Coagulotoxic effects by brown snake (*Pseudonaja*) and taipan (*Oxyuranus*) venoms, and the efficacy of a new antivenom

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Received: date; Accepted: date; Published: date

Abstract: Snakebite is a neglected tropical disease that disproportionately affects the poor. Antivenom is the only specific and effective treatment for snakebite, but its distribution is severely limited by several factors, including the prohibitive cost of some products. Papua New Guinea (PNG) is a snakebite hotspot but the high costs of Australian antivenoms (thousands of dollars per treatment) makes it unaffordable in PNG. A more economical taipan antivenom has recently been developed at the Instituto Clodomiro Picado (ICP) in Costa Rica for PNG and is currently undergoing clinical trials for the treatment of envenomations by coastal taipans (*Oxyuranus scutellatus*). In addition to potentially having the capacity to neutralise the effects of envenomations of non-PNG taipans, this antivenom may have the capacity to neutralise coagulotoxins in venom from closely related brown snakes (*Pseudonaja* spp.) also found in PNG. Consequently, we investigated the cross-reactivity of taipan antivenom across the venoms of all *Oxyuranus* and *Pseudonaja* species.

In addition, to ascertain differences in venom biochemistry that influence variation in antivenom efficacy, we tested for relative cofactor dependence. We found that the new ICP taipan antivenom exhibited high selectivity for *Oxyuranus* venoms and only low to moderate cross-reactivity with any *Pseudonaja* venoms. Consistent with this genus level distinction in antivenom efficacy were fundamental differences in the venom biochemistry. Not only were the *Pseudonaja* venoms significantly more procoagulant, but they were also much less dependent upon the cofactors calcium and phospholipid. There was a strong correlation between antivenom efficacy, clotting time and cofactor dependence. This study sheds light on the structure-function relationships of the procoagulant toxins within these venoms and may have important clinical implications including for the design of next-generation antivenoms.

Keywords: *Oxyuranus*, *Pseudonaja,* venom, antivenom, ICP antivenom, coagulotoxic, prothrombinase complex, coagulopathy, disseminated intravascular coagulation, venom induced consumptive coagulopathy

1. Introduction

Snakebite is a neglected tropical disease that disproportionately affects the poorest nations (Harrison et al., 2009). The prevalence of snakebite and resultant health repercussions in Papua New Guinea (PNG) renders residents of this economically disadvantaged nation at particularly high risk. Indeed, the death rate from snakebite in some parts of PNG is estimated to be an alarming 7.9 people per 100,000 inhabitants (Lalloo et al., 1995a), compared to 0.13 deaths per 100,000 people in Australia (Welton et al., 2017).

Snakebite in poor, rural settings in tropical regions is not only a serious public health hazard but also a data-deficient issue, as record keeping is either of poor quality or entirely absent. For example, while the Port Moresby (south eastern PNG) area has been the subject of intensive snakebite research (Lalloo et al., 1995b; Williams et al., 2007, 2005), which indicated that *O. scutellatus* is by far the greatest hazard regarding snakebite in that region, the incidence of *P. textilis* bites and resulting fatalities in Oro Province where these snakes are common (O'Shea, 1996) is still unknown and may be high.

The genera *Oxyuranus* and *Pseudonaja* form a monophyletic clade, containing snakes which are very dangerous to humans because their venom has evolved to incapacitate mammalian prey rapidly by inducing stroke, likely as a consequence of selection pressures to reduce the risk of retaliation by these dangerous prey animals. This acute effect on mammals is mirrored clinically in humans: people have collapsed from *Pseudonaja* envenoming in just 15 minutes (not due to anaphylaxis) (Sutherland and Tibballs, 2001).

