuckle, Kevin, Dobson, James, Koludarov, Ivan, Li, Bin, Hay, Chris, Dunstan, Nathan, Allen, Luke, Hendrikkx, Iwan, Kwok, Hang Fai, Fry, Bryan G., Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*), *Comparative Biochemistry and Physiology Part C* (2017), doi:[10.1016/j.cbpc.2017.04.007](https://doi.org/10.1016/j.cbpc.2017.04.007)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*)

Vittoria Cipriani¹, Jordan Debono¹, Jonathan Goldenberg¹, Timothy NW Jackson^{1,2}, Kevin Arbuckle³, James Dobson¹, Ivan Koludarov¹, Bin Li⁴, Chris Hay¹, Nathan Dunstan⁵, Luke Allen⁵, Iwan Hendrikx¹, Hang Fai Kwok³, Bryan G Fry^{1*}

1. Venom Evolution Lab, School of Biological Sciences, University of Queensland, St Lucia QLD, 4072 Australia
2. Australian Venom Research Unit, Department of Pharmacology and Therapeutics, University of Melbourne, Parkville Victoria 3010, Australia
3. Department of Biosciences, College of Science, Swansea University, Swansea SA2 8PP, UK;
4. Faculty of Health Science, University of Macau, Avenida da Universidade, Taipa, Macau SAR
5. Venom Supplies, Tanunda, South Australia 5352 Australia

* corresponding author bgfry@uq.edu.au

ABSTRACT

Venom is a key evolutionary trait, as evidenced by its widespread convergent evolution across the animal kingdom. In an escalating prey-predator arms race, venoms evolve rapidly to guarantee predatory or defensive success. Variation in venom composition is ubiquitous among snakes. Here, we tested variation in venom activity on substrates relevant to blood coagulation amongst *Pseudonaja* (brown snake) species, Australian elapids responsible for the majority of medically important human envenomations in Australia. A functional approach was employed to elucidate interspecific variation in venom activity and all nine currently recognised species of *Pseudonaja*. Fluorometric enzymatic activity assays were performed to test variation in whole venom procoagulant activity among species. Analyses confirmed the previously documented ontogenetic shift from non-coagulopathic venom in juveniles to coagulopathic venom as adults, except for the case of *P. modesta*, which retains non-coagulopathic venom as an adult. These shifts in venom activity correlate with documented ontogenetic shifts in diet among brown snakes from specialisation on reptilian prey as juveniles (and throughout the life cycle of *P. modesta*), to a more generalised diet in adults that includes mammals. The results of this study bring to light findings relevant to both clinical and evolutionary toxinology.

Keywords: *Pseudonaja* brown snake; venom; toxin; venom evolution; diet; enzymatic activity

ACCEPTED MANUSCRIPT

INTRODUCTION

Interspecific interactions have played a key role in shaping the biodiversity of Earth (Thompson, 1999). Many extraordinary adaptations are the results of millennia of continuous genomic modifications (Elena and Lenski, 2003), an excellent example of which is venom. Venom has independently emerged in multiple lineages and has provided fascinating evidence of its importance as a potential key innovation driving animals' ecological and evolutionary diversification (Fry et al. 2009, Casewell et al., 2013; Harris and Arbuckle, 2016; Sunagar et al., 2016).

Many venom toxins are hypothesised to be the result of a recruitment process in which a gene for a non-toxic protein, usually encoding a critical endophysiological function, is over-expressed in a venom gland, with subsequent venom gland specific duplication and diversification (Kini and Chan, 1999; Fry, 2005; Casewell et al., 2011; Vonk et al., 2013). This process, known as the "birth and death" mode of gene evolution (Nei et al., 1997) implies the presence of non-successful genes as well, which are degraded into non-functional pseudogenes (Kordis and Gubenek, 2000). Genes successfully recruited or restricted to venom glands are often amplified to create multigene families. Through the preservation of the core molecular scaffold of the ancestral protein and the modification of only the functional residues, multigene families acquire newly derived activities (de la Vega et al., 2003), which are the key to toxin diversification in animal lineages.

It has been demonstrated that specific toxins are correlated with particular diets (Daltry et al., 1996a, b; Fry et al., 2003, 2008, 2012; Pawlak et al., 2006; Gibbs and Mackessy, 2009; Casewell et al., 2013; Heyborne and Mackessy, 2013; Jackson et al., 2016; Yang et al., 2016). Variations in venom composition and activity exist not only among species but among life history stages within a single species. Ontogenetic and/or allometric shifts in venom are often correlated with similar shifts in diet (Mackessy et al., 2003; Jackson et al., 2016).

Australia contains a tremendous diversity of elapid snakes (Williams et al., 2006; Jackson et al., 2016) and members of the family account for over half of all snake species present in Australia (Cogger, 2014). It is hypothesised that Australian elapids derived from Asian species, which migrated to Australia during glacial periods (Wüster et al., 2005) when the Sunda and Sahul shelves were exposed as land bridges (Barber et al., 2000), thus facilitating their dispersal. The venoms of most elapids in Australia and elsewhere contain appreciable quantities of neurotoxins (Birrell et al., 2007). As a high relative abundance of neurotoxic peptides over enzymatic toxins has been correlated in elapid snakes with a diet dominated by reptilian prey (Jackson et al., 2016), this suggests that ancestral Elapidae may have fed predominantly on reptiles. During evolution however, several Australasian genera evolved potent prothrombin-activating coagulopathic venoms,

including *Hoplocephalus*, *Notechis*, *Oxyuranus*, *Pseudonaja* and *Tropidechis* (Earl et al., 2015; Trabi et al., 2015).

In particular, *Pseudonaja* is the most medically important snake genus in Australia, accounting for the majority of lethal human envenomations (Isbister, 2006). The systematics of this genus has long been debated and the identification of species can be difficult given the existence of extreme variability in colours and patterns even within each species (Shine, 1977). Attempts to use karyotype analyses to resolve the taxonomy were only partially successful (Mengden, 1985), however extensive phylogenetic analysis of mitochondrial DNA sequences has provided a stable phylogenetic framework for the major lineages (Skinner et al. 2005). *Pseudonaja* spp. are characterised by short fangs (present at the front of the maxilla, as in all elapid snakes) and small venom glands and associated compressor musculature. All brown snakes are active foragers and typically diurnal, although juveniles of all species and adults of tropical/arid zone species may forage nocturnally (Jackson et al. 2016). Juveniles of most species predate upon lizards while adults of most species predate upon mammals (Shine, 1977; Jackson et al., 2016). The sole exception is *Pseudonaja modesta*, an apparently neotenic species in this regard, which is a lizard specialist at all ages (Shine, 1977). Two other species, *P. inframacula* and *P. guttata* also differ from the others (although both include mammals in their diet as adults), in that the former continues to feed predominantly on reptiles throughout its life and the latter includes a large quantity of frogs in its diet (approximately 40% of total prey - Shine, 1977). Previous work linked the proteomics profile of brown snakes to dietary variation and demonstrated an ontogenetic shift in *P. affinis* and *P. textilis* from venom purely composed of peptide toxins in juvenile snakes to procoagulant venom including the prothrombinase toxin complex in adults (Jackson et al. 2016). In contrast, *P. modesta* maintains a venom profile similar to the juveniles of other species into adulthood, and neither clots plasma *in vitro* or expresses the prothrombinase complex in its venom gland transcriptome (Jackson et al. 2013; 2016). As coagulopathy is typically the most significant of the clinical sequelae resulting from human envenomation by *Pseudonaja*, it is the adult venom of the mammal feeders that is largely responsible for the clinical importance of brown snake envenomations. Transcriptomic works demonstrated extreme variability in the venom of adults of the *P. aspidorhyncha*, *P. nuchalis* and *P. textilis* (Reeks et al., 2016). Notably, however, while adult venom gland transcriptomes from all of the species included in that study studied transcribed prothrombin activator, the venom of smallest species of the three (*P. nuchalis*) contained a greater diversity of other components (Reeks et al., 2016). However, as transcriptomes only provide a “snapshot” of the gene expression of a tissue and are not identical to the secretory proteome in any case, in part due to the effect of microRNA silencing (Durban et al., 2013), the relative proportions

of various toxins present in these species' venom gland transcriptomes may not be reflective of the proportions in the secreted venom.

