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Catch a tiger snake by its tail: differential toxicity, co-factor dependence and antivenom efficacy in a procoagulant clade of Australian venomous snakes.

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Abstract

A paradigm of venom research is adaptive evolution of toxins as part of a predator-prey chemical arms race. This examined differential co-factor dependence, variations relative to dietary preference, and the impact upon relative neutralisation by antivenom of the procoagulant toxins in the venoms of a clade of Australian snakes. All genera were characterised by venoms rich in factor Xa which act upon endogenous prothrombin. Examination of toxin sequences revealed an extraordinary level of conservation, which indicates that adaptive evolution is not a feature of this toxin type. Consistent with this, the venoms did not display differences on the plasma of different taxa. Examination of the prothrombin target revealed endogenous blood proteins are under extreme negative selection pressure for diversification, this in turn puts a strong negative selection pressure upon the toxins as sequence diversification could result in a drift away from the target. Thus this study reveals that adaptive evolution is not a consistent feature in toxin evolution in cases where the target is under negative selection pressure for diversification. Consistent with this high level of toxin conservation, the antivenom showed extremely high-levels cross-reactivity. There was however a strong statistical correlation between relative degree of phospholipid-dependence and clotting time, with the least dependent venoms producing faster clotting times than the other venoms even in the presence of phospholipid. The results of this study are not only of interest to evolutionary and ecological disciplines, but also have implications for clinical toxinology.

Keywords: venom; toxin; adaptive evolution; coagulopathy; disseminated intravascular coagulation; antivenom; elapid

Introduction

Venom is a bioactive secretion produced in specialised cells which is injected into a target animal through a wound (Fry et al. 2009a; Fry et al. 2009b). Venom typically consists of a complex combination of enzymes, toxic proteins, peptides, organic and inorganic ions and salts (Fry et al. 2009a; Fry et al. 2009b) and is an evolutionary innovation typically utilised for either a defensive or predatory purpose. Once injected, the numerous toxic compounds act to facilitate subjugation of prey by disrupting homeostasis through the perturbation of normal action of vital enzymes, receptors, and/or ion channels (Calvete et al., 2009).

Venomous reptiles evolved within the clade Toxicofera—the common ancestor of the Iguania lizards, Anguimorpha lizards, and snakes possessed the substrate from which divergent venom systems later evolved (Fry et al., 2006; Fry et al., 2012). Venom has been hypothesised as the key initiator behind the explosive radiation of the advanced snakes (Caenophidia) (Daltry et al., 1996; Vidal et al. 2009) and indeed the evolution of venom seems to be associated with increased diversification rates across all groups of tetrapods (Harris and Arbuckle, 2016).

Globally, Australia is the only continent whose snake fauna is dominated by front-fanged venomous snakes (Keogh et al., 2002). The *Notechis* genus is among the most medically important species of Australian elapid snakes and historically has been responsible for many bites and fatalities (Sutherland and Tibballs 2001). While snake identification can be critical for snakebite treatment, species classification across tiger snake populations has long been a complex taxonomic issue. For example, previous studies used body size and colour variation as the two major characteristics to classify tiger snake populations into subspecies (Rawlinson 1991; Cogger 2000); however, Keogh et al. (2005) used molecular data to reveal that neither of these characteristics are phylogenetically useful in the taxonomy of this group. This supported earlier suggestions by Schwaner (1985a, 1985b) and Schwaner and Sarre (1988) that distinctions based on colour or size were arbitrary and that all populations of *Notechis* comprise a single, polymorphic species with extremely low levels of sequence diversification. As tiger snakes are amongst the most well-known and best studied of Australian elapid taxa, the clade which includes *Notechis* and the closely related genera *Austrelaps, Tropidechis, Hoplocephalus* and *Paroplocephalus* is known as the "tiger snake clade". *Austrelaps* is basal to all other genera in this clade (Lee et al. 2016).

Variation in the composition and activity of snake venoms can have serious implications for snakebite treatment and can be attributed to a number of factors, one of which is population isolation. Detailed studies of sea levels have helped determine the age of *N. scutatus* populations (Keogh et al., 2005). Prior to the last inter-glacial period, *N. scutatus* presumably formed a continuous series of interbreeding populations, from Western Australia through to Queensland (Rawlinson 1974; Schwaner 1985a; Keogh et al., 2005). Rising sea levels 6,000-10,000 years ago inundated the coastal plain in South Australia, causing tiger snake populations to become fragmented (Rawlinson 1974).

Species with fragmented populations are excellent models for the study of venom evolution, as geographically isolated populations with differing environments may evolve significant variation in venom composition (Chippaux et al. 1991, Daltry et al., 1996). This may lead to geographically distinct clinical patterns observed in envenomed humans (Daltry et al., 1997; Barlow et al., 2009; Gibbs et al. 2011; Sunagar et al., 2014). Consequently, even when the offending snake has been correctly identified, the indicated antivenom may provide effective treatment in one region, but could have limited effectiveness or even fail to neutralise venoms of the same species from different regions. This has serious implications for snakes in the *Notechis* clade, as tiger snake antivenom is currently used to treat *Notechis, Austrelaps, Hoplocephalus, and Tropidechis carinatus. Pseudechis porphyriacus* is not a member of this clade but is also treated with tiger snake antivenom because this the venom of species is also rich in FXa (White 2001). Thus, furthering the understanding of venomous taxa and the factors influencing evolution and bioactivity of their venoms is imperative to improving the therapeutic management and medical effectiveness of antivenoms (Calvete et al., 2009; Gutiérrez et al., 2009).

Dietary specialisation is another important factor thought to be a major selective force shaping venom composition, as venom directly relates to a species' ability to subjugate prey (Daltry et al., 1996; Pough et al., 2001; Vonk et al., 2011; Jackson et al. 2016a). Specialisation of diet is more common in snakes than in other reptiles (Toft 1985) and has played a fundamental part in their

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adaptive radiation (Greene 1983). The genus *Austrelaps* has a specialist diet of ectothermic prey (Shine 1987b) and early studies suggested that anurans are the primary prey species for mainland *N. scutatus* (Shine 1977; Shine 1987a; Tan et al., 1993), whereas, island populations consume primarily endotherms (Schwaner 1985a). *Hoplocephalus* species, conversely, exploit more densely forested habitats compared to *Notechis*, and the diet of the two more derived species is predominantly lizards and small mammals, whilst *H. bitorquatus* continues to primarily feed upon frogs (Shine 1983; Webb & Shine, 1998). Sixty percent of the lizards consumed by the highly specialised broad-headed snake (*H. bungaroides*) are a single species of gecko (*Oedura lesueurii*) (Webb & Shine, 1998), and *Hoplocephalus stephensii* diets include significant quantities of small mammals (rats and pygmy possums) (Fitzgerald et al., 2004). The nocturnal predator *Tropidechis carinatus* feeds primarily on both mammals and anurans (Shine & Charles 1982). Thus, there is clearly variable dietary specialisation within the "tiger snake" clade, and this may correlate with geographical variation in the venoms of these species.

Studies that focus on the variability of venom composition are critical for the research and production of antivenoms, which is currently the only specific treatment for snakebite (Maduwage & Isbister, 2014). Antivenom contains antibodies which bind to the toxic components (via the recognition of binding sites – epitopes) in the venom, thereby neutralising them and preventing or reversing clinical effects (Isbister 2010; Maduwage & Isbister, 2014). However, the effects of venom-induced consumption coagulopathy (VICC) resulting from Australasian elapid envenomations have been shown to be irreversible and cannot be neutralised by the antivenom once the damage has already occurred (Isbister et al., 2009; Tanos et al., 2008). The antivenom thus serves to prevent further coagulopathy from developing.

Australian elapid snake venoms produce deleterious effects in humans, sometimes resulting in the collapse of central and peripheral nervous systems, failure of the cardiovascular system, blood coagulation pathways, or disrupting other critical homeostatic systems (Sutherland and Tibballs 2001). Australian elapid snakes are unique amongst elapids in their potent procoagulant effects. These actions are driven by a venom-specific form of factor Xa, the recruitment of which into the venom proteome occurred near the base of the Australian elapid radiation but was only amplified to high levels in divergent genera (Trabi et al., 2015). Factor Xa binds with activated factor V (FVa), which together cleaves prothrombin (factor II) to form thrombin (factor IIa), with thrombin activating fibrinogen to fibrin resulting in clot formation (St Pierre et al., 2005). An exception to the requirement of endogenous FVa as a co-factor is the *Oxyuranus/Pseudonaja* clade, which have additionally recruited FVa into their venom and thus no-longer need to interact with endogenous FVa (Kini et al., 2001).

Snake venom prothrombin-activators are either metalloproteases or serine proteases and are grouped into four categories based on their cofactor requirement; A, B, C and D (Kini et al., 2001). Australian elapids are limited to groups C and D based upon their relative need for endogenous FVa as a co-factor (Joseph & Kini, 2001; Joseph et al., 1999; Rao et al., 2003; Reza et al., 2005). Group C venoms contain both FXa and FVa, therefore, the complete 1:1 prothrombin-activating complex (FVa:FXa) is already expressed in their venoms, and they only require calcium and phospholipid for full activity. The FXa:FVa complex constitutes a high percentage of the total venom protein content in group C; approximately 10-20% of the total *Oxyuranus scutellatus* (coastal taipan) venom (Lavin & Masci, 2009) and 20-40% of *Pseudonaja textilis* (eastern brown snake) venom (Lavin & Masci, 2009; Rao & Kini, 2002). Group D snake venoms contain only FXa, which must bind with endogenous FVa present in the envenomed prey's plasma, as well as calcium and phospholipid for optimal activity (Kini & Chow, 2001; Reza et al., 2006). Group D FXa accounts for approximately 5% the total venom content in *N. scutatus* (Kini 2005). Consequently, the relative rate of action for group D venoms is slower than that of the group C venoms due to lower amounts

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of FXa and their requirement for endogenous FVa and the rate-limiting step of FVa:FXa binding. Expression levels of FXa are highest in endotherm specialists (e.g. *Notechis* and *Oxyuranus* species), with reduced expression levels in extreme lizard specialists like *Pseudonaja modesta* and juveniles of other *Pseudonaja* species (White et al., 1987; Jackson et al. 2016b; Cipriani et al. 2017).

The adaptive evolution of procoagulant toxins in venom has remained under-investigated. For example, previous studies have analysed the venom of *N. scutatus* from only a small subset of different populations (e.g. Williams et al., 1988; Francis et al., 1991; St. Pierre et al., 2005; Munawar et al., 2014). Thus intraspecific variations within this clade are as unknown as interspecific and intragenera variations. Therefore this study will test 1) if diet is a selection pressure operating on prothrombin-activating toxins by comparing the coagulation ability, 2) the efficacy of tiger snake antivenom on an *in vitro* procoagulant test across all venoms in this clade, and 3) the relative calcium and phospholipid co-factor dependency for each venom.