77 The etiology of “rapid collapse” following brown snake envenoming is poorly understood,
78 but bites from these snakes typically result in severe disruption of hemostasis (Chaisakul et
79 al., 2013; Lalloo et al., 1995b), characterised by venom-induced consumptive coagulopathy
80 (VICC) (Allen et al., 2012; Isbister et al., 2010b). In humans, this irreversible and life-
81 threatening condition is caused by procoagulant toxins that consume essential blood
82 coagulation proteins and results in a net anticoagulant effect with occasional hemorrhaging
83 (Isbister, 2009; Maduwage and Isbister, 2014; Sutherland and Tibballs, 2001). These
84 sequelae persist until blood clotting factors have been replaced (resynthesised) by the body,
85 and death may result from internal bleeding such as cerebral hemorrhage.
86 *Oxyuranus* and *Pseudonaja* species contain in their venoms activated forms of proteins
87 homologous to the blood coagulation factors X (FXa) (Trabi et al., 2015) and V (FVa) (Earl
88 et al., 2015), which together form the prothrombinase complex responsible for the
89 aforementioned coagulopathy. These powerful procoagulant toxins share 48-54% sequence
90 identity with human endogenous prothombinase (Bos and Camire, 2010; St. Pierre et al.,
91 2005) and have acquired several unique gain-of-function features that make them more
92 potent than the endogenous FXa:FVa prothrombinase complex (Bos et al., 2009; Lavin and
93 Masci, 2009). For instance, the FVa subunit of these toxins has been shown to escape
94 hemostatic downregulation by activated protein C (Bos et al., 2009), the natural inactivator
95 of endogenous FVa. While a procoagulant toxin derived from FXa was likely present in the
96 venoms of the common ancestor of most Australian elapids (Fry et al., 2008), the action of
97 the plesiotypic toxic form of FXa is rate-limited by its requirement for the endogenous
98 cofactor FVa to cleave prothrombin (Factor II) into thrombin (Factor IIa) to trigger blood
99 clotting. However, FVa was additionally recruited into the venom of the
100 *Oxyuranus*+*Pseudonaja* clade (Fry et al., 2008) thereby enabling injection of the complete
101 prothrombinase complex (FXa:FVa), without the need to bind to endogenous FVa (Kini,
102 2005; Palta et al., 2014); this event increased the efficiency of coagulotoxic effects by
103 eliminating this rate limiting step.
104 Another key efficiency adaptation in the *Oxyuranus*+*Pseudonaja* clade is the reduced
105 dependence of this FXa:FVa prothrombinase toxin complex upon the cofactors calcium and
106 phospholipid relative to that of the endogenous FXa:FVa complex. While calcium and
107 phospholipid are often required as cofactors by toxins to initiate their disruption of
108 haemostasis (Kini et al., 2002; Rosing et al., 1980), the FXa:FVa prothrombinase complex
109 in the venoms of this clade can function in their absence (Bos et al., 2009; Kini et al.,
110 2002). The recruitment of FVa into these venoms and their lessened cofactor dependence
111 are key evolutionary innovations which likely contribute to the high coagulotoxicity of the
112 venoms of snakes in these genera compared to other venomous species (Broad et al., 1979;

Mirtschin and Davis, 1982). Furthermore, this procoagulant complex makes up a large percentage of the total protein composition of these venoms: 10-20% of the total *O. scutellatus* venom (Lavin and Masci, 2009) and 20-40% of *P. textilis* venom (Lavin and Masci, 2009; Rao and Kini, 2002).

To counter the rapid and devastating pathology in envenomed patients bitten by *Oxyuranus* and *Pseudonaja* species, early treatment with effective antivenom is required (Winkel et al., 2006). However, in Papua New Guinea the high cost of Australian-produced antivenom has historically limited its availability and accessibility. Up to 80% of health facilities have no antivenom supplies at all, and in some areas of high snakebite burden there is only enough antivenom available each year to treat 15% of snakebite patients (Williams et al., 2005). This large gap between supply and demand sometimes leads to theft or unscrupulous marketing of antivenoms specific to other regions (Warrell, 2008). In addition, treatment is sometimes delayed within hospitals in an effort to avoid exhausting this precious product; this delay can have devastating effects because early administration is crucial to favourable clinical outcomes (Trevett et al., 1995).