Previous bioactivity testing of *Pseudonaja* venoms has focussed upon the medically important *P. textilis* and *P. affinis*, which are the species responsible for most bites to humans (Judge et al., 2002; Millers et al., 2009; Skejitch and Hodgson, 2013). In particular, Eastern brown snakes (*P. textilis*) have been deeply studied with the aim to extract valid components for drug design and therapeutics (Masci et al., 1988; Filippovich et al., 2002; Rao and Kini, 2002). However, functional variation of venom across the rest of the genus has remained relatively poorly investigated. This study aims to fill this knowledge gap by extensively testing the functional variation in brown snake venoms and comparing this with dietary variation.

MATERIALS AND METHODS

Species studied

Venoms were sourced commercially from Venom Supplies Pty Ltd, or were part of the long-term research collection of the Venom Evolution Laboratory. Snakes were milked using the pipette method, as described in Mirtschin et al. (2006), specifically developed for low yielding snakes species such as *Pseudonaja* ssp. After milking, the venom was immediately flash frozen in liquid nitrogen. Species and localities are: *P. affinis* (Perth, Western Australia), *P. aspidorhyncha* (St George, Queensland), *P. guttata* (Longreach, Queensland), *P. inframacula* (Yorke Peninsula, South Australia), *P. ingrami* (Kununurra, Western Australia), *P. mengdeni* (Alice Springs, Northern Territory), *P. modesta* (Sandstone, Western Australia), *P. nuchalis* (Darwin, Northern Territory) and *P. textilis* (Gold Coast, Queensland). Juvenile specimens of *P. affinis*, *P. nuchalis* and *P. textilis* were captive bred at Venom Supplies Pty Ltd and adult venoms of these three species were sourced from the parents of these juveniles, for direct comparison within the present study.

Enzymatic activity studies

Aliquots of 1 mg for enzymatic activity testing were suspended in a solution made of 50% glycerol and 50% water and were kept at -20°C. A Thermo Scientific™ Fluoroskan Ascent™ Microplate Fluorometer was employed to test variation in procoagulant activity across all *Pseudonaja* species. In order to compare whole venom enzymatic activity within the genus, different concentrations of venom from adult individuals of all the species were tested with various substrates. Juvenile venom was also tested where available (*P. affinis*, *P. nuchalis*, and *P. textilis*). Activity of the serine proteases present in the venom or activated by it was monitored *in vitro* using 6-amino-1-naphthalene-sulfonamide-based (ANSN), and coumarin derivatives, such as 7-amino-4-

methylcoumarin (AMC) and 7-amino-4-(trifluoromethyl) coumarin (AFC) fluorogenic substrates. Once cleaved by specific factors, the fluorescent group exhibits an increase of fluorescence, which was monitored. Specific substrates were used to investigate different factors active in the venom or activated by the venom, as indicated in Table 1. The use of different substrates allowed assessing the effect of venom on different components of the coagulation cascade.

Table 1 Substrates applied in the tests. In the table the factors which specifically cleave the fluorescent group of each substrate are indicated, as well as the type of reaction monitored by Thermo Fluoroskan Ascent™ 2.6.

Fluorogenic Substrate	Target	Factors involved in the experiment	Reaction measured	In vitro activity
SN-7 (Haematologic Technologies Inc.)	Factor Xa	Venom + substrate	Hydrolysis of ANSN group	Activity of factor Xa present in the venom
SN-5 (Haematologic Technologies Inc.)	Plasmin	Venom + human plasmin + substrate	Hydrolysis of ANSN group	Inhibition of prey Plasmin
ES011 (Bio-technic/R&D systems™)	Coagulation Factor VIIa	Venom + fVII + substrate	Hydrolysis of Arg-AMC amide bond	Activation of prey's Factor VII in Factor VIIa Activation of
SN-17a (Haematologic Technologies Inc.)	Thrombin	Venom + human prothrombin + substrate	Hydrolysis of ANSN group	prey's Prothrombin in Thrombin

In each experiment the samples were tested under identical conditions and simultaneously in a 384-well plate at 37°C. All assays were repeated in triplicate. Blank wells were measured to establish the lower limit and positive and negative controls were employed to ensure success of the assay and to eliminate potential cross reactivity of the venom-substrate in activation assays. Specific buffer (150mM NaCl and 50mM Tri-HCl, pH 7.4) was freshly prepared and used in all the assays. Reactions (the relative change in fluorescence) were measured following the instructions of the substrates' manufacturer (filter pairs: excitation and emission) with the software Thermo Fluoroskan Ascent™ 2.6. Relative enzymatic activity was calculated as an increase in absorbance corresponding to the cleavage of the fluorescent group. Finally, the raw data were normalised to meet analysis assumptions and processed with GraphPad Prism 7.0.

Factor Xa activity assay

Factor Xa rapidly catalyses prothrombin conversion to thrombin either as prothrombinase complex coupled with the co-factor Va or independently. Hence, it plays an essential role in the clotting cascade. Relevantly, it is present in the venom of *Pseudonaja* ssp. in this active form, and is largely responsible for the clinical relevance of this genus, which is well known to cause blood disruptions in bite victims due to the consumptive coagulopathy in human victims versus stroke formation in prey items. This differential effect is due to the venom being diluted into a larger blood volume in humans with the subsequent formation of microthrombi, versus the more concentrated venom: blood ratio in prey facilitating the formation of large blood clots. Variation between all species and further for three species in adults *versus* juveniles was investigated for the first time. For this test, 90µl of substrate SN-7 (1.35 µl per well - 15µl per 1 mL of buffer) were added to 10µl of venom in three different amounts (0.5µg, 1µg and 2µg per well). Human Factor Xa (5.9 mg/ml HCXA -OO60 - R&D systems) was used as positive control (0.073µg per well). Filter pairs were set at 355/460 nm (excitation/emission) and the reaction was measured every 20 seconds over 60 minutes.

Factor VII activation assay

Factor VIIa has a major role in the clotting cascade, as it converts Factor X to Factor Xa, which either creates (with fVa) the prothrombinase complex, which converts large amounts of prothrombin to thrombin, or directly converts prothrombin, resulting in clot formation. This study investigated the venoms' potential to activate this important factor. For this assay, 10µl of venom (1µg) and 10µl of Human Factor VII (0.1µg per well - Haematologic Technologies Inc. HCVIIA-0030) were mixed in a 384-well plate (Thermo Fisher Scientific) and immediately 80µl of ES011 substrate (0.9µl per well - 11.25µl per 1ml of buffer) were dispensed. The hydrolysis reaction was monitored at 390/ 460 nm (Ex/Em). Increase in fluorescence was measured over 100 minutes every 30s. Controls were established as follows: human Factor VIIa (0.1µg in 20µl of buffer - Haematologic Technologies Inc. HCVIIA-0031)/substrate was used as positive control, moreover, another positive control was established utilising coastal taipan (*Oxyuranus scutellatus*) venom, which is well known to convert Factor VII to Factor VIIa (as already described for the samples, 1µg in 10µl of venom/0.1µg in 10µl of Human Factor VII/substrate) (Nakagaki et al., 1992). Factor VII (0.1µg in 20µl of buffer) substrate alone was used as negative control. Additionally, each venom (1µg in 20µl of buffer) was mixed with the substrate to quantify the venom cross-reactivity. Positive conversion of Factor VII to Factor VIIa from the venom was determined by the increase of fluorescence given resultant from the cleavage of the substrate, which is specific for Factor VIIa.