Methodology

Sample collection and preparation

Venom samples were sourced from the pre-existing, cryogenic venom research collection of the Venom Evolution Lab with additional venoms purchased from Venom Supplies (Tanunda 5352, South Australia). A total of 25 venom samples from the *Notechis* clade were used in this study *Austrelaps ramsayi* (Paddys River ACT); *Hoplocephalus bitorquatus* (Texas QLD); *Hoplocephalus bitorquatus* (Brigalow NSW); *Hoplocephalus bungaroides* (Sydney NSW); *Hoplocephalus stephensii* (Brisbane QLD); *Notechis scutatus* (Melbourne VIC); *Notechis scutatus* (Melbourne 1935); *Notechis scutatus* (Werribee VIC); *Notechis scutatus* (Barmah VIC); *Notechis scutatus* (Chappell Island); *Notechis scutatus* (Tasmania); *Notechis scutatus* (Kangaroo Island); *Notechis scutatus* (Perth WA); *Notechis scutatus* (Margaret River WA); *Notechis scutatus* (Coomalbidgup WA); *Notechis scutatus* (Bunya Mt QLD); *Notechis scutatus* (Bruny Island); *Notechis scutatus* (New Year Island); *Notechis scutatus* (Franklin Island); *Notechis scutatus* (Mt Gambier SA); *Notechis scutatus* (Lake Alexandrina SA); *Paroplocephalus atriceps* (Lake Cronin WA); *Tropidechis carinatus* (Brisbane QLD); *Tropidechis carinatus* (Eumundi QLD); and *Tropidechis carinatus* (Numbinbah Valley QLD).Venom variation may occur between individuals in the same population due to differences in sex and age. To account for individual variation in expression level, pooled samples were used when possible (Table 1). For populations which were not represented by pooled venom samples, multiple samples from different individuals were used where available (Table 1).

Lyophilized crude venom was resuspended in deionized water before being centrifuged. It was then passed through a 0.45nm filter (0.45µm Econofltr PES, Agilent Technologies, China) and prepared in a 1:1 mixture as a 1mg/mL stock concentration in 50% glycerol (to prevent freezing) and stored at -20°C. Bioactivity of venoms was preserved during venom preparation by keeping the samples on ice at all times. Human plasma (Batch# 398892, citrate 3.2%) was donated by Australian Red-Cross (research approval# 16-04QLD-10) and was aliquoted into 2mL tubes, flash frozen in liquid nitrogen and stored at -80°C until use. When required, plasma was rapidly thawed in a Thermo Haake ARCTIC immersion bath circulator SC150-A40 at 37°C and immediately used for experimentation.

STA-R MAX

Procoagulation tests were performed using a STA-R-Max® (Stago, Asnières sur Seine, France) with a analyser viscosity-based (mechanical) detection system to measure clotting time of human plasma as a proxy for bioactivity of prothrombin-activating toxins. The efficacy of antivenom in

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prolonging coagulation times was also tested using the analyser.

Procoagulation tests

Prior to experimentation, the viability of human plasma was tested daily as a positive control using a standard activated Partial Thromboplastin Time (aPTT) test. For these tests, kaolin (adapted from STA C.K Prest standard kit, Stago Catalog#00597) which is a coagulation-activator, was added to plasma and incubated for 120 seconds before $CaCl_2$ (0.025M, Stago Catalog#00367) was added and clot formation was measured. All tests were performed in triplicate. Human plasma which clotted within 45-50 seconds was deemed healthy (reference range determined by testing coagulation times on the plasma batch daily for 30 experimentation days). For negative controls, 1:1 water/glycerol stock (same volume as venom stock, with water in replacement of venom) was used to ensure that the plasma did not self-clot. The normal range for this control was deemed to be 340-400 seconds (reference range determined by testing coagulation times on the plasma batch for 30 experimentation days).

A 10-point dilution series with incremental, decreasing concentrations of venom (from 20µg/ml to 0.05µg/ml final concentrations) were used to quantitatively compare the relative coagulation capacity across all venoms in this study. Owren Koller (OK) Buffer (Stago Catalog#00360) was used to dilute the venom. $CaCl_2$ (50µL) was added with 50µL Phospholipid (cephalin prepared from rabbit cerebral tissue adapted from STA C.K Prest standard kit, Stago Catalog#00597, and solubilized in 5ml of OK Buffer). OK Buffer (25µL) was then added to the cuvette and incubated at 37°C for 120 seconds before adding 75µL of plasma (also heated to 37°C), with an end total volume of 250µL. Coagulation time started immediately and was continuously measured until the plasma had clotted or reached the maximum allotted time (999 seconds). Once the venom sample was loaded into the STA-R-Max® analyser, the pipetting, incubation, and recording was all automated by the analyser.

All reagents were from Stago (Asnières sur Seine, France) and clotting times were recorded in seconds. A 10-dilution series starting at 20µg/ml and ending in 0.05µg/ml was performed in triplicate. To test for co-factor dependency, 20µg/ml screenings were also performed with and without phospholipid and $CaCl_2$. To keep the final volume (250µL) constant, OK buffer was used as a substitute.

Antivenom tests

Monovalent Tiger Snake Antivenom (lot# 0550-09401; expiry 2003) from Commonwealth Serum Laboratories (CSL) Limited (Parkville 3052, Victoria) used. A previous study showed that antivenom is effective long past expiry date and therefore is useful for research purposes (O'Leary et al. 2009). Antivenom was centrifuged (using Allegra^{TM} X-22R Centrifuge, Beckman Coulter, USA) at 12000rpm for 10 minutes at 4^{o}C. The supernatant fluid was removed, filtered (0.45µm Econofltr PES, Agilent Technologies, China), then aliquoted into 2ml Eppendorf tubes and stored at 4^{o}C until use. Immediately before use, antivenom was diluted into a 5% antivenom/95% OK Buffer solution. The protocol for procoagulation tests was modified so that 25µL of OK Buffer was replaced with 25µL of the antivenom/OK buffer solution before being incubated so as to not change the final 250µL sample volume. To determine if the antivenom had any additional effects on the plasma, a negative control was performed by measuring the plasma clotting times of antivenom with water in replacement of venom in the sample mixture.

Calibrated Automated Thrombogram (CAT)

Thrombin generation was measured by a Calibrated Automated Thrombogram (CAT, Stago), using the method previously described by Hemker et al. (2002; 2003). The following was pipetted into a 96-well round-bottom microtiter plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA):

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a starting volume of 80µL of pre-warmed human plasma (37°C), 10µL of venom, and 10µL of phospholipid. The plate was then inserted into a Thermo Fisher Fluoroskan fluorometer (Thermo Labsystem, Helsinki, Finland). The default 10-minute incubation step was removed so as to not omit the initial thrombin formation. Prior to starting, dispensing lines were washed and then primed with pre-warmed 2.5 mmol/l fluorogenic substrate in 5 mmol/l CaCl2 (FluCa-Kit; Diagnostica Stago). The test was then automatically initiated by dispensing 20µl of the fluorogenic substrate into each well and run at 37°C for 60-minutes, with readings automatically taken every 20 seconds.

For each venom, three different venom amounts (0.000001µg, 0.00001µg, and 0.0001µg) were added to an enzymatic buffer (150mM NaCl & 50mM Tris-HCL, pH 7.4) and manually pipetted into the wells for final concentrations of (0.0000083µg, 0.000083µg, and 0.00083µg/ml). Tests were performed in triplicate for each experiment with three calibrators per half plate. Calibrators adjusted for any internal filter effects and eliminated any variation in plasmas. For the calibrator assays, phospholipid and venom trigger was substituted with a thrombin-α2-macroglobulin complex solution of a known concentration (thrombin calibrator, Stago, US).

For each thrombin generation measurement, the following parameters were recorded by the Thrombinoscope software (Maastricht, The Netherlands): the endogenous thrombin potential (ETP), represented by the activity of free thrombin multiplied by the time it remains active in the plasma (area under the curve); maximum concentration of thrombin (peak); and lag-time (time to start). The RAW data was then converted by the Thrombinoscope software into thrombin activity (nM) corresponding to the calibrator (Hemker et al., 2002).

Statistical analysis

Coagulation times (seconds) for each of the venoms and antivenoms were graphed using Prism 7.0 software (GraphPad Software Inc, La Jolla, CA, USA) to produce concentration response curves. CAT thrombin generation times were also graphed over time (minutes) using GraphPad Prism. Calculation of EC_{50} (concentration of venom at which 50% of the effect is observed) values for the venom and antivenom concentration curves for each dataset were performed using Prism 7.0 software (GraphPad Software Inc, La Jolla, CA, USA). Data is expressed as mean±SD. After EC_{50}'s were calculated, the relative antivenom efficacy was calculated using the formula:
x=(((antivenom EC_{50} x-axis)/(venom EC_{50} x-axis))*((antivenom EC_{50} y-axis)/(venomEC_{50} y-axis)))*(antivenom maximal clotting time/venom maximal clotting).

The relationships between the different tests were analysed using phylogenetic Generalized Least Squares (PGLS). The phylogenetic tree used was based upon (Keogh et al 2005; Lee et al 2016) and created using Mesquite software (version 3.2) which was then imported into Rstudio using the APE package (Paradis et al., 2004). Ancestral states were estimated for all traits (including the proportional shift of the relative co-factor dependency and relative antivenom efficacy) using maximum likelihood as implemented in the contMap function of the R package phytools (Revell, 2012). To calculate the correlation between diet and the different tests, diet preference for each species and population was determined from previous ecological studies (e.g. Shine 1977; Schwaner 1985b; Shine 1987a; Shine 1987b; Schwaner & Sarre, 1988; Tan et al., 1993; Fitzgerald et al., 2004; Fearn et al., 2012) (Table 1). Dietary preference was then ranked according to metabolism and circulatory rates for preferred prey, the highest metabolism and circulation rate was ranked as 1 and the lowest circulation rate levels ranked as 6. PGLS models (Symonds and Blomberg, 2014) were fit in Caper (Orme et al., 2015) with coagulation time as the response variable and diet class as the explanatory variable to assess whether metabolic attributes of prey predict haemotoxicity of the venom. P-values ≤0.05 were considered statistically significant.

Results

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Clotting time

The basal species *A. ramsayi* showed very weak procoagulant properties (222.22±3.4 second clotting time at 20µg/ml concentration relative to the 300-350 second spontaneous clotting time of plasma without venom), the dilution curve was therefore not carried out. All other venoms displayed potent procoagulant effects, with *Notechis* and *Tropidechis* being more potent than *Hoplocephalus* and *Paroplocephalus* (ANOVA: $F_{2,33}$=44.82, P<0.001) (Table 2, Figure 1). An historical sample of *N. scutatus* venom (collected in 1935) displayed equipotent activity to freshly obtained venom, thus validating previous studies which demonstrated the stability of venoms over time. This result demonstrates that even samples which are decades old may be valuable bioresources (Jesupret et al. 2014).

Although there was variation in clotting activity among *Notechis* populations (ANOVA: $F_{15,53}$ =14.94, P<0.001), there was no particular geographical pattern to this variation. The Tasmanian population had the fastest coagulation times within *N. scutatus* (9.43±0.38 seconds) while also maintaining their potency throughout the dilution series, with only a 3.2-fold decrease in coagulation time from the 20µg/ml to 0.05µg/ml concentrations. With only intermediate clotting times compared to the rest of *Notechis* and *Tropidechis*, the Chappell Island population venom exhibited the least amount of activity loss during the dilution series with only a 2.8-fold decrease from the 20µg/ml to 0.05µg/ml concentrations. *Hoplocephalus bungaroides* venom was the fastest clotting of any amongst the *Hoplocephalus* or *Paroplocephalus* species (Table 2). *H. stephensii, H. bitorquatus*, and *P. atriceps* were the least coagulant species overall at the initial 20µg/ml concentration, while also losing the most amount of procoagulant activity during the dilution series. *P. atriceps* venom had a 6.2-fold decrease in clotting times during the dilution series.