While Seqirus (formerly CSL Limited, Australia) taipan (monovalent) antivenom is available in PNG at a cost of ~US$1,270/vial, Seqirus brown snake (monovalent) antivenom is not available. The only current specific treatment for *Pseudonaja* envenomations is Seqirus polyvalent antivenom, but one vial can cost up to US$1,670 in PNG, which is almost 60% of the per capita GDP. The high cost of Australian-made antivenoms substantially impacts availability, but fortunately a new antivenom for taipan envenoming (*Papuan taipan antivenom ICP*; Instituto Clodomiro Picado, Costa Rica) which performed well in preclinical (Vargas et al., 2011) and clinical trials and is expected to cost less than 25% of the current product price may ease this crisis.

Antivenom is a plasma-derived product which contains antibodies produced by an immunised animal (usually a horse or sheep) that neutralise particular venom components. Homogeneity in toxin compositions and toxin structure/function sometimes means that an antivenom produced using venom from one snake species may afford paraspecific cross-protection against the venoms of other, generally closely related species of snake due to extensive shared evolutionary history. For example, substantial cross-reactivity of tiger snake antivenom was recently demonstrated across multiple closely-related genera diverging 8–10 million years ago (Lister et al., 2017). *Oxyuranus* and *Pseudonaja* are sister taxa diverging <15 million years ago (Sanders et al., 2008). This close phylogenetic relatedness is echoed by similar venom composition and sequence homology in the FXa:FVa prothrombinase complex (93% similarity of the FXa subunits and 97% similarity of the FVa subunits). Thus, the new ICP taipan antivenom may also neutralise toxins in the

venoms of other *Oxyuranus* species, and perhaps also those of *Pseudonaja* venoms, albeit to variable degrees since the venoms may contain significantly different levels of FXa and FVa (Barber et al., 2012).

In this study, we used crude venom from adult snakes in functional tests to 1) quantify the relative procoagulant activity across all recognised species in the medically significant *Oxyuranus*+*Pseudonaja* clade, 2) use cofactor dependence tests as a probe to better understand the structure-function relationship differences in the FXa:FVa prothrombinase complex of *Oxyuranus* and *Pseudonaja* venoms, and 3) determine the relative efficacy of ICP taipan antivenom in neutralising the coagulotoxic effects of *Oxyuranus* and *Pseudonaja* venoms. We hypothesised that 1) the *Pseudonaja* venoms would be more coagulotoxic than *Oxyuranus* venoms, 2) lessened dependence upon calcium and/or phospholipid cofactors would potentiate the coagulotoxic effect of all 28 venoms in this study and 3) due to the close evolutionary relationship between the *Oxyuranus* and *Pseudonaja* genera which is reflected in very high sequence similarity between their toxins, the ICP Taipan antivenom would cross-react with *Pseudonaja* venoms.

2. Materials and Methods

2.1 *Venom collection and preparation*

We studied whole venoms from nine *Oxyuranus* samples and 19 *Pseudonaja* samples (Table 1), covering all currently recognised species (three and nine, respectively) within these two genera. Our samples included several populations from most species to account for intraspecific venom variation; this can be important, as *P. textilis* venoms may vary in toxicity across their range (Flight et al., 2006). To account for possible individual variation in expression levels (Chippaux, 1998), we were able to represent 10 out of the 12 species in this study with at least two individuals, with the exception of one sample each from *P. guttata* and *P. modesta*, as they are less commonly maintained in captivity.