Prothrombin activation assay

Given the numerous feedback activations of other cofactors endorsed by thrombin generation, this serine protease assumes a key role in the clotting cascade. The central reaction, thrombin generation from the zymogen prothrombin, was tested. For this analysis, 10µl of venom (1µg) and 10µl of human prothrombin (0.075µg per well - Haematologic Technologies Inc. HCP-0010) were mixed in a 384-well plate (Thermo Fisher Scientific) and immediately 80µl of SN-17a substrate (0.9 µl per well - 11.25µl per 1ml of buffer) were dispensed. The hydrolysis reaction was monitored at 355/ 460 nm (Ex/Em). Increase in fluorescence was measured over 200 minutes every 30s. Controls were established as follows: human alpha-thrombin (0.075µg in 20µl of buffer - Haematologic Technologies Inc. HCT-0020)/substrate was used as positive control to ensure the functionality of the substrate; Human Prothrombin (0.075µg in 20 µl of buffer)/substrate as negative control; moreover, each venom (1µg in 20 µl of buffer) was mixed with the substrate to quantify the venom cross-reactivity, which was subtracted from the measurements if found. Proteolytic activation of the zymogen prothrombin to thrombin from the venom was determined by the increase of fluorescence given by the cleavage of the substrate, which is thrombin-specific.

Plasmin inhibition assay

The serine protease plasmin actively dissolves fibrin blood clots, a counterproductive mechanism for procoagulant venom. To investigate the venom's potential inhibition of this regulatory component of the coagulation cascade in order to promote stable and long lasting clots, plasmin inhibition tests were performed. For this assay, 10µl of venom (2 µg) and 10µl of human plasmin (0.1µg - Haematologic Technologies Inc. HCPM-0140) were incubated in a 384-well plate (Thermo Fisher Scientific) at 37°C for 5 minutes, followed by the addition of 80µl of fluorogenic substrate SN-5 (0.9µl per well - 11.25µl per 1ml of buffer) for a total volume of 100µl. The positive control was plasmin/substrate in the same quantity, whereas the negative control was venom/substrate. Plasmin activity was monitored over 1 hour every 20 seconds at 355/460 nm (Ex/Em). Inhibitory activity was determined by a decrease in plasmin activity compared to the positive control (plasmin + substrate).

Phylogenetic Comparative Analyses

A phylogeny was assembled using Skinner et al. 2005 as this is currently the most comprehensive phylogeny available for *Pseudonaja* and was used for all further analyses conducted in R v3.2.5 (R-Core-Team, 2011) using the ape package (Paradis et al., 2004) for general handling of phylogenetic and trait data. Ancestral states were estimated and reconstructed over the tree in

order to investigate the evolutionary history of the traits and consequently their relation to one another over time. The continuous functional traits were reconstructed by maximum likelihood in the contMap function in phytools (Revell, 2012).

We then fit pGLS models (Symonds and Blomberg, 2014) in caper (Orme et al., 2015) to test for relationships between the different functional activities, and between each functional activity (as the response variable) and diet classes (generalist versus reptile specialists). Due to sample size constraints we restricted each model to a single explanatory variable and in each case we arcsine square-root transformed the response variable to improve model fit (since the functional activity values are bound between 0 and 1). Furthermore, for the models considering the relationship between different activities, we removed the three juveniles from the tree for two reasons. Firstly, their presence poses conceptual issues as they have not evolutionarily diverged from conspecific adults, and secondly, since ontogenetic shifts are already known to occur, comparing relationships between activities amongst only adults ensures better comparability (comparing ‘like for like’). In contrast, we included juveniles in models for dietary relationships by adding them to the tree as sister taxa to adult conspecifics with very short branch lengths (0.01 million years). This decision was driven by the fact that of the four reptile specialists in our dataset, all were juveniles which exhibited ontogenetic shifts in diet, except one (*P. modesta*). Therefore, inclusion of juveniles was necessary to have enough contrasts to avoid analytical issues, though these results should be interpreted with slightly more caution than the previous set of analyses.

RESULTS

Factor Xa activity assay

Increases in absorbance, which are correlated with increases in fluorescence and thus to substrate consumption, indicate the presence of Factor Xa in the venom of all *Pseudonaja* adults (Figure 1A), except for that of *P. modesta* (mean \pm SE = 0.17 ± 0.02). *P. affinis*, *P. nuchalis* and *P. textilis* juveniles have an average increase approaching zero (0.57 ± 0.01 ; 0.38 ± 0.02 ; 0.4 ± 0.01 , respectively), indicating that juvenile venoms possess almost no fXa activity. *P. mengdeni*, *P. textilis* and *P. nuchalis* venoms consume more of the substrate at each concentration than those of their congeners. Normalised values are plotted in Figure 1A, showing that *P. mengdeni* venom causes the highest rate of absorbance increase, with the *P. nuchalis* rate approximately half as rapid.

Factor VII activation assay

Normalised absorbance values for the Factor VII activation assay values are plotted in Figure 1B to compare increase of absorbance rate among species. *P. guttata* venom exhibits the fastest

conversion of Factor VII to its active form Factor VIIa. *P. affinis*, *P. aspidorhyncha* and *P. inframacula* venom exhibit a rate that is less than half of the *P. guttata* rate. Venom from juveniles of all *Pseudonaja* species does not activate Factor VII and neither does that of *P. modesta*.

Prothrombin activation assay

Normalised absorbance values for the prothrombin activation assay values are plotted in Figure 2B. There was a significant difference amongst species in increases of absorbance at the same venom concentrations. *P. guttata*, *P. ingrami*, *P. mengdeni*, *P. nuchalis* and *P. textilis* venoms cleaved all substrate in less than 20 minutes. In contrast, over the 3 hour assay *P. affinis* and *P. aspidorhyncha* venoms exhibited a slow conversion rate of prothrombin to thrombin. *P. modesta* and juveniles of other species did not convert prothrombin into thrombin.

Phylogenetic generalised least squares regression

Analyses were conducted based upon phylogenetic generalised least squares regression (pGLS). Factor Xa activity of the venom predicts the ability of the venom to activate Factor VII (pGLS: $t=3.9573$, $df=1$, $P=0.0027$) (Figure 1), and Factor Xa activity is extremely positively related to prothrombin activation (pGLS: $t=21.8440$, $df=1$, $P=9.047e-10$) (Figure 2). The ability of factor Xa to activate factor VII and also prothrombin is reflected in the correlation between these two factors (Figure 3), with factor VII activation by the venom predicting prothrombin activation (pGLS: $t=6.8513$, $df=1$, $P=4.451e-05$).

As these venoms are potently procoagulant, it would be expected that there would be a selection pressure for the co-evolution of procoagulation and plasmin inhibition as the latter activity would extend the effects of the former. However, this was shown to not be the case. The relative degree of plasmin inhibition was not correlated with Factor Xa activity (pGLS: $t=1.320$, $df=1$, $P=0.229$) (Figure 4), Factor VII activation (pGLS: $t=0.246$, $df=1$, $P=0.813$) (Figure 5) or prothrombin activation (pGLS: $t=0.365$, $df=1$, $P=0.726$) (Figure 6).

Our analysis of dietary classes revealed that specialisation on reptile prey in *P. modesta* or juveniles of other species was linked to lower Factor Xa activity (pGLS: $t=2.925$, $df=1$, $P=0.015$), Factor VII activity (pGLS: $t=3.892$, $df=1$, $P=0.003$), lower prothrombin activity (pGLS: $t=3.052$, $df=1$, $P=0.012$) and lower plasmin activity (pGLS: $t=2.830$, $df=1$, $P=0.018$).