Co-factor dependency

In addition to variation in the speed at which they induced clot formation, the venoms also displayed inter-genus phylogenetic patterns in relation to co-factor dependency (Tables 2 and 3, Figure 2). Clotting times were significantly shorter for all venoms when calcium was added (Tables 2 and 3), though *Notechis* and *Tropidechis* venoms were less calcium dependent than those of *Hoplocephalus* and *Paroplocephalus*. In addition, there was significant intraspecific variation between different *Notechis* populations (ANOVA: $F_{15,53}$=87.48, p<0.001). When phylogenetic relationships were considered, there was a significant relationship between the presence of phospholipids and maximum clotting time even when all co-factors were present (PGLS: t=2.304, df=1, p=0.03) (Figure 3). On the other hand, the effect of calcium was found to be non-significant (PGLS: t=0.3625, df=1, p=0.72). Thus, when calcium was present clotting was initiated sooner, but phospholipids had a stronger effect on the time taken for complete clot formation. While both co-factors were required for maximum clotting activity, the venoms studied demonstrated ability to cleave prothrombin in the absence of the co-factors, albeit less efficiently.

In the majority of venoms there was also a correlation between co-factors in that venoms that had a strong dependency on one co-factor also exhibited a strong dependency on the other and vice versa (Figure 2). In contrast, all three populations of *T. carinatus* had a relatively strong dependency on calcium, but only weak dependency on phospholipid.

Calibrated Automated Thrombogram

Initial examination of multiple concentrations of *N. scutatus* venom as a representative demonstrated some thrombin production by the higher venom concentrations at the start of the experiment prior to the commencement of plate reading during the short period of time (seconds) between substrate dispensing and plate reading (Figure 4). This was particularly acute for the less calcium dependent venoms. Thus lower concentrations were used in the testing of all species. Consistent with the clotting time tests, lag times differed between *Notechis/Tropidechis* and

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Hoplocephalus/Paroplocephalus (Figure 5). The Eumundi population of *T. carinatus* exhibited one of the shortest lag-times (9.98±2.01 minutes), supporting the results from the coagulation tests. There was no association between diet and lag-time for either specialist or generalist feeding ecologies (PGLS: t=0.77, df=1, P=0.45 and PGLS: t=0.37, df=1, P=0.72, respectively). Peak height and increased thrombin activity, which was assessed as the ETP, showed no clear trends between species or populations. As expected the lag-time and time-to-peak were shortened in higher concentrations of venom, however, the venoms showed a general trend of increased thrombin peaks at the lowest venom concentration (0.0000083µg/ml).

Relative taxa specific effects and influence of dietary specialisation

The association between dietary preference and coagulation times was investigated under two scenarios: 1) *Notechis* all scored as generalists, or 2) individual populations scored according to specific (but limited) feeding ecology study results. In the first scenario, there was a significant association between dietary preference and coagulation time (PGLS: t=2.93, df=1, p=0.008), with venoms of species that feed on mammals/endotherms exhibiting quicker clotting times than those of reptile feeders. There was a marginally non-significant association between dietary preference and coagulation time when each population of *Notechis* was ranked utilising the limited amount of data available on the feeding ecologies of each population (PGLS: t=2.05, df=1, p=0.052).

When comparing the venom effects of a bird specialist (Chappell Island population of *N. scutatus*) to that of a frog specialist (*T. carinatus*) upon cane toad, chicken and human plasmas, no significant differences were observed: *N. scutatus* p-values were cane toad versus chicken 0.9939, cane toad versus human 0.9996, chicken versus human 0.9542; *T. carinatus* p-values were cane toad versus chicken 0.9997, cane toad versus human 0.7401, chicken versus human 0.9727 (Figure 6). This points towards the venoms acting upon conserved cleavage sites in prothrombin, which was confirmed by prothrombin sequence alignment (Figure 7). As the target was so highly conserved, we aligned the sequences of the FXa toxins from *Hoplocephalus*, *Notechis* and *Tropidechis*, which were revealed to have a 98.5% conservation (Figure 7) despite these three genera having been separated for 6 million years (Lee et al. 2016).

Antivenom efficacy

Consistent with the unusually highly conserved toxins as a consequence of the target site being under negative selection pressure for diversification, CSL tiger snake antivenom was not only generally efficient against *Notechis* venoms but displayed strong cross-reactivity with *Hoplocephalus* and *Paroplocephalus* venoms, with the clotting curve shifting significantly for each of the venoms (Figures 8). Of all venoms, the monovalent tiger snake antivenom showed the greatest efficacy against *P. atriceps* venom. When clotting time was tested as an explanatory variable for antivenom neutralisation, the correlation was non-significant (PGLS: t=0.615, df=1, P=0.544), indicating that the efficacy of antivenom is not dependent on the procoagulant activity of the venom. There was also no statistically strong correlation between antivenom efficacy and either relative calcium dependence (PGLS: 1.76, df=f, p=0.089) or phospholipid dependence (PGLS: t=0.1, df=1, p=0.92).

Testing of an expired vial of CSL antivenom from 1959 which had largely been stored at refrigerated temperatures, but had spent 2 years at room temperature demonstrated an efficacy similar to that of 2003 (also expired) antivenom (Figure 9). It is possible that the slight difference may not in fact be due to degradation, as the vial did not display any turbidity, but rather the antivenoms being raised against different venoms. Regardless, this demonstrates that expired antivenom may be effective at least for laboratory work (but of course other factors besides efficacy must be considered in a clinical setting).

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Discussion

Using coagulation times and thrombin production, we were able to 1) determine that there are no taxa specific effects of the toxins types but that there may be a correlation between FXa expression levels in the venom and diet, 2) establish relative dose-dependent neutralisation efficacy of tiger snake antivenom across all venoms in this clade, and 3) determine the relative calcium and phospholipid co-factor dependency for each venom. Overall, this study provides the most comprehensive investigation of the procoagulant activity of venoms from snakes in the *Notechis* clade to date, with venom from 16 populations of *N. scutatus* as well as *Austrelaps ramsayi, Hoplocephalus bitorquatus, H. bungaroides, H. stephensii, Paroplocephalus atriceps,* and *Tropidechis carinatus* investigated.

Coagulation Activity

In other studies, venom composition has been shown to sometimes change significantly between populations, depending on the selective advantage conferred due to differences in prey availability, use of habitat type, and seasonal variation in prey abundance (*c.f.* Daltry et al., 1996; Aguilar et al., 2007, Margreaves et al. 2016). However geographical variation in venom function in this study was found to be minor in comparison to interspecific and intergeneric variation, thus facilitating a phylogeny based comparison of venom features across the taxa studied. The *N. scutatus* populations underwent rapid divergence in the last 6,000-10,000 and remain extremely genetically similar despite the extensive variation in morphology and colour between the different populations (Keogh et al., 2005). Consistent with this, the venoms were highly similar to each other relative to that of other genera in this same clade. *Hoplocephalus* spp., and *Paroplocephalus atriceps* venom showed prolonged clotting times in comparison to *Notechis* venom, which is consistent with their venoms being known to express lower levels of prothrombin-activators (St Pierre et al., 2005). Keogh et al. (2000) placed *Paroplocephalus* as a sister group to the genus *Hoplocephalus*, and the similar coagulotoxicity of *Hoplocephalus* and *Paroplocephalus* venoms is reflective of this close relationship (Figure 1).

The results from this study support the hypothesis that *Austrelaps* are basal to the *Notechis* clade, thus evolving from an *Austrelaps*-like ancestor (e.g. Lee et al., 2016). *A. ramsayi* venom showed very weak procoagulation expression in the clotting tests, suggesting that they diverged before the up-regulation of prothrombin-activating toxins in this clade. *Hoplocephalus/Paroplocephalus* likely diverged after the up-regulation of FXa toxins in the last common ancestor shared with *Notechis*/*Tropidechis* after the split with *Austrelaps*. *Notechis+Tropidechis* thus represent the most derived state, in terms of coagulotoxicity relative to the inferred *Austrelaps*-like ancestral state. These data support the findings of St. Pierre et al. (2005), which demonstrated that *H. stephensii* express moderate levels of FXa in their venom, whereas FXa is expressed at relatively elevated levels in *Notechis/Tropidechis* venoms.

Group D procoagulant toxins target components of the coagulation cascade which are themselves structurally constrained, thus the FXa toxins characteristic of this snake clade likely remain conserved under strong negative selection pressure against diversification (Figure 7). Indeed, the primary structure of group D procoagulant toxins varies very little and share an overall amino acid identity of 98.5% with one another (Figure 7). Any changes to the functional sites may result in reducing this homology with endogenous FXa, thereby minimising the efficiency of the toxin in activating prothrombin. This also explains the similar action of FXa in every species in this clade in cleaving prothrombin at identical sites to form thrombin (Joseph & Kini, 2001; Rao et al., 2003; Reza et al., 2005; Sajevic et al., 2011). Group D procoagulants are a fascinating example of toxins that preserve the primary function of the ancestral gene (the endophysiological blood factor Xa) from which they descend, but have co-opted that function for use as a toxin. The genomes of snakes expressing group D procoagulants in their venoms thus encode a pair of paralogous proteins, which,

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despite preserving a high degree of structural similarity, perform completely different functions at the organismal level – the "parent" gene maintains haemostasis and the "child" gene facilitates prey subjugation (by disrupting the haemostasis of envenomated prey animals) (Reza et al., 2006). Thus, group D procoagulant toxins might be considered examples of either sub- or neo-functionalisation, depending upon the "level of analysis" – at the molecular level, since they largely preserve the "function" of the ancestral gene, they may be considered sub-functionalised, but at the organismal level, since they have acquired a completely new role in their deployment as toxins, they should be considered neo-functionalised. These toxins are therefore also fascinating examples of exaptation, in which an ancestral function (maintenance of haemostasis) is co-opted for an entirely novel function (prey subjugation), which, prior to the gene's duplication and its selective expression in the venom gland (i.e. its "recruitment" as a venom toxin), had not featured in the gene's selective history (Reza et al., 2006; Jackson et al. 2016a).

Co-factor Requirement

Group D venoms require mammalian FVa, calcium ions, and phospholipids for maximum procoagulant activity (Kini et al., 2001; Isbister 2010; Sajevic et al., 2011). These co-factors increase the maximum velocity of the enzymatic reaction and the catalytic efficiency of the enzymes (Rosing et al., 1980). In the present study, coagulation experiments with and without required co-factors revealed more variation in co-factor dependency than expected (Figure 2). Although the literature suggests that the co-factor dependency of venoms is relatively simply explained in binary terms – either a given co-factor is required for efficient activity, or it is not (i.e. Kini et al., 2001; Steen 2002; Isbister 2010; Sajevic et al., 2011) – our results indicate that the degree to which group D prothrombin activators rely on its co-factor for optimal efficacy is highly variable even between different populations of the same species (Figure 2).

The addition of calcium to venom significantly decreased coagulation time compared to only venom, whereas phospholipid had less of a significant effect on the initiation of coagulation. Coagulation tests which included both calcium and phospholipid, however, had the quickest clotting times. This confirms that the initiation of procoagulation activity is calcium-dependent, and that venom, to some extent, is able to bind with FVa with a degree of efficiency in the absence of phospholipid. These findings differ from studies which previously reported that Group D venoms require both calcium and phospholipids (e.g. Joseph & Kini, 1999; Kini et al., 2001; Isbister 2010; Sajevic et al., 2011). While the catalytic process was significantly shorter in the presence of calcium, its presence was not necessary to create a clot. That is, the *Notechis* clade venoms were shown here to have the ability to 'highjack' the coagulation cascade without the need for calcium, with populations of *N. scutatus* apparently independently evolving a greater ability to produce calcium-independent venoms. This fits with the trend seen in other species (e.g. *Pseudonaja* and *Oxyuranus* species) (Nakagaki et al., 1992), While there was some correlation between calcium-dependency and clotting time, the strongest correlation between overall clotting time (as distinguished from the speed of clot initiation) and co-factor dependence was in relationship to phospholipids (Figure 3). Phospholipids play an important role in the coagulation cascade and can alter efficiency of clotting reactions by several orders of magnitude (Steen 2002). Phospholipids are a co-factor in forming the prothrombinase (FVa:FXa) complex because a negatively charged phospholipid is required for FVa to bind, thereby creating a binding site with high-affinity for FXa (Steen 2002). In this study, the less phospholipid-dependent venoms were also shown to be the fastest clotting venoms.