Only adult venoms were used to avoid ontogenetic variation in venoms, particularly since this has been previously documented in *Pseudonaja* (Cipriani et al., 2017). All venoms were extracted, flash-frozen, then lyophilised, and later reconstituted in deionised water, centrifuged (5 min., 4°C 14,000 RCF), and the supernatant was diluted to a concentration of 1 mg/mL with 50% glycerol to prevent freezing at -20°C. Protein concentrations were determined in triplicate using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermofisher, Sydney, NSW, Australia) at an absorbance of 280 nm.

Venoms were sourced from individual snakes (captive and wild-caught) from either the long-term cryogenic collection of the Venom Evolution Laboratory, Venom Supplies Pty Ltd, or Reptile Kingdom Australia. We did not consider venoms from either wild-caught or captive individuals to be a confounding variable in our study because McCleary et al.

(2016) found captivity had no effect on venom composition over time in *Pseudonaja*. Snakes were milked using traditional methods (membrane method) or via the pipette-tip method for low-yielding *Pseudonaja* species (Mirtschin et al., 2006).

2.2 *Antivenom*

A monospecific taipan antivenom (Lot#: 5330913TALQ; Exp: 09/16) manufactured at Instituto Clodomiro Picado (ICP) was used. This antivenom is a whole IgG preparation obtained from the fractionation by caprylic acid of plasma from horses immunised with the venom of *O. scutellatus* from PNG (Vargas et al., 2011).

2.3 *Plasma collection and preparation*

Human plasma was collected from healthy human donors, with sodium citrate used as an anticoagulant, and donated by Australian Red Cross (research approval #16-04QLD-10; 44 Musk Street, Kelvin Grove, Queensland 4059). Two batches of pooled plasma (Label #3991594 (O-) and label #3985833 (B+), citrate 3.2%) were further pooled and aliquoted, then flash-frozen in liquid nitrogen and immediately stored at -80°C until required. When required, plasma was rapidly thawed at 37°C in a Thermo Haake ARCTIC immersion bath circulator SC150-A40 and immediately used for experimentation. Plasma aliquots were replaced every hour at maximum to maintain freshness.

2.4 Procoagulation tests

Clotting times of human plasma by venom were automatically measured using a STA-R Max® analyser (Stago, Asnières sur Seine, France) in an 8-point series of decreasing venom concentrations (μg/mL: 20, 10, 4, 1.6, 0.66, 0.25, 0.125, 0.05). Once the venom sample was loaded into the machine, pipetting (and diluting, when appropriate) into small cuvettes occurred automatically, according to our pre-programmed experimental methodologies. Venom and plasma were replaced every 15–30 min to minimise enzymatic degradation. All tests were performed in triplicate.

For each test performed, 50 μL phospholipid (cephalin prepared from rabbit cerebral tissue adapted from STA C.K Prest standard kit, Stago Catalog # 00597, solubilised in 5 mL of Owren Koller (OK) Buffer [Stago Cat# 00360]), 25 μL OK buffer and 50 μL $CaCl_2$ (0.025 M, Stago Catalog # 00367) were added to 50 μL venom (0.1 mg/mL) in the cuvette, as they are known cofactors usually required for activity (Nakagaki et al., 1992; St. Pierre et al., 2005). For other dilution points, the 50 μL of venom (0.1 mg/mL) was automatically diluted by the machine to complete the 8-point dilution series of decreasing venom concentrations (20–0.05 μg/mL). Once all reagents were added, the cuvette was shaken

briefly by the machine to mix the components and then incubated at 37°C for 120 seconds (to allow for antivenom to bind to the venom in antivenom tests) before plasma (75 µL) was added immediately prior to the clotting-time, which was measured in seconds for a maximum of 999 seconds. Reagents were kept at 15–19°C in the machine.

Positive controls were performed daily to test the quality of the plasma and reagents. For these tests, Kaolin (a coagulation activator) (Stago C.K Prest standard kit, Stago Catalog #00597) replaced venom in the cuvette. The average clotting time for these controls was 56.8 ± 2.2 s (n=35), as determined by daily tests performed over an approximately one-month period.