DISCUSSION

All enzymatic activity assays were performed with the aim of understanding interspecific variation in the activity of brown snake venoms and to investigate the recent hypothesis correlating the apparent absence of prothrombin activators in the venom of *P. modesta* and juveniles of other *Pseudonaja* ssp. with the feeding ecology of these snakes (Jackson et al., 2016).

The Factor Xa assay (Figure 1A) confirmed the possession of this activity by the venom of adults of all species of *Pseudonaja* barring *P. modesta*, corroborating previous proteomic, transcriptomic, pharmacological and clinical data (White et al., 1986; Jackson et al., 2013; 2016). Adult *P. mengdeni*, *P. nuchalis* and *P. textilis* venoms consumed the substrate most rapidly, indicating the presence of large quantities of Factor Xa in their venom.

The high rate of Factor VII activation caused by (adult) *Pseudonaja* venoms may explain the rapid rate of coagulopathy induced by *Pseudonaja* envenomations to humans (Sutherland and Leonard, 1994) (Figure 1B). This is due to the feedback loop in which Factor VIIa in turn activates more of the bite victim's endogenous Factor X, thus increasing the rate of overall prothrombin activation. The high levels of Factor Xa activity, prothrombin activation activity and Factor VII activation activity recorded here for the previously unstudied *P. guttata* venom suggest it may be a species capable of inflicting lethal human envenomations, despite being one of the smallest brown snakes (only slightly larger than *P. modesta*).

It is clear that there is a correlation between Factor Xa activity and prothrombin activity, with juveniles and *P. modesta* lacking both (Figure 2B). The relative role of Factor Xa's co-factor Factor Va (present in the venom of *Pseudonaja* ssp. as part of the prothrombinase toxin complex) in driving the relative prothrombin activation rate should be the subject of future studies.

Previously only *P. textilis* venom had been examined for plasmin inhibition activity and tests had been conducted only using the (isolated) kunitz peptides that are responsible for this activity (Filippovich et al., 2002). The current study revealed that this activity is widespread within the genus (Figure 4C). However there was not a direct correlation between plasmin inhibition and the procoagulant activities of the venom, indicating a lack of a direct co-evolutionary selection pressure between toxin types (Figures 4, 5, and 6). This is consistent with kunitz peptides and prothrombin activators being under differential gene expression control. The strong plasmin inhibition of the adult venoms would cause blood clots to persist, thus potentiating prey immobilisation by stroke formation. Therefore, it is very intriguing to note the apparent absence of this activity in adult *P. ingrami* venom despite it possessing potent prothrombin activation activity.

That *P. guttata*, a species that includes large quantities of frogs in its diet (Shine 1977), should have potentially procoagulant venom correlates with the presence of procoagulant toxins in the venom of many other ranivorous Australian elapid snakes (e.g. *Notechis scutatus*, *Tropidechis carinatus*, *Pseudechis porphyriacus* etc.). This may relate to the elevated metabolic rates of calling

male frogs encountered by the snakes increasing the frog's blood flow, to the difficulties faced by interclass prey organisms in evolving inhibitor-mediated defences against enzymatic toxins of venomous predators, or to peculiarities of the poorly studied coagulation cascade of amphibians. This general trend warrants further investigation. On the other hand, all of these species at least occasionally (if not frequently) also feed on mammals, and the warm blood and elevated metabolic rates of mammals make haemotoxins an attractive choice for mammal-eating snakes. Other variations in activity on specific substrates are intriguing and may also be linked to diet. As rodents and small marsupials are capable of inflicting significant defensive bites, the venoms of mammal-eating snakes would also likely be shaped by prey retaliation potential, as has been seen for other snakes such as the long-glanded coral snake *Calliophis bivirgatus* (Yang et al. 2016). This strong selection pressure may drive the expression of procoagulant venoms in Australian elapid snakes, even if they include relatively small quantities of mammals in their diet (e.g. *P. inframacula*, included in the present study).

As Factor Xa was recruited to the venom system of Australian elapid snakes very early in their radiation (Jackson et al. 2013), its absence from the venom of many species may be an even more intriguing question than its over-expression in the venom systems of many others. Jackson et al. (2016) discussed in detail the possible reasons for the absence of procoagulant toxins in venoms of juvenile *Pseudonaja* and *P. modesta*. Briefly, these may include not only the ineffectiveness of procoagulant toxins on dormant skinks with depressed metabolic rates, but also the interference of such toxins (via the formation of clots around the bite site) with the effective delivery of neurotoxic peptides to their target receptors. The evolvability of peptide toxins may also make them excellent weapons for venomous predators engaged in arms races with prey organisms (Lobkovsky, et al., 2010, Fry et al., 2009).

Overall, the results of the present study make a contribution to our understanding of the evolutionary history of Australian elapid snake venom. Moreover, the activity of the venom of *P. guttata* was analysed for the first time and the results suggest that this species could be medically important given the effect of its venom prothrombin activator enzymes on human coagulation factors. The demonstration of considerable variation in coagulopathic activity among the venoms of *Pseudonaja* species and its correlation with dietary variation corroborates earlier studies that argued for the role of adaptive differentiation as an influence on venom composition and activity within this genus.

FUNDING

VC was funded by an European Commission Scholarship. JD was funded by an Australian Postgraduate Award PhD Scholarship. BGF was funded by a University of Queensland Major

Equipment and Infrastructure grant. B.L. is in receipt of PhD studentship from the Faculty of Health Science, University of Macau and H.F.K. was supported by a University of Macau Multi-Year Grant (MYRG2015-00025-FHS).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

Barber, P.H., Palumbi, S.R., Erdmann, M. V., Moosa, M.K., 2000. Biogeography: a marine Wallace's line? 406, 692–694. doi:10.1038/35021135

Birrell, G.W., Earl, S.T.H., Wallis, T.P., Masci, P.P., de Jersey, J., Gorman, J.J., Lavin, M.F., 2007. The diversity of bioactive proteins in Australian snake venoms. *Mol. Cell. Proteomics* 6, 973–986. doi:10.1074/mcp.M600419-MCP200

Casewell, N.R., Wagstaff, S.C., Harrison, R.A., Renjifo, C., Wüster, W., 2011. Domain loss facilitates accelerated evolution and neofunctionalization of duplicate snake venom metalloproteinase toxin genes. *Mol. Biol. Evol.* 28, 2637–2649. doi:10.1093/molbev/msr091

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. doi:10.1016/j.tree.2012.10.020

Cogger, H. G., 2014. Reptiles and amphibians of Australia. Seventh edition. CSIRO PUBLISHING.

Daltry, J.C., Ponnudurai, G., Shin, C.K., Tan, N.-H., Thorpe, R.S., Wuster, W., 1996a. Electrophoretic profiles and biological activities: intraspecific variation in the venom of the Malayan Pit Viper (*Calloselasma rhodostoma*). *Toxicon* 34(1), 67-79.

Daltry, J.C., Wuster, W., Thorpe, R.S., 1996b. Diet and snake venom evolution. *Nature*. doi:10.1038/379537a0

de la Vega, R. C. R., Merino, E., Becerril, B., Possani, L. D., 2003. Novel interactions between K⁺ channels and scorpion toxins. *Trends Pharmacol. Sci.*, 24(5), 222-227. doi:10.1016/S0165-6147(03)00080-4

Durban, J., Pérez, A., Sanz, L., Gómez, A., Bonilla, F., Rodríguez, S., Chacón, D., Sasa, M., Angulo, Y., Gutiérrez, J.M., Calvete, J.J., 2013. Integrated “omics” profiling indicates that miRNAs are modulators of the ontogenetic venom composition shift in the Central American rattlesnake, *Crotalus simus simus*. *BMC Genomics* 14, 234. doi:10.1186/1471-2164-14-234

Earl, S., Sunagar, K., Jackson, T. N. W., Reeks, T. and Fry, B. G. (2015). Factor Va Proteins. In Fry, B. G., (Ed.). *Venomous reptiles and their toxins: evolution, pathophysiology and biodiscovery* (pp. 255-260). Oxford University Press.