Venom and plasma stocks used throughout experimentation were citrated but there may have been trace calcium ions remaining. The experimental design to determine calcium dependency may, therefore, have been limited due to the possibility that experiments without calcium added were not completely calcium free. Additionally, the venom itself may contain trace amounts of calcium.

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However, we deemed the relative change in calcium concentration with added calcium was sufficient to show calcium requirement. This has shed more light on venom bioactivity within the *Notechis* clade and the relationship between venom evolution and prey.

Calibrated Automated Thrombogram

Total lag-times were congruent with the results of the coagulation tests, for example with *Notechis* having significantly less lag-time compared to *Hoplocephalus* (t-test: t=3.6, df=25, p=0.001). However, dietary preference was found to have no significant association with lag-time, which was unexpected. When venom concentration was increased there was a significant drop in peak height with the 8.3µg/ml concentration having no measurable thrombin activity. This possibly indicates a limitation in the thrombogram methodology; ETP and peak height are highly sensitive to hypercoagulability caused by high levels of prothrombin in the blood (Machlus et al., 2009). Therefore, oversensitivity to high prothrombin concentrations could reduce accuracy of results. Another possibility is that higher venom concentrations cause the initiation of the coagulation pathway to react sooner due to the calcium independence of the action, thus thrombin generation occurred before the thrombogram started measuring. The latter consideration was supported by results using higher concentrations (Figures 4 and 5), with it being clear that the venom was rapidly activating prothrombin in the absence of calcium reducing the ETP prior to the commencement of the reaction readings.

Dietary Specialisation

Because prey subjugation is the dominant force in venom evolution, it is likely that dietary preference may play a role in co-factor dependence. This study found that the populations of *N. scutatus* which are less calcium-dependent are also less phospholipid-dependent (Figure 2) and that these are also the populations producing the most potently procoagulant venoms (Figures 1 and 3). These populations are known to include large quantities of endotherms in their diet (e.g. tiger snakes from Chappell Island and Tasmania) (Schwaner & Sarre, 1988). Interestingly, *Tropidechis* venom exhibited a strong dependence on calcium for activity but relatively low dependence on phospholipids. Independency of one or both co-factors removes rate limiting steps in the pathway, thus making venoms more effective at targeting a prey's circulatory system.

The evolution of venom specificity results from natural selection on predatory venomous taxa fine-tuning their toxin arsenal for effective targeting of their prey's physiology (Daltry et al., 1996; Barlow et al., 2009; Vonk et al., 2011). Indeed, bioactivity studies have shown that the toxic properties of venoms from different species are typically most effective on their preferred prey (Da Silva & Aird, 2001; Richards et al., 2012), although coevolutionary arms races with prey may lead to toxin resistance which can counteract this in some cases (Holding et al., 2016).

This study found a relationship between prey type and relative prothrombin quantity but without adaptive evolution of the toxins. In early ecology studies the diets of mainland *N. scutatus* were found to consist primarily of anurans (Shine 1977; 1987a). In contrast, Fearn et al. (2012) found that *N. scutatus* is an opportunistic generalist predator, having the most varied diet of any Australian elapid, consisting of mammals, birds, anurans, reptiles, and fish. The only *N. scutatus* populations which may be classed as specialists are those which inhabit islands off the southern cost of Australia where prey is limited to only a few species. For example, the Chappell Island population predominately feeds almost exclusively on the mutton bird *Puffinus tenuirostris* and Cape Barren goose *Cereopsis novaehollandia*. Note that this 'specialist' feeding ecology is due to a lack of alternative prey on the islands they inhabit and so may not reflect genuine specialisation but rather the behaviour of generalists with limited opportunities. Current knowledge of dietary preferences in the tiger snake clade is incomplete, which is reflected in our alternative treatments of diet in the statistical analyses. When each *N. scutatus* population was ranked as a generalist, while other

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species' diets were scored as reported in the literature, we found a significant relationship between venom which promotes faster clotting and predation upon prey with higher metabolic rates (e.g. endotherms). Similarly, when each specific *Notechis* population diet was considered and ranked according to metabolism and circulatory rates the relationship was only marginally non-significant, hence these analyses still provide some evidence of an association between venom activity and diet (though this effect seems to be stronger in interspecific comparisons than among populations of *Notechis*).

Venoms from snakes which consume predominately reptiles had slower clotting times compared to those which feed on endotherms (Figure 1). Many viperid and some elapid snakes tend to rely on their procoagulant venom for subjugating prey by inducing stroke (Fry et al. 2009; Jackson et al., 2016b; Cipriani et al. 2017). Endotherms are highly sensitive to this predatory strategy due to their extremely high blood circulatory rates and metabolism relative to ectotherms. It has been conjectured that because of the slow-moving circulatory systems of lizards they are generally not as vulnerable to procoagulant toxins (Jackson et al., 2016b) and that therefore there may be selection against expression of large quantities of procoagulant toxins in the venom of lizard specialists (Jackson et al., 2013; 2016b). Instead, venoms from Australian elapids that specialise as reptile feeders are dominated by low-molecular weight peptides (Jackson et al., 2016b). An interesting exception to this trend in the present study was *H. bungaroides*, which had the fastest coagulation time of any *Hoplocephalus* spp., and on average clotted plasma only 1.49-fold slower than *N. scutatus* venom. This was unexpected, as they are highly specialised lizard feeders. This may reflect their feeding ecology in that *H. bungaroides* aren't typical foragers like many other lizard specialists amongst Australian elapids (e.g. *Brachyurophis* and *Pseudonaja modesta*), which prey upon skinks when they are asleep and thus when their metabolism is at its lowest (Jackson et al., 2016b). In contrast, *H. bungaroides* ambush active geckos (Webb & Shine, 1998), when their metabolic activity and blood flow is elevated and, therefore, stroke-induction is possibly a viable envenomation strategy. Adult *H. stephensii* feed predominantly on small mammalian prey while lizards constitute majority of the diet in neonates and juveniles (Fitzgerald et al., 2004). A possible explanation for the relative increase in potency in *Notechis* along with decreased co-factor dependence may be that *N. scutatus* targets a wide range of mammalian prey, some of which defend themselves when attacked (Fearn 2011). As such, injecting potent FXa-rich venom to quickly subdue the prey likely limits the risk of injury from prey retaliation. Whereas, *H. stephensii* tend to feed only on small mammals and most likely would not need to rely on high expression levels of FXa to subdue their prey.

Globally, the venom of extant elapids is typically dominated by neurotoxins (Birrell et al., 2007), which is associated with a diet rich in reptilian prey (Jackson et al., 2016b). Therefore, when Australian elapids dispersed from Asia (Wüster et al., 2005) it has been suggested that these ancestral elapids primarily fed upon reptiles (Jackson et al., 2016b). The recruitment of FXa into the venom proteome is presumed to have occurred near the base of the Australian elapid radiation (Fry *et* al., 2008; Jackson et al., 2013; Trabi et al., 2015) and the FXa toxin appears to have played a vital role in the evolutionary history of Australian elapids that predates the last common ancestor of both the *Pseudonaja* and *Oxyuranus* clade and *Notechis* clade. The expression of this toxin (and the related group C procoagulants in the venoms of *Pseudonaja* and *Oxyuranus*) has been up- (e.g. *Notechis*, adult *Pseudonaja*) and down-regulated (e.g., *Austrelaps*, juvenile *Pseudonaja*) in various lineages of Australian elapids. Within the tiger snake clade, up-regulation of group D procoagulant toxin expression, and thus an increase in the procoagulant potency of the venoms, may have aided the transition from a diet consisting primarily of reptiles and frogs (*Austrelaps*, *Hoplocephalus* spp., and *P. atriceps*) to one including larger quantities of (potentially dangerous) endothermic prey (*N. scutatus* and *T. carinatus*).

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While there was a correlation between diet and effect on plasma, there was no taxon-specific effect of the venoms when their activity was compared in a range of plasmas (Figure 6). Thus, while diet drives expression levels, the venom FXa appears to target conserved prothrombin regions (i.e. at the molecular level the function of all FXa toxins is essentially the same, as discussed above). The conservation of function is mirrored by the high degree of sequence similarity of the target and toxin sequences (Figure 7).

Antivenom efficacy

The effect of genetic drift on the molecular scaffolds of the toxins may also be responsible for the observed variation in responses to antivenom neutralisation. Variation in antivenom efficacy may thus provide information regarding the existence of variations in the surface chemistry of FXa toxins among these genera. In this study, CSL tiger snake antivenom effectively neutralised the clotting activity of all venoms studied (Figures 8 and 9), consistent with the standard treatment protocol for envenomations by these species (Sutherland and Tibbals 2001, Gan et al., 2009). From these data, it is evident that the monovalent antivenom had the highest efficacy against *P. atriceps* and *H. stephensii* venoms, although there were significant differences between the *H. stephensii* venoms that were not reflected in clotting time or co-factor dependency variance.

As there was no correlation between antivenom efficacy and clotting times, this suggests that mutations in the epitopes of the FXa toxins are functionally inconsequential for the overall action of the toxin. Such mutations would therefore occur in antivenom-binding sites distinct from functional (active) sites of the toxin molecule. For antivenoms to be effective against coagulopathic symptoms of envenomation, the antibodies are required to bind to the prothrombinase complex which then prevents the cleavage of prothrombin to thrombin (Isbister et al., 2007). These minor structural changes in the epitope regions of the FXa toxin in the prothrombinase complex may affect the ability of the antivenom to bind the complex, which limits the ability of the antivenom to prevent the cleavage of prothrombin. This suggests that only slight variation within the molecular structure of procoagulant toxins may be required to reduce antivenom efficacy. Nevertheless, our results are encouraging in that the antivenom was found to be effective against all of the venoms tested – the differences we have detected are merely relative differences and not indicative of a lack of efficacy of current antivenom products against any of the snake venoms tested here.

It must be noted that once VICC develops, it has been demonstrated that larger doses or repeated doses of antivenom do not reduce recovery time, which is dependent on the re-synthesis of clotting factors (Isbister et al., 2009; Isbister et al., 2012). We recognise that there are limitations in extrapolating clinical recommendations from laboratory data, for example, studies which incubate antivenom with venom *in vitro* before adding venom to the plasma may demonstrate efficacy in preventing the procoagulant activity of the venoms, but not provide an accurate measure of clinical effectiveness (Currie 2006). However, absence of efficacy under such ideal scenarios would certainly predict a lack of clinical usefulness. Our study suggests that CSL tiger snake antivenom is of widespread effectiveness against envenomation by members of the tiger snake clade, and this is also congruent with clinical outcome reports (Sutherland & Tibballs, 2001) which indicates that our results likely hold relevance for medical applications.

Conclusion

Venom ecology is an understudied area of research and much remains to be discovered concerning the ways in which snakes use their venom in nature. To investigate varying physiological responses to envenomations between prey species, working with natural prey items is ideal (Richards et al., 2012). Unfortunately, natural prey species are logistically difficult and/or expensive to obtain in sufficient quantity for experimental purposes, necessitating the use of various model taxa as proxies. The focus of future studies should be on the examination of procoagulant activity in the

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plasma of natural prey, under different treatment groups (e.g. at different temperatures) that reflect the possible range of metabolic states in which prey may be encountered in nature. It is notable, however, that for the venoms and plasmas studied here, there was no strong variation in activity among plasma types. This is congruent with the venom FXa enzymes targeting highly conserved regions of prothrombin. Hence these toxins being a notable exception to the paradigm put forth that evolutionarily young animals will have toxins undergoing adaptive evolution (Sunagar and Moran 2015). This is due to the toxins in this study targeting physiological proteins that themselves are under extreme negative selection for adaptive evolution. Thus if the target is constrained, there is a reciprocal negative selection pressure for diversification put onto the toxins too.