Negative controls were also performed daily, where 1:1 water/glycerol replaced venom in the tests. The average clotting time for these controls was 496.9 ± 124.4 s (n=36). Venoms were considered non-coagulant if the highest concentration of venom (20 µg/mL) produced clotting times within the standard error of the negative control (i.e. over 372.5s). For these venoms, dose-response curves were not performed.

2.5 Cofactor dependence tests

To determine the level of cofactor dependence of the venoms, the aforementioned coagulation tests were conducted again at the highest venom concentration (20 µg/mL)—also in triplicate—with either calcium or phospholipid being replaced with 50 µL OK Buffer. In addition, to determine whether cofactor dependence trends observed at 20 µg/mL of venom persisted at a lower concentration of venom, we repeated these tests at 5 µg/mL. Calcium and phospholipid dependence values were calculated for each venom using the results from the 20 µg/mL tests. These dependence values were calculated by dividing the clotting times for each venom without the cofactor by clotting times with the cofactor present in the assay.

2.6 Antivenom efficacy tests

The efficacy of ICP antivenom was tested against all venoms in this study by substituting in the aforementioned venom dose-response curves 25 µL of a mixture with antivenom diluted 1:10 in OK Buffer (2.5uL antivenom in 25uL total), which would have a final dilution of 1/100 (2.5uL antivenom in 250uL total volume). This 1/100 concentration of antivenom was chosen based on the neutralising effect that 2.5uL of antivenom (out of a total 250uL volume) was observed against a 20ug/mL concentration of the immunising venom (*O. scutellatus* (PNG locality)). To calculate antivenom efficacy, the AUC values for the venom+antivenom dose-response curves were divided by the venom-only dose-response curve AUC values, then subtracted by 1 (so that no shift in AUC values (a value

of 1 divided by 1) would have a value of 0 instead of 1). Negative controls using a 10% antivenom solution instead of venom were run to determine whether antivenom had any effect on the plasma. The average clotting time for these controls was 650.6 ± 115.5 s (n=21), which was significantly different than the venom negative control tests stated above (496.9 ± 124.4 s (n=36)).

2.7 Factor Xa activity test

We tested the relative factor Xa activity of all venoms using a Thermo Scientific™ Fluoroskan Ascent™ Microplate Fluorometer by adding the following (per venom in triplicate) to a 384-well plate (black, Lot#1171125, nunc™ Thermo Scientific, Rochester, NY, USA) kept at 37°C: 10 µL each of $CaCl_2$ (0.025 M, Stago Catalogue # 00367) and phospholipid (as described in section 2.4), and 10 µl venom (1 ng/µL). Immediately thereafter, 60 µL of quenched fluorescent substrate (20 µL/5 mL OK buffer) was automatically dispensed into each well (total volume of 100 µL in each well) using the following substrate: ES002 (Mca-R-P-K-P-V-E-Nval-W-R-K(Dnp)-NH_2) (Lot#: DKC10, www.rndsystems.com).

The plate was shaken automatically for three seconds before each reading and the fluorescence (a measure of the venom's action on the substrate) was recorded for 300 measurement cycles (400 minutes or until activity ceased) following the instructions of the substrate manufacturer (filter pairs: 320/405 nm, excitation/emission) using the software Thermo Fluoroskan Ascent™ 2.6.

Human Factor Xa enzyme (0.01 µg/µL) (7.5 mg/mL HCXA -OO60 - R & D systems) was used as positive control to ensure functionality of the substrate. The measurement of blank wells with substrate and OK buffer only was subtracted from all other measurements to eliminate any background activity (ie. fluorescence by the substrate and/or OK buffer alone). Raw data were organised using Excel 2016 and then analysed using Prism 7.0. All data were normalised for presentation purposes.