Elena, S.F., Lenski, R.E., 2003. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nat Rev Genet* 4, 457–469.

Filippovich, I., Sorokina, N., Masci, P.P., De Jersey, J., Whitaker, A.N., Winzor, D.J., Gaffney, P.J., Lavin, M.F., 2002. A family of textilinin genes, two of which encode proteins with antihemorrhagic properties. *Br. J. Haematol.* 119, 376–384. doi:10.1046/j.1365-2141.2002.03878.x

Fry, B. G., 2005. From genome to “venome”: molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. *Genome Res.* 15, 403–420. doi:10.1101/gr.3228405

Fry, B.G., Casewell, N.R., Wüster, W., Vidal, N., Young, B., Jackson, T.N.W., 2012. The structural and functional diversification of the Toxicofera reptile venom system. *Toxicon* 60, 434–448. doi:10.1016/j.toxicon.2012.02.013

Fry, B.G., Roelants, K., Champagne, D.E., Scheib, H., Tyndall, J.D.A., King, G.F., Nevalainen, T.J., Norman, J.A., Lewis, R.J., Norton, R.S., Renjifo, C., de la Vega, R.C.R., 2009. The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. *Ann. Rev. Genom. Hum. Genet.*, 10, 483–511. doi:10.1146/annurev.genom.9.081307.164356

Fry, B.G., Scheib, H., van der Weerd, L., Young, B., McNaughtan, J., Ramjan, S.F.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, 215–46. doi:10.1074/mcp.M700094-MCP200

Fry, B.G., Wüster, W., Ryan Ramjan, S.F., Jackson, T., Martelli, P., Kini, R.M., 2003. Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: evolutionary and toxinological implications. *Rapid Commun. Mass Spectrom.* 17, 2047–62. doi:10.1002/rcm.1148

Gibbs, H.L., Mackessy, S.P., 2009. Functional basis of a molecular adaptation: Prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. *Toxicon* 53, 672–679. doi:10.1016/j.toxicon.2009.01.034

Harris, R.J., Arbuckle, K., 2016. Tempo and mode of the evolution of venom and poison in tetrapods. *Toxins*, 8, 193.

Heyborne, W.H., Mackessy, S.P., 2013. Identification and characterization of a taxon-specific three-finger toxin from the venom of the Green Vinesnake (*Oxybelis fulgidus*; family Colubridae). *Biochimie* 2013, 95, 1923–1932.

Isbister, G., 2006. Snake bite: a current approach to management. *Aust. Prescr.*, 29(5), 125-129

Jackson, T.N.W., Sunagar, K., Undheim, E.A.B., Koludarov, I., Chan, A.H.C., Sanders, K., Ali, S.A., Hendriks, I., Dunstan, N., Fry, B.G., 2013. Venom down under: Dynamic evolution of Australian elapid snake toxins. *Toxins (Basel)*. 5, 2621–2655. doi:10.3390/toxins5122621

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., Hendriks, 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 (Basel)*. 8, 1–23. doi:10.3390/toxins8110309

Judge, R.K., Henry, P.J., d'Aprile, A.C., Lynch, D., Jelinek, G.A., Wilce, M.C., Wilce, J.A., 2002. Identification of PLA(2) and alpha-neurotoxin proteins in the venom of *Pseudonaja affinis* (dugite). *Toxicol Appl Pharmacol* 181, 184–191. doi:10.1006/taap.2002.9416

Kini, R. M. and Chan, Y. M., 1999. Accelerated evolution and molecular surface of venom phospholipase A2 enzymes. *J. Mol. Evol.*, 48(2), 125–132.

Kordis, D, and Gubenek, F., 2000. Adaptive evolution of animal toxin multigene families. *Gene*, 261, 43–52.

Lobkovsky, A.E., Wolf, Y.I., Koonin, E.V., 2010. Universal distribution of protein evolution rates as a consequence of protein folding physics. *Proc. Natl. Acad. Sci. USA*, 107, 2983–2988.

Mackessy, S.P., Williams, K., Ashton, K.G., 2003. Ontogenetic Variation in Venom Composition and Diet of *Crotalus oreganus concolor*: a case of venom paedomorphosis?. *Copeia* 2003(4), 769–782. doi:http://dx.doi.org/10.1643/HA03-037.1

Masci, P.P., Whitaker, A.N., and De Jersey, J., 1988. Purification and characterization of a prothrombin activator from the venom of the Australian brown snake, *Pseudonaja textilis textilis*. *Biochem. Int.*, 17(5), 825-835.

Millers, E. K. I., Trabi, M., Masci, P. P., Lavin, M. F., de Jersey, J., Guddat, L. W., 2009. Crystal structure of textilinin-1, a Kunitz-type serine protease inhibitor from the venom of the Australian common brown snake (*Pseudonaja textilis*). *FEBS journal*, 276(11), 3163-3175.

Mirtschin, P.J., Dunstan, N., Hough, B., Hamilton, E., Klein, S., Lucas, J., Millar, D., Madaras, F., Nias, T., 2006. Venom yields from Australian and some other species of snakes. *Ecotoxicology* 15, 531–538. doi:10.1007/s10646-006-0089-x

Mengden, G. A., 1985. Australian elapid phylogeny: A summary of the chromosomal and electrophoretic data. In Grigg, G. C., Shine, R., and Ehmann, H. (Eds.) *Biology of Australasian frogs and reptiles*, (pp. 185-192), Royal Zool. Soc. N.S.W., Sydney.

Nakagaki, T., Lin, P., and Kisiel, W., 1992. Activation of human Factor VII by the prothrombin activator from the venom of *Oxyuranus scutellatus* (Taipan snake). *Thromb. Res.*, 65(1), 105-116.

Nei, M., Gu, X., and Sitnikova, T. (1997). Evolution by the birth-and-death process in multigene families of the vertebrate immune system. *Proc. Natl. Acad. Sci. U. S. A* 94(15), 7799-7806.

Orme, D., Freckleton, R., Thomas, G., Petzoldt, T., Fritz, S., Isaac, N., Pearse, W., 2015. Caper: Comparative Analyses of Phylogenetics and Evolution in R, R Package Version 0.5.2, Available online: <https://CRAN.R-project.org/package=caper> (accessed on 01/02/17).

Paradis, E., Claude, J., and Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*, 20(2), 289-290.

Pawlak, J., Mackessy, S.P., Fry, B.G., Bhatia, M., Mourier, G., Fruchart-Gaillard, C., Servent, D., Ménez, R., Stura, E., Ménez, A., Kini, R.M., 2006. Denmotoxin, a three-finger toxin from the colubrid snake *Boiga dendrophila* (mangrove catsnake) with bird-specific activity. *J. Biol. Chem.* 281, 29030–29041. doi:10.1074/jbc.M605850200

R-Core-Team, R. A Language and Environment for Statistical Computing; R Foundation for Statistical Computing: Vienna, Austria, 2011. Available online: <https://www.R-project.org/> (accessed on 27 January 2017).

Rao, V.S. and Kini, R.M., 2002. Pseutarin C, a prothrombin activator from *Pseudonaja textilis* venom: its structural and functional similarity to mammalian coagulation Factor Xa–Va complex. *Thromb. Haemost.* 88(4), 611–9.

Reeks, T., Lavergne, V., Sunagar, K., Jones, A., Undheim, E., Dunstan, N., Fry, B., Alewood, P.F., 2016. Deep venomics of the *Pseudonaja* genus reveals inter- and intra-specific variation. *J. Proteomics* 133, 20–32. doi:10.1016/j.jprot.2015.11.019

Revell, L. J., 2012. Phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.*, 3(2), 217-223.