The results presented in this study have demonstrated significant variation in coagulopathic activity among the venoms of the clade consisting of *Austrelaps, Hoplocephalus, Notechis, Paroplocephalus* and *Tropidechis.* Each genus (other than *Austrelaps*) has procoagulant venom, but the relative potency of this activity varies among them, suggesting that strong selection pressures driven by diet play a central role in determing the expression levels of group D prothrombin activators in these venoms but that due to the structural constraints upon the endogenous prothrombin target, the FXa toxins themselves are under similar negative selection pressure for diversification.

The adaptive evolution of procoagulant toxins in snake venom has remained under-investigated. Due to the existence of closely related genera which have evolved to inhabit different niches with differing feeding ecologies, the tiger snake clade is an ideal model for the investigation of functional variations among venoms containing group D prothrombin activators. This study not only contributes substantially to existing knowledge of the bioactivity of these venoms across geographically isolated populations but also to knowledge of the efficacy of currently available monovalent antivenoms. Our results demonstrate the efficacy of the antivenom in neutralising the procoagulant effects of *N. scutatus*, as well as cross-reactivity with *Hoplocephalus* spp., *Paroplocephalus atriceps,* and *Tropidechis carinatus* venoms, with these results strongly suggestive of clinical efficacy.

Antivenoms also acted as selective probes to delineate venom divergence between species and populations and revealed that genetic drift is likely responsible for variations in the epitopes of FXa toxins recognised by antivenoms. Thus, whilst the functional sites of FXa toxins remain unchanged, their immunogenicity may change, in the absence of selection pressures. On the other hand, coagulation times and thrombinoscope revealed that the venoms of snakes within this clade vary significantly in bioactivity, with diet acting as a selection pressure on expression levels of FXa toxins. Thus, our results reveal that, in terms of group D prothrombin activators within the tiger snake clade, selection primarily appears to act upon on relative toxin expression levels whereas changes in the immunogenicity of the toxins are likely epiphenomenal – unselected byproducts of random change (i.e. unconstrained drift).

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Table 1: Species (including any previous classifications) and localities of venom samples used in this study, and number of venom samples used from each population. Feeding ecology and whether the samples were from individual milking's or pooled is also outlined by the table.

Species	Previous Classification	Location	Known Feeding Ecology	Number of Samples	Pooled or Individual Samples
Austrelaps ramsayi		Paddys River ACT	Lizards & Anurans	1	Pooled
Hoplocephalus bitorquatus		Texas QLD	Lizards & Small Mammals	1	Individual
Hoplocephalus bitorquatus		Brigalow NSW	Lizards & Small Mammals	1	Individual
Hoplocephalus bungaroides		Sydney NSW	Lizards	3	both
Hoplocephalus stephensii		Brisbane QLD	Lizards & Small Mammals	3	Individual
Notechis scutatus	*Notechis scutatus scutatus*	Melbourne VIC	Anurans	2	Both
Notechis scutatus	*Notechis scutatus scutatus*	Melbourne 1935	Anurans	1	Pooled
Notechis scutatus	*Notechis scutatus scutatus*	Werribee VIC	Anurans	1	Pooled
Notechis scutatus	*Notechis scutatus scutatus*	Barmah VIC	Anurans	1	Pooled
Notechis scutatus	*Notechis ater serventyi*	Chappell Island	Endotherms	3	Both
Notechis scutatus	*Notechis ater humphreysi*	Tasmania	Generalist	3	Both
Notechis scutatus	*Notechis ater niger*	Kangaroo Island	Endotherms	2	Pooled
Notechis scutatus	*Notechis scutatus occidentalis*	Perth WA	Anurans	1	Pooled
Notechis scutatus	*Notechis scutatus occidentalis*	Margaret River WA	Generalist	2	Individual
Notechis scutatus	*Notechis scutatus occidentalis*	Coomalbidgup WA	Generalist	1	Individual
Notechis scutatus		Bunya Mt QLD	Mammals & Anurans	1	Individual
Notechis scutatus		Bruny Island	Endotherms	1	Pooled
Notechis scutatus		New Year Island	Endotherms	1	Pooled
Notechis scutatus		Franklin Island	Endotherms	1	Pooled
Notechis scutatus		Mt Gambier SA	Mammals & Anurans	1	Pooled
Notechis scutatus		Lake Alexandrina SA	Anurans	2	Pooled
Paroplocephalus atriceps		Lake Cronin WA	Lizards	1	Individual
Tropidechis carinatus		Brisbane QLD	Mammals & Anurans	1	Individual
Tropidechis carinatus		Eumundi QLD	Mammals & Anurans	1	Individual
Tropidechis carinatus		Numbinbah Valley QLD	Mammals & Anurans	1	Individual

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Table 2: Mean clotting times at the 20µg/ml venom concentration for each of the different co-factor dependency coagulation tests. Proportional shift for calcium and phospholipid tests is the shift in clotting time compared to venom with both calcium and PPL. Time is in mean±SD seconds. PPL=Phospholipid.

Species	Venom with both calcium and PPL	Venom with calcium, no PPL	Venom with calcium Proportional Shift	Venom with PPL, no calcium	Venom with PPL Proportional Shift
H. bitorquatus Brigalow NSW	31.03±0.26	74.26±0.97	2.39	256.49±5.86	8.26
H. bitorquatus Texas QLD	24.4± 0.4	67.99±0.94	2.79	240.61±3.41	9.86
H. bungaroides Sydney NSW	16.98±0.38	33.04±0.56	1.95	153.28±0.77	9.03
H. bungaroides Sydney NSW	18.96±0.69	34.95±0.31	1.84	174.11±1.5	9.18
H. stephensi Brisbane QLD	26.02±1.23	48.88±1.01	1.88	236.7±30.61	9.1
H. stephensi Brisbane QLD	21.49±0.29	38.48±0.88	1.79	168.2±5.92	7.83
H. stephensi Brisbane QLD	18.51±0.24	35.48±0.25	1.92	202.03±10.7	10.92
N scutatus Bunya Mt QLD	10.51±0.20	16.52±0.23	1.57	80.78±1.16	7.69
N. scutatus Barmah VIC	10.74±0.44	19.15±0.17	1.78	62.96±0.99	5.86
N. scutatus Bruny Island	13.99±0.42	22.07±0.49	1.58	98.91±1.73	7.07
N. scutatus Chappell Island	13.98±0.33	20.07±0.29	1.44	58.1±0.35	4.15
N. scutatus Coomalbidgup WA	14.35±0.29	24.7±0.44	1.72	126.84±0.51	8.84
N. scutatus Franklin Island	11.1±0.14	18.02±0.44	1.62	70.95±0.34	6.39
N. scutatus Kangaroo Island	12.2±0.77	23.09±0.53	1.89	122.86±1.27	10.07
N. scutatus Lake Alexandrina SA	9.95±0.22	13.97±0.11	1.4	46.35±1.69	4.66
N. scutatus Margaret River WA	10.36±0.98	13.59±0.23	1.31	40.74±0.55	3.93
N. scutatus Melbourne VIC	11.33±0.21	21.76±0.08	1.92	91.89±1.29	8.11
N. scutatus Melbourne VIC 1935	14.68±0.21	25.02±0.46	1.7	112.28±2.12	7.65
N. scutatus Mt Gambier SA	13.26±0.51	23.04±0.55	1.74	153.74±2.09	11.6
N. scutatus New Year Island	12.13±0.19	21.03±0.25	1.73	89.06±1.66	7.34
N. scutatus Perth WA	14.11±0.48	22.01±0.4	1.56	101.55±2.05	7.19
N. scutatus Tasmania	9.43±0.24	13.5±0.15	1.43	50.67±0.81	5.38
N. scutatus Werribee VIC	11.21±0.05	19.83±0.17	1.77	78.56±2.07	7.01
P. atriceps Lake Cronin WA	21.73±0.87	47.33±0.77	2.18	304.82±2.84	14.03
T. carinatus Brisbane QLD	10.77±0.34	17.33±0.21	1.61	122.91±1.06	11.42
T. carinatus Eumundi QLD	9.3±0.04	12.49±0.61	1.34	85.96±0.87	15.3
T. carinatus Numbinbah Valley QLD	9.72±0.11	15.05±0.35	1.55	142.39±2.65	8.84

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Figure legends

Figure 1: Ancestral state reconstruction of clotting times, where warmer colours represent faster clotting times. Bars indicate 95% confidence intervals for the estimate at each node, Note that due to the high dynamicity of venom evolution the ranges quickly become broad as one moves down the tree. Numbers at tips are clotting time averages (Table 2).

Figure 2: Calibrated Automated Thrombogram curve for the Melbourne population of *Notechis scutatus* at seven different final concentrations. The decrease in thrombin production at higher concentrations is indicative of prothrombin activation occurring prior to the commencement of the experiment. X-axis is time (seconds) and y-axis is nM of thrombin production.

Figure 3: Calibrated Automated Thrombogram curves. Coloured lines represent each of the three different venom concentrations: green represents 0.00083µg, red 0.000083µg, and blue 0.0000083µg/ml. X-axis is time (seconds) and y-axis is nM of thrombin production.

Figure 4: Ancestral state reconstruction of relative co-factor dependency as indicated by the proportional shift in clotting time when co-factor is not added, where warmer colours represent less dependency. Numbers at tips are means from N=3 tests indicating the x-fold shift in clotting time caused by co-factor absence (Table 2). Bars indicate 95% confidence intervals for the estimate at each node, Note that due to the high dynamicity of venom evolution the ranges quickly become broad as one moves down the tree.

Figure 5: Ancestral state reconstruction of clotting time versus relative phospholipid dependency, where warmer colours represent faster clotting times. Numbers at tips are means from N=3 tests indicating the x-fold shift in clotting time caused by co-factor absence (Table 2). Bars indicate 95% confidence intervals for the estimate at each node, Note that due to the high dynamicity of venom evolution the ranges quickly become broad as one moves down the tree.

Figure 6. Comparison of a bird specialist (Chappell Island population of *N. scutatus*) and a frog specialist (*T. carinatus*) effects upon amphibian, avian and mammalian plasmas shown as concentration curve views (left panels) and normalised logarithmic transformed views (right panels). X-axis is concentration µg/ml) and Y-axis is time (seconds).

Figure 7: The extremely high levels of conservation of the prothrombin targets from 1) mammal (accession code P00734), 2) avian (accession code A0A1L1RV31) and 3) amphibian (accession code Q5FVW1) and the FXa toxins from 1) *H. stephensii* (accession code AY940208), 2) *N. scutatus* (accession code DQ104218) and 3) *T. carinatus* (accession code AY769963). Cysteines are shown in black highlight and signal peptides in lowercase. Conserved cleavage sites indicated by * and green highlighting.

Figure 8: Coagulation dose-response curve views. Red lines represent venom in optimal conditions (i.e. with calcium and phospholipid) while blue line represents the clotting after preincubation with CSL tiger snake antivenom and then run under the same conditions as the red line protocol. X-axis is concentration µg/ml) and Y-axis is time (seconds).

Figure 9: Coagulation normalised logarithmic transformed views. Red lines represent venom in optimal conditions (i.e. with calcium and phospholipid) while blue line represents the clotting after

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preincubation with CSL tiger snake antivenom and then run under the same conditions as the red line protocol.