2.8 Statistical analyses

All dose-response curves, as well as cofactor dependence tests, were analysed using Prism 7.0 software (GraphPad Software Inc, La Jolla, CA, USA) and Rstudio. ANOVAs and t-tests were conducted using GraphPad Prism 7.0a. ANOVAs were conducted to test whether multiple groups (e.g. *P. textilis* localities) were significantly different. T-tests were used to assess whether two groups being compared (e.g. *Oxyuranus* vs. *Pseudonaja*) were significantly different. A D'Agostino-Pearson normality test was conducted to determine normality of the data prior to analyses. When the data were not normally distributed, a Kruskal-Wallis test or Mann-Whitney U-test was used instead of ANOVAs or t-tests,

respectively. Area under the curve (AUC) values for the venom and antivenom concentration curves were calculated using Prism 7.0 software. For all statistical tests, p-values $\leq$ 0.05 were considered statistically significant. The results are expressed as the mean ± SD.

The phylogenetic tree used for visual representation of the data was based on previously published trees (Sanders et al., 2008; Skinner et al., 2005) and re-created manually using Mesquite software (version 3.2). This tree was then used for all comparative analyses in Rstudio using the APE package for basic data manipulation (Paradis et al., 2004). In order to investigate the evolutionary relationships of traits, ancestral states were estimated over the tree using maximum likelihood in the contMap function of the R package phytools (Revell, 2012). We then fit PGLS (phylogenetic generalised least squares) models (Symonds and Blomberg, 2014) in caper (Orme et al., 2015) to test whether dependence on either calcium or phospholipid cofactors influenced clotting time, and also whether phospholipid dependence is related to calcium dependence. Coding for the R-analyses is in Supplementary File 1.

3. Results

3.1 *Procoagulant venom activity*

We conducted a total of 150 dose-dependent coagulation curves (25 in triplicate each for coagulation curves and antivenom curves) across all currently recognised *Pseudonaja* species (9) and *Oxyuranus* species (3). Whether compared by clotting time at maximal concentration (20 μg/mL) or full clotting curve via Area Under the Curve (AUC), the same phylogenetic patterns were observed (PGLS $df = 1$, $t = 11.4880$, $p = 1.1 \times 10^{-11}$) in that *Pseudonaja* venoms were appreciably more toxic than *Oxyuranus* venoms (Figure 1). Consistent with data reported by Jackson et al. (2016), we found only very weak procoagulant properties in *P. modesta* venoms (337.0 ± 4.4 s clotting time at the 20 μg/mL concentration relative to the spontaneous clotting time of plasma without venom (496.9 ± 20.74 s SD, n = 36)). In agreement with Barber et al. (2014), we also found a similar lack of discernable procoagulant effects for *O. temporalis* venom (>421.0 s with 20 μg/mL venom vs 496.9 ± 20.74 s SD without venom) (Table 1). All other venoms displayed potent procoagulant effects, with a non-parametric Mann-Whitney U-test revealing that *Pseudonaja* venoms exhibited significantly more potent coagulotoxic effects than *Oxyuranus* venoms (Mann-Whitney U-test = 4, N=25, P < 0.0001).

In contrast to data reported by Flight et al. (2006) and Skejic and Hodgson (2013), we found no significant difference (Kruskal-Wallis test: p = 0.52) among the procoagulant activities of *P. textilis* venoms samples from four localities up to 1,785km apart.

3.2 *Cofactor dependent clotting*

Our PGLS (phylogenetic generalised least squares) analyses revealed that calcium dependence and phospholipid dependence both predict clotting time, such that venoms with greater cofactor independence produced faster clotting times (Fig. 2; calcium: PGLS: $df = 1$, $t = 4.3042$, $p = 2.11 \times 10^{-04}$; phospholipid: $df = 1$, $t = 1.9902$, $p = 0.05718$, Fig. 3). Calcium dependence was found to be an extremely strong predictor of phospholipid dependence (PGLS: $df = 1$, $t = 7.7003$, $p = 3.592 \times 10^{-08}$; Fig. 4), such that venoms either relied heavily on both cofactors or neither.