Shine, R., 1977. Habitats, diets, and sympatry in snakes: a study from Australia. *Can. J. Zool.* 55, 1118–1128. doi:10.1139/z77-144

Skejitch, J., Hodgson, W.C., 2013. Population Divergence in Venom Bioactivities of Elapid Snake *Pseudonaja textilis*: Role of Procoagulant Proteins in Rapid Rodent Prey Incapacitation. *PLoS One* 8, 1–10. doi:10.1371/journal.pone.0063988

Skinner, A., Donnellan, S.C., Hutchinson, M.N., Hutchinson, R.G., 2005. A phylogenetic analysis of *Pseudonaja* (Hydrophiinae, Elapidae, Serpentes) based on mitochondrial DNA sequences. *Mol Phylogenet Evol* 37, 558–571. doi:10.1016/j.ympev.2005.06.020

Sunagar, K., Casewell, N.R., Varma, S., Kolla, R., Antunes, A., Moran, Y., 2016. Deadly Innovations: Unraveling the Molecular Evolution of Animal Venoms. *Venom Genomics and Proteomics*, 1-27. doi:10.1007/978-94-007-6649-5_49-1

Sutherland, S. K., Leonard, R. L., 1994. Snakebite deaths in Australia 1992-1994 and a management update. *Med. J. Aust*, 163(11-12), 616-618.

Symonds, M. R., and Blomberg, S. P., 2014. A primer on phylogenetic generalised least squares. In *Modern phylogenetic comparative methods and their application in evolutionary biology* (pp. 105-130). Springer Berlin Heidelberg.

Thompson, J.N., 1999. The evolution of species interactions. *Science*, 284(5423), 2116-2118.

Trabi, M., Sunagar, K., Jackson, T. N. W., and Fry, B. G. (2015). Factor Xa Enzymes. In Fry, B. G., (Ed.) *Venomous reptiles and their toxins: evolution, pathophysiology and biodiscovery* (pp. 261-266). Oxford University Press.

Vonk, F.J., Casewell, N.R., Henkel, C. V., Heimberg, A.M., Jansen, H.J., McCleary, R.J.R., Kerckamp, H.M.E., Vos, R.A., Guerreiro, I., Calvete, J.J., Wüster, W., Woods, A.E., Logan, J.M., Harrison, R.A., Castoe, T.A., de Koning, A.P.J., Pollock, D.D., Yandell, M., Calderon, D., Renjifo, C., Currier, R.B., Salgado, D., Pla, D., Sanz, L., Hyder, A.S., Ribeiro, J.M.C., Arntzen, J.W., van den Thillart, G.E.E.J.M., Boetzer, M., Pirovano, W., Dirks, R.P., Spaink, H.P., Duboule, D., McGlenn, E., Kini, R.M., Richardson, M.K., 2013. The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system. *Proc. Natl. Acad. Sci. U. S. A.* 110, 20651–20656. doi:10.1073/pnas.1314702110

White, J., Williams, V., Passahl, J. H., 1986. The five-ringed brown snake, *Pseudonaja modesta* (Gunther): report of a bite and comments on its venom. *Med. J. Aust.* 147(11-12), 603-605.

Williams, D., Wüster, W., Fry, B.G., 2006. The good, the bad and the ugly: Australian snake taxonomists and a history of the taxonomy of Australia's venomous snakes. *Toxicon* 48, 919–930. doi:10.1016/j.toxicon.2006.07.016

Wüster, W., Dumbrell, A.J., Hay, C., Pook, C.E., Williams, D.J., Fry, B.G., 2005. Snakes across the Strait: Trans-Torresian phylogeographic relationships in three genera of Australasian snakes (Serpentes: Elapidae: *Acanthophis*, *Oxyuranus*, and *Pseudechis*). *Mol. Phylogenet. Evol.* 34, 1–14. doi:10.1016/j.ympev.2004.08.018

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., Peng, K.L.K., Frank, N., Vetter, I., Fry, B.G., 2016. The snake with the scorpion's sting: novel three-finger toxin sodium channel agonists from the venom of the long-glanded blue coral snake. *Toxins* 8(10), 303. doi:10.3390/toxins8100303

ACCEPTED MANUSCRIPT

Figure 1: Ancestral state reconstructions over branches for A) Factor Xa activity and B) Factor VII activation where warmer colours represent higher normalized activity. Bars indicate 95% confidence intervals for the estimate at each node, note that due to the high dynamicity of venom evolution these quickly become broad as you move down the tree. Phylogeny follows Skinner et al. 2005.

Figure 2: Ancestral state reconstructions over branches for A) Factor Xa activity and B) prothrombin activation where warmer colours represent higher normalized activity. Bars indicate 95% confidence intervals for the estimate at each node, note that due to the high dynamicity of venom evolution these quickly become broad as you move down the tree. Phylogeny follows Skinner et al. 2005.

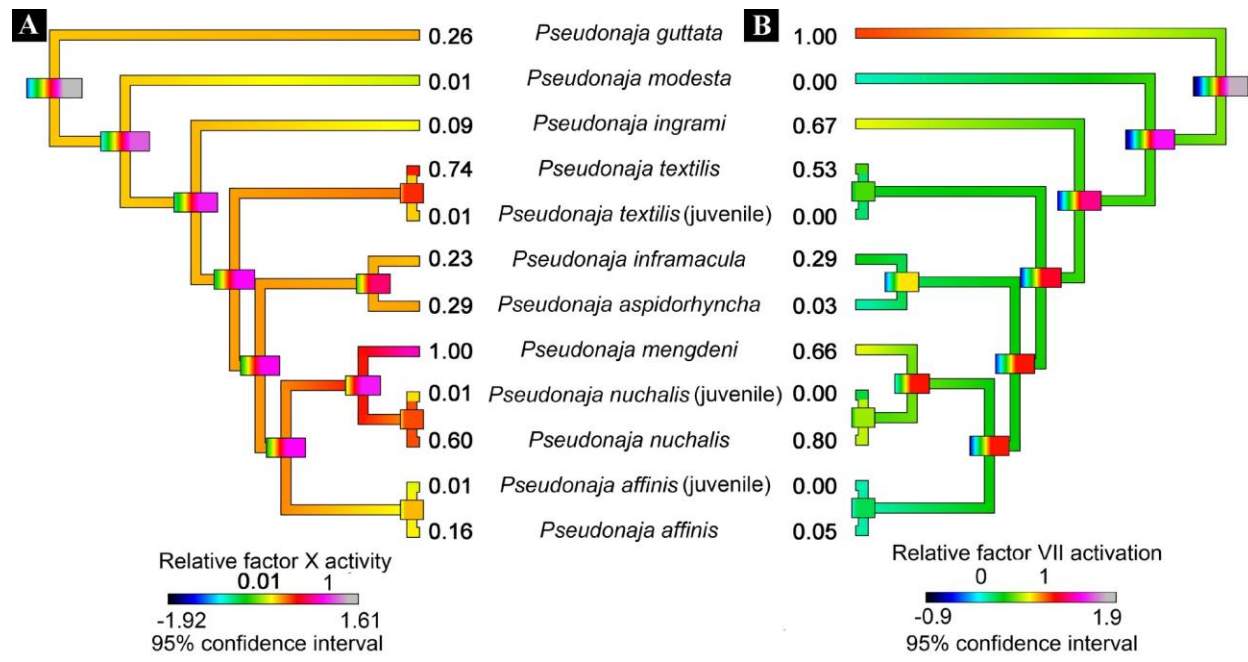
Figure 3: Ancestral state reconstructions over branches for A) Factor VII activation and B) prothrombin activation where warmer colours represent higher normalized activity. Bars indicate 95% confidence intervals for the estimate at each node, note that due to the high dynamicity of venom evolution these quickly become broad as you move down the tree. Phylogeny follows Skinner et al. 2005.