Figure 10: Comparative efficacy of 2003 and 1959 antivenoms as concentration curve views (left panel) and normalised logarithmic transformed views (right panel). X-axis is concentration µg/ml) and Y-axis is time (seconds).

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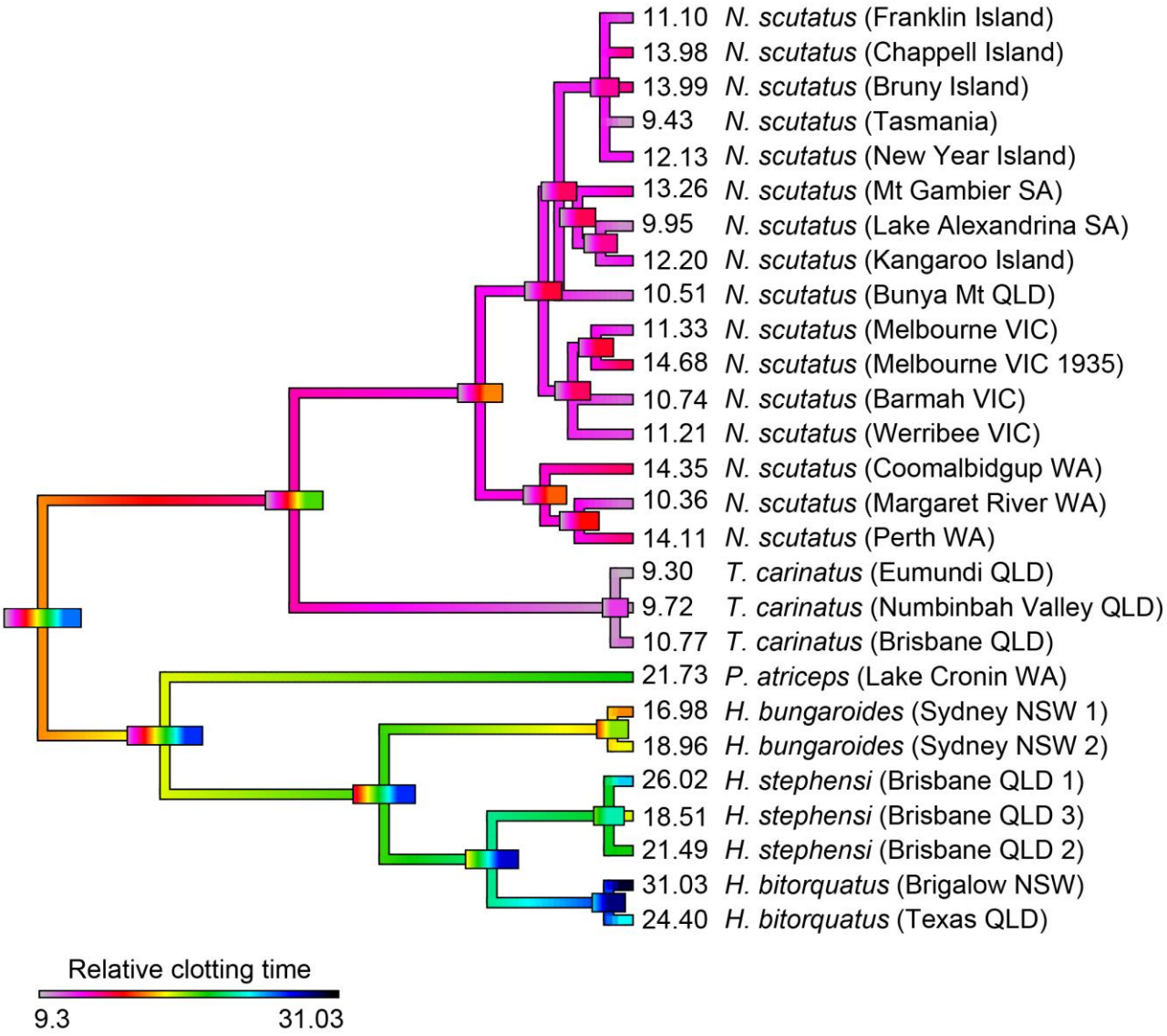
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13.98 N. scutatus (Chappell Island)
13.99 N. scutatus (Bruny Island)
9.43 N. scutatus (Tasmania)
12.13 N. scutatus (New Year Island)
13.26 N. scutatus (Mt Gambier SA)
9.95 N. scutatus (Lake Alexandrina SA)
12.20 N. scutatus (Kangaroo Island)
10.51 N. scutatus (Bunya Mt QLD)
11.33 N. scutatus (Melbourne VIC)
14.68 N. scutatus (Melbourne VIC 1935)
10.74 N. scutatus (Barmah VIC)
11.21 N. scutatus (Werribee VIC)
14.35 N. scutatus (Coomalbidgup WA)
10.36 N. scutatus (Margaret River WA)
14.11 N. scutatus (Perth WA)
9.30 T. carinatus (Eumundi QLD)
9.72 T. carinatus (Numbinbah Valley QLD)
10.77 T. carinatus (Brisbane QLD)
21.73 P. atriceps (Lake Cronin WA)
16.98 H. bungaroides (Sydney NSW 1)
18.96 H. bungaroides (Sydney NSW 2)
26.02 H. stephensi (Brisbane QLD 1)
18.51 H. stephensi (Brisbane QLD 3)
21.49 H. stephensi (Brisbane QLD 2)
31.03 H. bitorquatus (Brigalow NSW)
24.40 H. bitorquatus (Texas QLD)
Relative clotting time
9.3
31.03

Figure 1

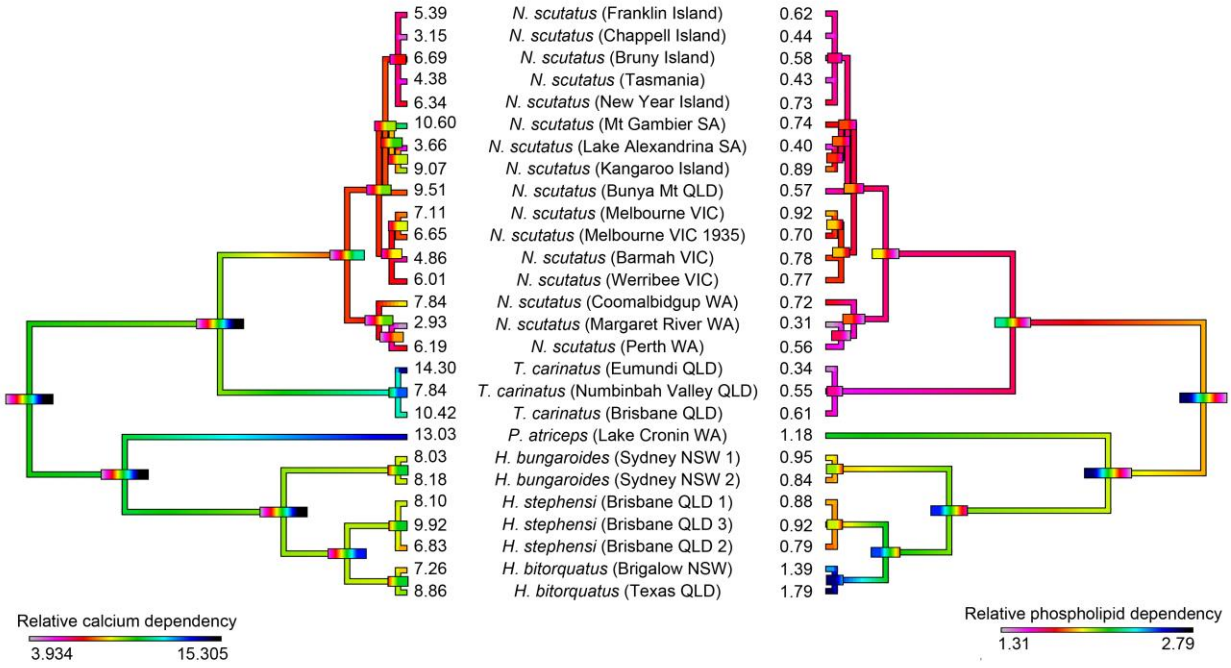

Figure 2

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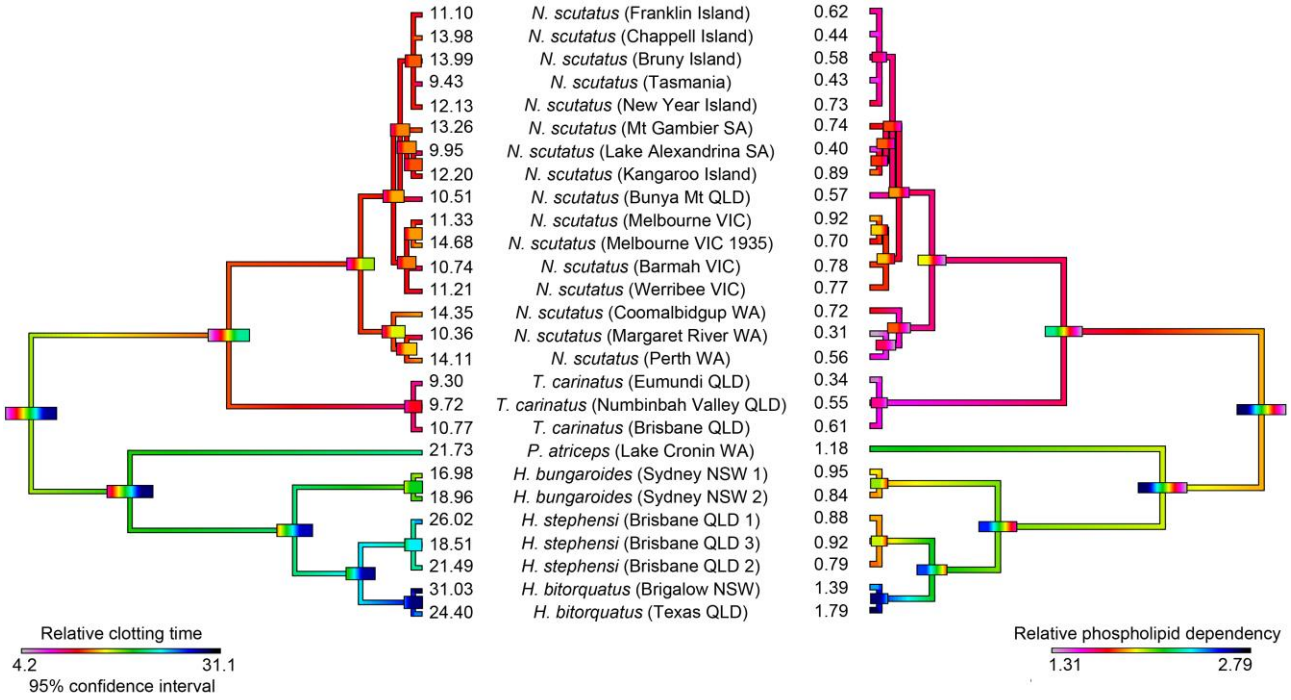