Surprisingly, not all venoms were potentiated with the addition of phospholipid. In fact, the opposite effect was observed for some *Pseudonaja* venoms, which showed faster clotting times in the absence of phospholipid (but were still faster than *Oxyuranus* venoms even in the presence of phospholipid) (Table 1, Fig. 3). The exception to this within the *Pseudonaja* was the monophyletic clade of *P. affinis, P. aspidorhyncha,* and *P. inframacula* that showed phospholipid dependence.

Cofactor dependence tests performed at a lower concentration (5 μg/mL) revealed that all venoms shifted towards more cofactor dependence at the lower venom concentration (Supplementary Figures S1 and S2). Only slight variations were observed in the most dependent venoms, whereas the least dependent venoms remained so at both venom concentrations.

3.3 Antivenom efficacy

The taxonomical selectivity of ICP Taipan antivenom for *Oxyuranus* venoms was consistently higher compared to *Pseudonaja* venoms (PGLS: $df = 1$, $t = 4.9364$, $p = 3.974 \times 10^{-5}$; Fig. 5). Specifically, the antivenom neutralised all *Oxyuranus* venom samples but performed comparably poorer against the *Pseudonaja* species. Examination of the well-neutralised *Oxyuranus* clade revealed no correlation between antivenom efficacy and clotting time (PGLS: $df = 1$, $t = -0.0670$, $p = 0.9492$). Thus the poor neutralisation of *Pseudonaja* venoms was likely due to surface biochemistry rather than speed of action.

3.4 *Factor Xa substrate activity*

P. guttata venom had the highest activity upon the synthetic FXa substrate, with *P. textilis* venoms having slightly lower activity, and the two procoagulant *Oxyuranus* species

showed the lowest activity levels. For all venoms, higher substrate activity was typically related to faster clotting times, and this was significant (PGLS: df = 1, t = -1.9682 (a negative value due to the inverse relationship between clotting time and substrate cleavage), p = 0.0598; Fig. 6). The greater variability for substrate cleavage versus clotting time reflected the fact that the substrate was an artificial environment.

4. Discussion

We set out to determine the relative procoagulant activity (Fig. 1; Table 1), cofactor dependence (Fig. 2-4; Table 1), ICP Taipan antivenom efficacy (Fig. 5; Table 1), and FXa substrate specificity (Fig. 6) across all species within the medically significant genera *Oxyuranus* and *Pseudonaja*. Within species displaying coagulotoxicity (all other than *O. temporalis* or *P. modesta* that showed no such activity), all species of *Pseudonaja* were significantly more procoagulant than *Oxyuranus* spp., with *P. textilis* populations having the most procoagulant venoms. Cofactor dependence varied according to a significant phylogenetic signal and was significantly positively correlated with clotting time, meaning that the relative dependence of venoms on cofactors corresponded with clotting time patterns. Cofactor dependence patterns across the venoms still persisted at a lower venom concentration (5 μg/mL, compared to 20 μg/mL). The ICP Taipan antivenom (made using only Papua New Guinea *O. scutellatus* venom in the immunising mixture, in 'naïve' horses with no prior exposure to other venom antigen mixtures) exhibited highly significant selectivity for *Oxyuranus* venoms and displayed little or no cross-reactivity with *Pseudonaja* venoms. Within the well-neutralised *Oxyuranus* clade, clotting times (ie. potency of venoms) was not a significant predictor of antivenom efficacy (t = -0.0670, p = 0.9492). Factor Xa activity on artificial substrate moderately corresponded with relative clotting times on whole plasma.