Figure 4: Ancestral state reconstructions over branches for A) Factor Xa activity and B) plasmin inhibition where warmer colours represent higher normalized activity. Bars indicate 95% confidence intervals for the estimate at each node, note that due to the high dynamicity of venom evolution these quickly become broad as you move down the tree. Phylogeny follows Skinner et al. 2005.

Figure 5: Ancestral state reconstructions over branches for A) Factor VII activation and B) plasmin inhibition where warmer colours represent higher normalized activity. Bars indicate 95% confidence intervals for the estimate at each node, note that due to the high dynamicity of venom evolution these quickly become broad as you move down the tree. Phylogeny follows Skinner et al. 2005.

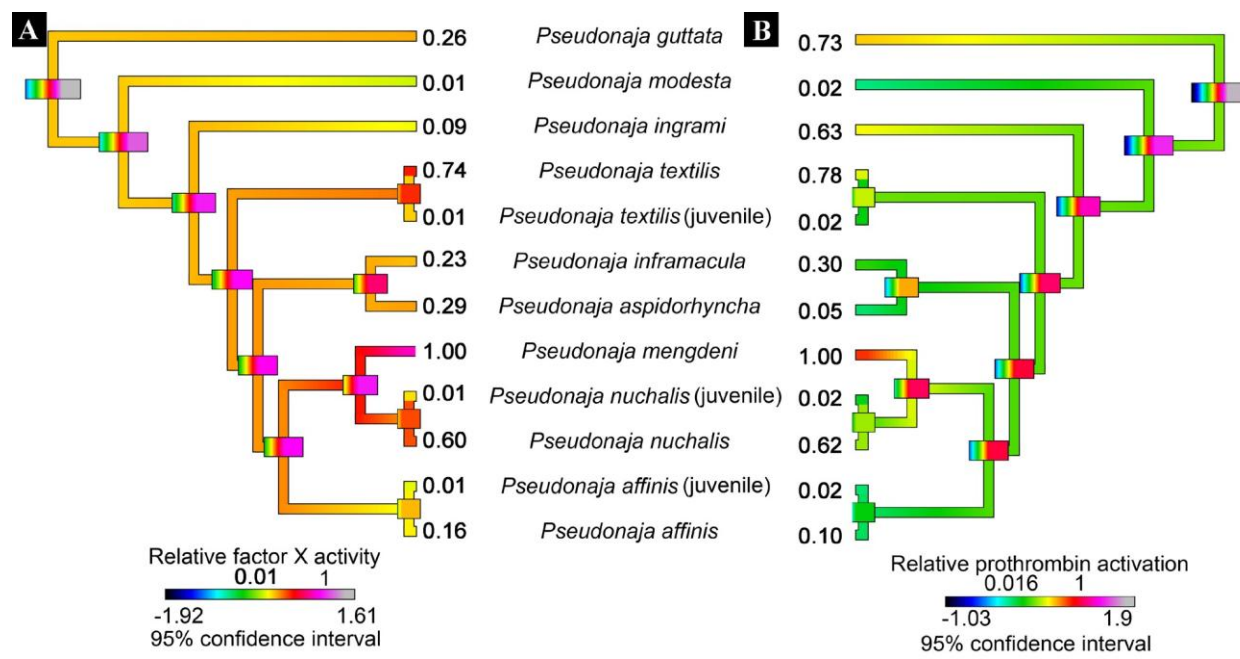
Figure 6: Ancestral state reconstructions over branches for A) prothrombin activation and B) plasmin inhibition where warmer colours represent higher normalized activity. Bars indicate 95% confidence intervals for the estimate at each node, note that due to the high dynamicity of venom evolution these quickly become broad as you move down the tree. Phylogeny follows Skinner et al. 2005.