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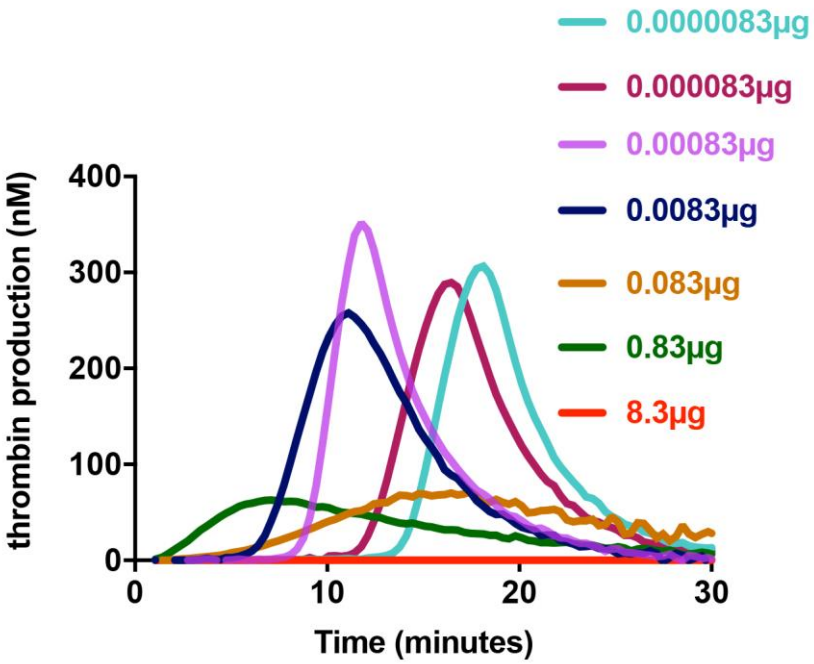

Figure 4

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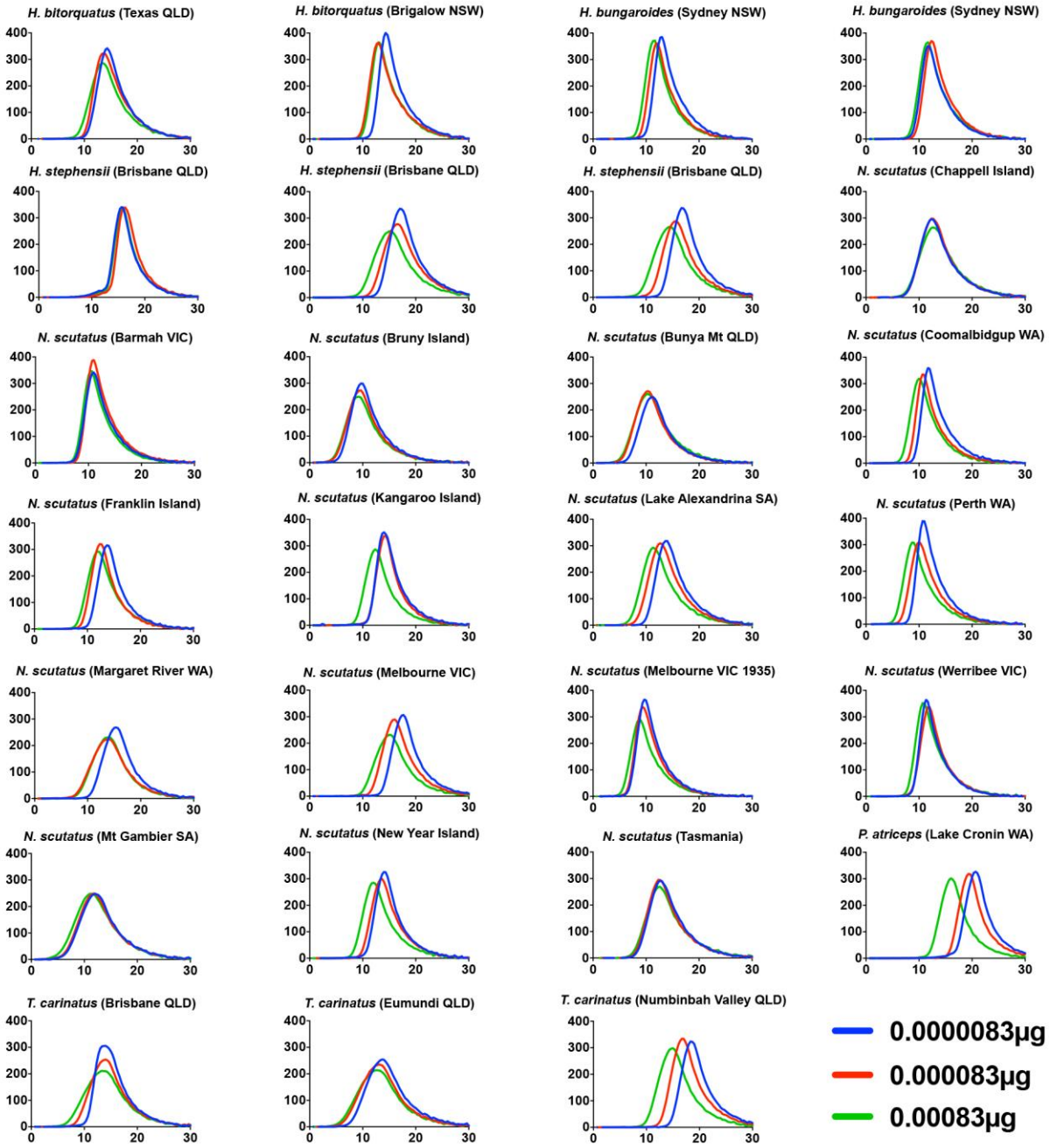
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H. bitorquatus (Brigalow NSW)
H. bungaroides (Sydney NSW)
H. bungaroides (Sydney NSW)
H. stephensii (Brisbane QLD)
H. stephensii (Brisbane QLD)
H. stephensii (Brisbane QLD)
N. scutatus (Chappell Island)
N. scutatus (Barmah VIC)
N. scutatus (Bruny Island)
N. scutatus (Bunya Mt QLD)
N. scutatus (Coomalbidgup WA)
N. scutatus (Franklin Island)
N. scutatus (Kangaroo Island)
N. scutatus (Lake Alexandrina SA)
N. scutatus (Perth WA)
N. scutatus (Margaret River WA)
N. scutatus (Melbourne VIC)
N. scutatus (Melbourne VIC 1935)
N. scutatus (Werribee VIC)
N. scutatus (Mt Gambier SA)
N. scutatus (New Year Island)
N. scutatus (Tasmania)
P. atriceps (Lake Cronin WA)
T. carinatus (Brisbane QLD)
T. carinatus (Eumundi QLD)
T. carinatus (Numbinbah Valley QLD)
0.0000083µg
0.000083µg
0.00083µg

Figure 5

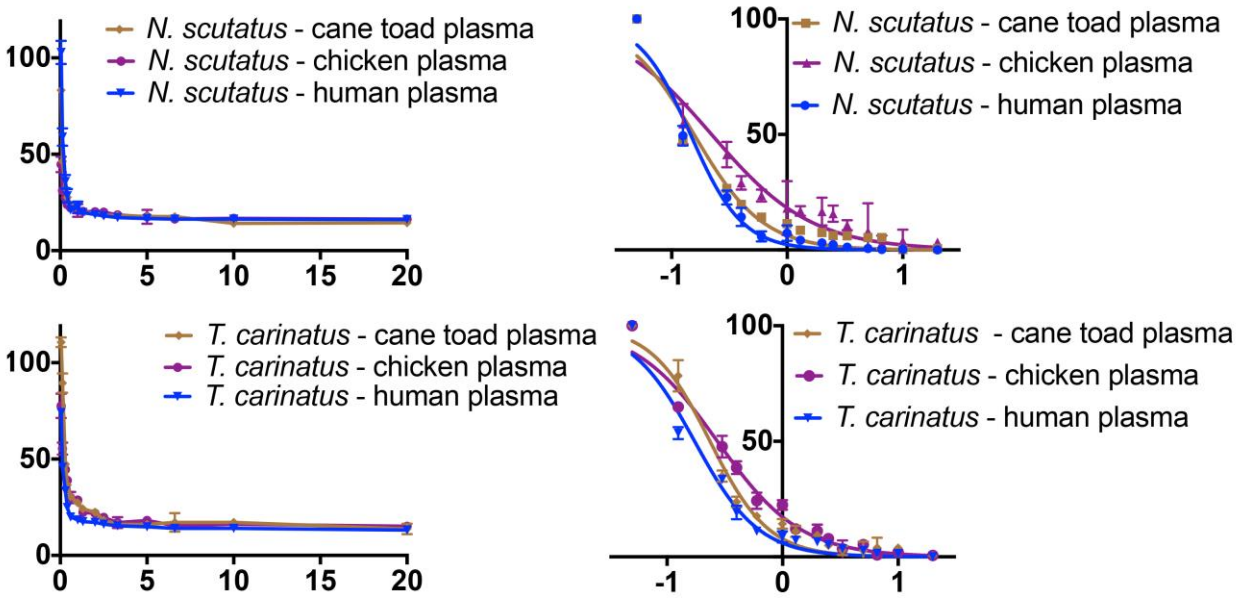

Figure 6

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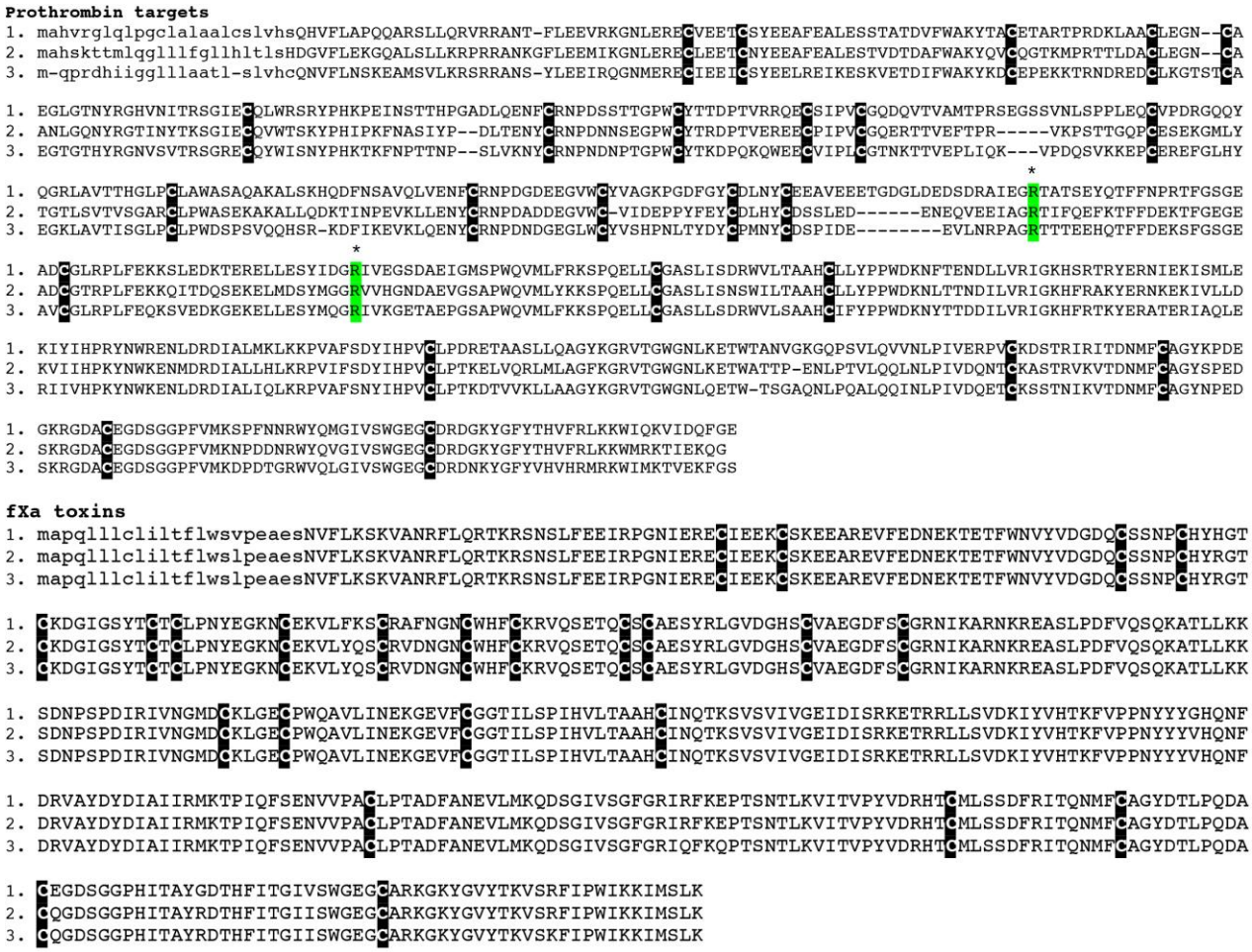

Figure 7

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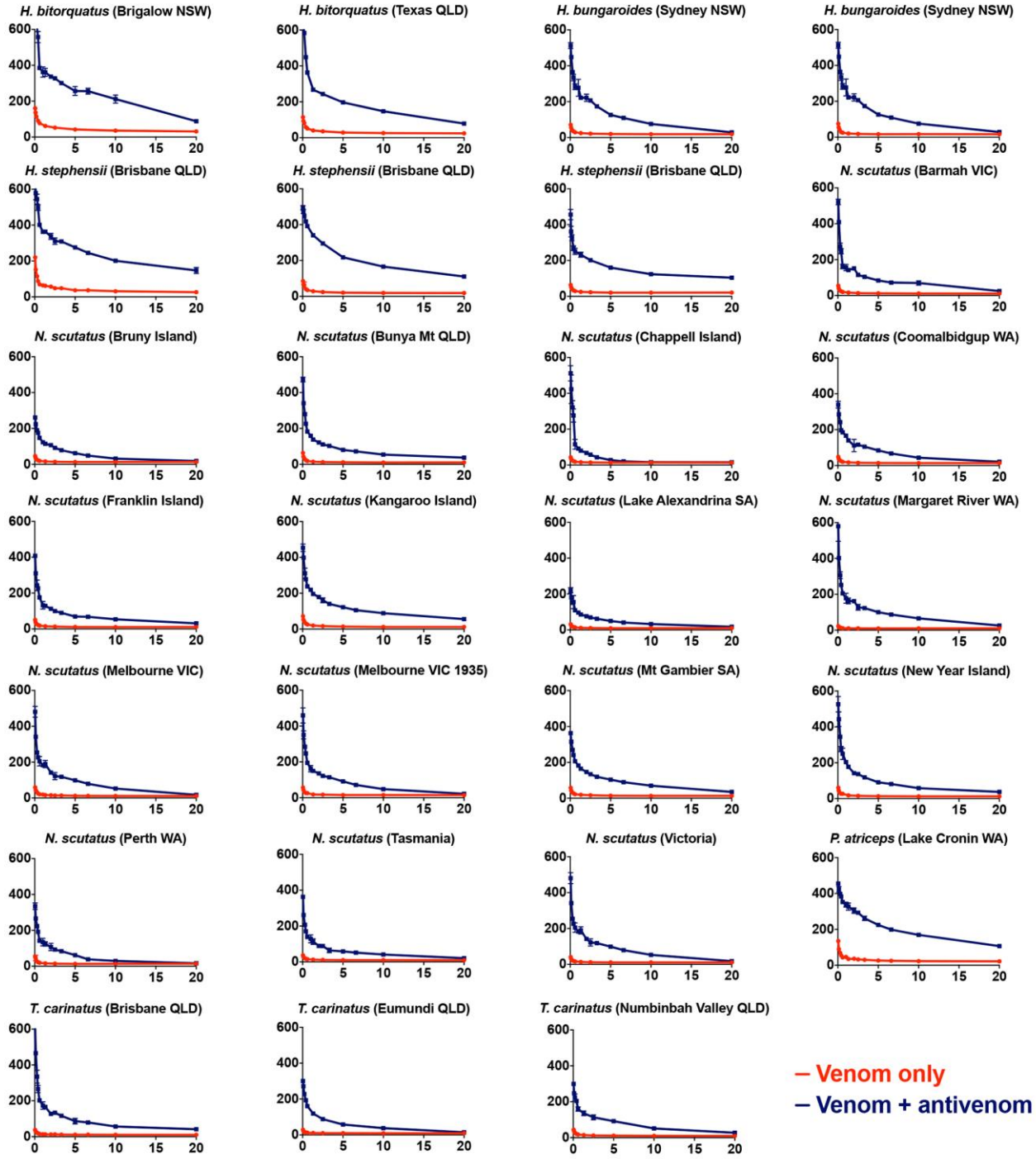
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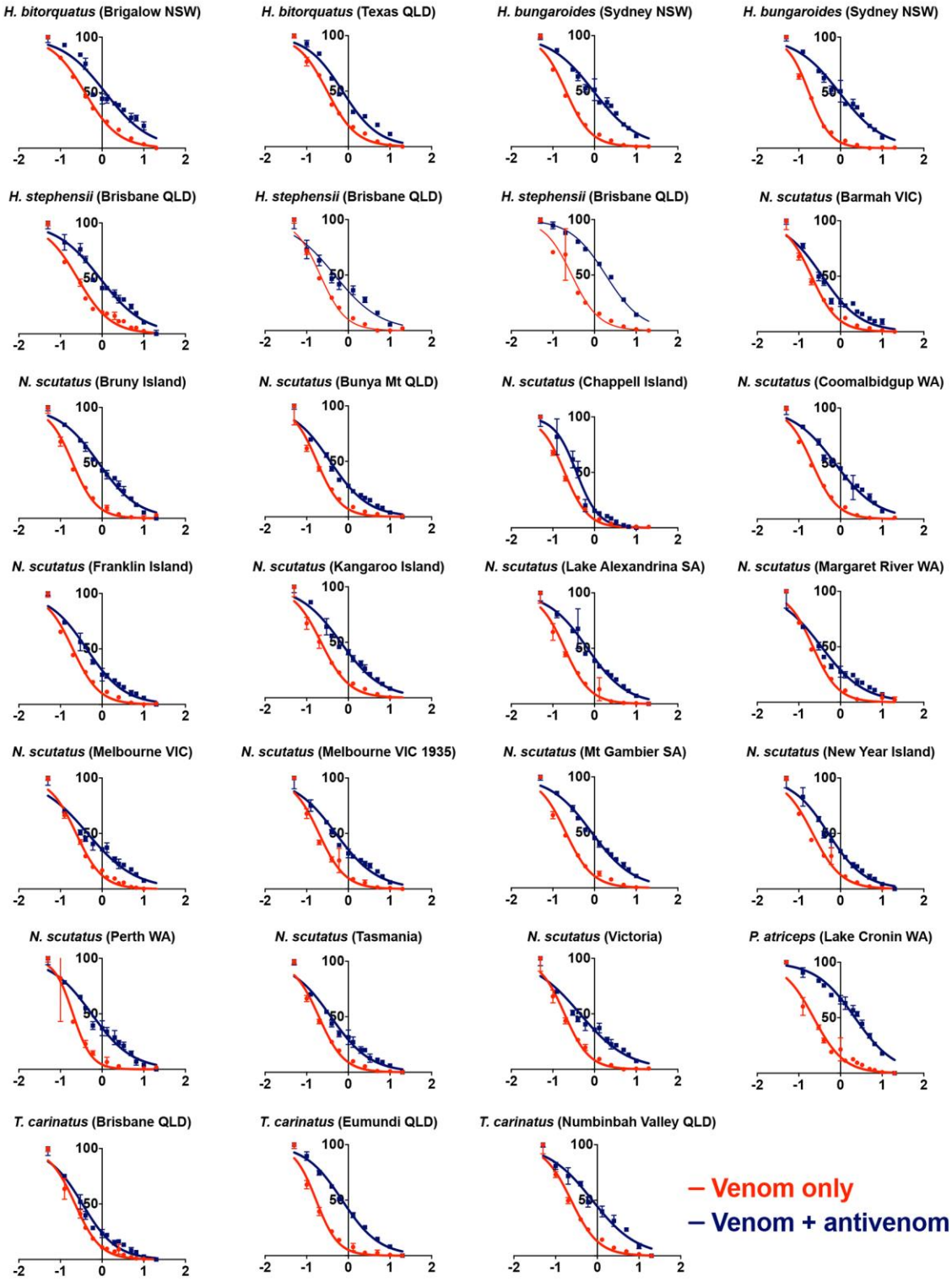
H. bitorquatus (Texas QLD)
H. bungaroides (Sydney NSW)
H. bungaroides (Sydney NSW)
H. stephensii (Brisbane QLD)
H. stephensii (Brisbane QLD)
H. stephensii (Brisbane QLD)
N. scutatus (Barmah VIC)
N. scutatus (Bruny Island)
N. scutatus (Bunya Mt QLD)
N. scutatus (Chappell Island)
N. scutatus (Coomalbidgup WA)
N. scutatus (Franklin Island)
N. scutatus (Kangaroo Island)
N. scutatus (Lake Alexandrina SA)
N. scutatus (Margaret River WA)
N. scutatus (Melbourne VIC)
N. scutatus (Melbourne VIC 1935)
N. scutatus (Mt Gambier SA)
N. scutatus (New Year Island)
N. scutatus (Perth WA)
N. scutatus (Tasmania)
N. scutatus (Victoria)
P. atriceps (Lake Cronin WA)
T. carinatus (Brisbane QLD)
T. carinatus (Eumundi QLD)
T. carinatus (Numbinbah Valley QLD)
– Venom only
– Venom + antivenom

Figure 8


H. bitorquatus (Brigalow NSW)
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H. bungaroides (Sydney NSW)
H. bungaroides (Sydney NSW)
H. stephensii (Brisbane QLD)
H. stephensii (Brisbane QLD)
H. stephensii (Brisbane QLD)
N. scutatus (Barmah VIC)
N. scutatus (Bruny Island)
N. scutatus (Bunya Mt QLD)
N. scutatus (Chappell Island)
N. scutatus (Coomalbidgup WA)
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N. scutatus (Lake Alexandrina SA)
N. scutatus (Margaret River WA)
N. scutatus (Melbourne VIC)
N. scutatus (Melbourne VIC 1935)
N. scutatus (Mt Gambier SA)
N. scutatus (New Year Island)
N. scutatus (Perth WA)
N. scutatus (Tasmania)
N. scutatus (Victoria)
P. atriceps (Lake Cronin WA)
T. carinatus (Brisbane QLD)
T. carinatus (Eumundi QLD)
T. carinatus (Numbinbah Valley QLD)
– Venom only
– Venom + antivenom

Figure 9

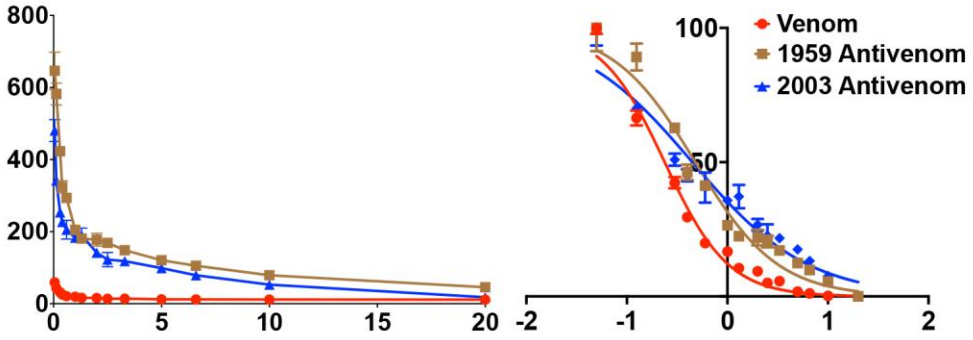

Figure 10

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