Figure 1



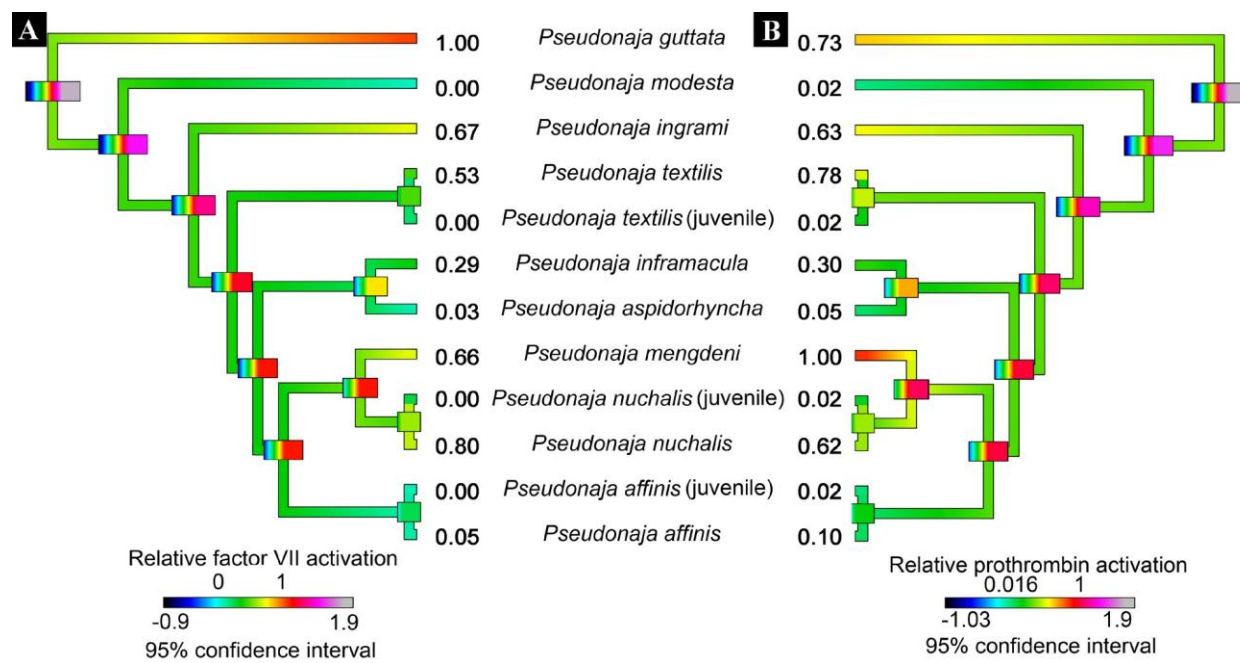
ACCEPTED MANUSCRIPT

Figure 2



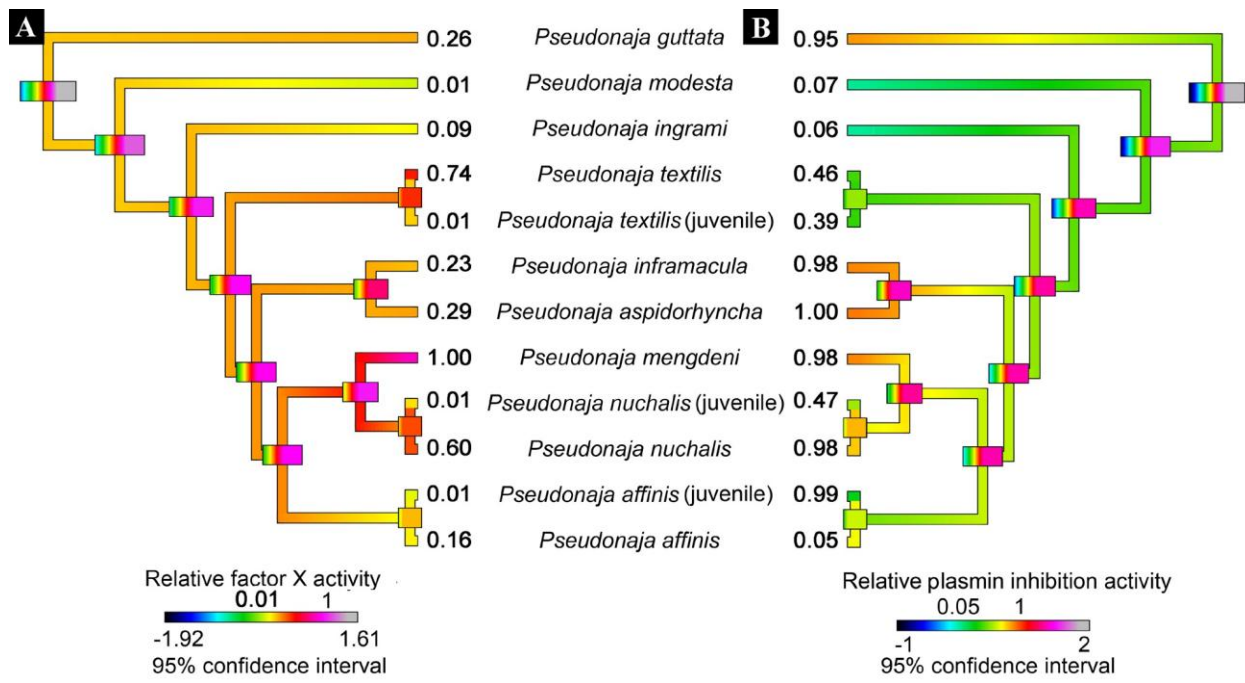
ACCEPTED MANUSCRIPT

Figure 3



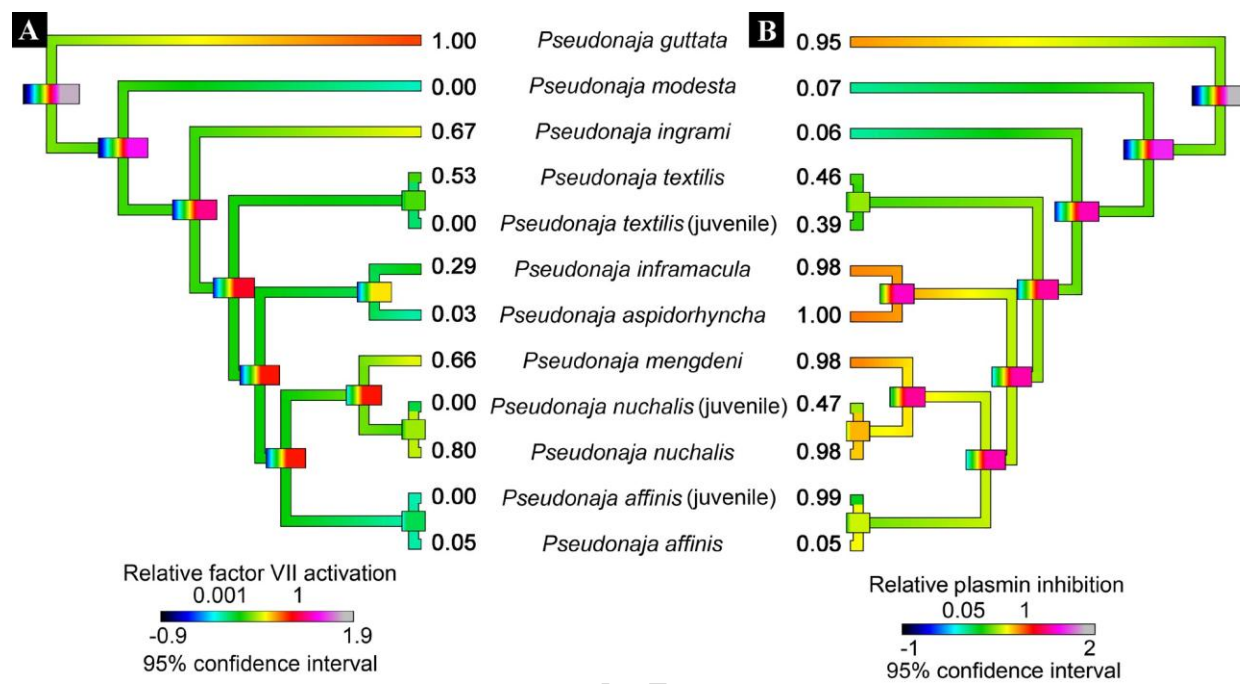
ACCEPTED MANUSCRIPT

Figure 4



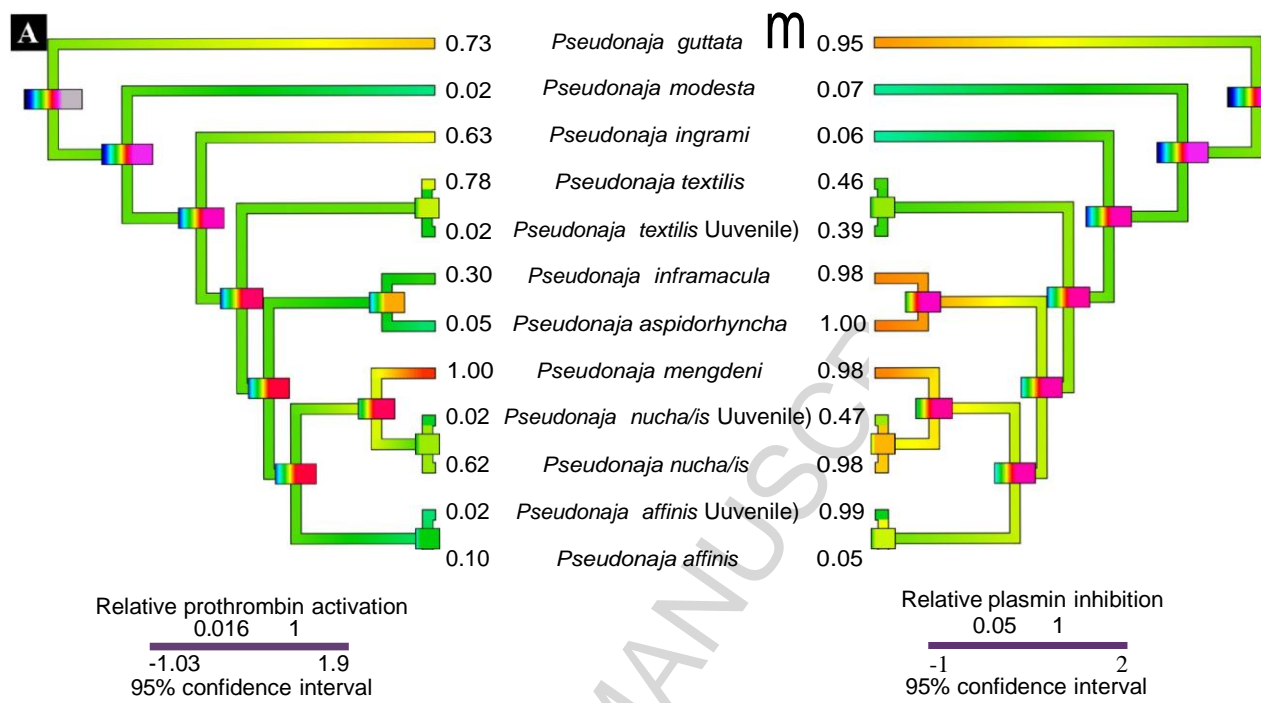
ACCEPTED M

Figure 5



ACCEPTED MANUSCRIPT

Figure 6



ACCEPTED MANUSCRIPT