4.1 Procoagulant venom activity

The rank order of procoagulant potency of *Oxyuranus* venoms in this study was: *O. scutellatus* were on average slightly faster than *O. microlepidotus,* while *O. temporalis* was non-coagulotoxic. For *Pseudonaja* venoms, the order was: *P. textilis* > *P. ingrami* > *P. guttata* > *P. mengdeni* > *P. nuchalis* > *P. affinis* > *P. aspidorhyncha* > *P. inframacula,* while *P. modesta* was non-coagulotoxic (See Table 1).

Despite the FXa:FVa complex being characteristic of *Oxyuranus* and *Pseudonaja* venoms (relative to other coagulotoxic Australian elapids which contain only FXa in their venom), the fact that all venoms exhibited varying clotting times within each genus

suggests possible dissimilarities in the functional residues across species. While FVa was recruited into the common *Oxyuranus*/*Pseudonaja* ancestor (Fry et al., 2008), venom speed of action was substantially faster within *Pseudonaja* (Fig. 1). This parallels the reduction in cofactor dependence in the same phylogenetic positions (see Fig. 2 and 3 and section 3.2).

While *Pseudonaja* venoms in this study were markedly more procoagulant than *Oxyuranus* venoms (Fig. 1; Table 1), it is important to note that Barber et al. (2012) found *Oxyuranus* venoms were more neurotoxic when tested in the chick biventer cervicis nerve-muscle preparations. This is consistent with our knowledge of clinical syndromes of envenoming by *Oxyuranus* consisting of neurotoxicity and coagulopathy, whereas envenoming by *Pseudonaja* is coagulopathic (Flight et al., 2006; Sutherland and Tibballs, 2001).

Interestingly, although some studies found a significant difference in procoagulant activity from the venoms of northern (QLD) and southern (SA) localities of *P. textilis* (Flight et al., 2006; Skejić and Hodgson, 2013), we found no evidence of variation in procoagulant activity among four very disparate localities spanning 1,785Km. While more venoms and data are required for a robust test of geographical variation in procoagulant activity of *P. textilis* venoms, our results suggest that there may not be a functional difference in the procoagulant enzyme levels across the species' distribution. Instead, the discrepancy may be due to the ontogenetic changes in *Pseudonaja* venoms from neurotoxic to coagulotoxic from juveniles to adults (Cipriani et al., 2017; Jackson et al., 2016). Previous geographical studies may have used venom from subadult snakes—no snake sizes were reported—for the South Australian populations which would be consistent with the lower coagulotoxicity. In contrast, this study used venom only from adult snakes in order to make meaningful comparisons across ranges and between species.

4.2 Variability in cofactor dependence

In vivo, plasma contains platelets (among other additional blood components), which possess calcium and phospholipids that assist in the biochemical reactions of the blood coagulation cascade. Because citrated plasma (calcium removed) used in *in vitro* testing is separated from and void of platelets, it is particularly important to reintroduce the cofactors calcium and phospholipid in order to best model and test these systems. Indeed, our cofactor dependence results indicate that the omission of these cofactors from venom assays will result in skewed data. While some previous venom studies included calcium and phospholipid cofactors in their clotting assays (Bos et al., 2016; Chester and Crawford, 1982; Owen and Jackson, 1973; Sousa et al., 2018), others failed to include either phospholipid (Isbister et al., 2010b; Nielsen et al., 2017; O'Leary and Isbister, 2010; Still et

al., 2017; Vargas et al., 2011), or both calcium and phospholipid (Ainsworth et al., 2018; Williams et al., 1994).

We found that relative cofactor dependence was proportional to clotting time, with the fastest clotting venoms also being the least dependent (Fig. 2-3; Table 1). The elimination of biochemical steps in the coagulation cascade could be advantageous by increasing catalytic activity, thereby potentiating progress towards a fibrin clot. This may explain why more calcium- and phospholipid-independent venoms are more potently coagulotoxic. We also found that calcium dependence and phospholipid dependence positively co-varied (Fig. 4). This relationship was expected because calcium mediates the interaction between blood coagulation proteins and the negatively charged phospholipid surface (Bos et al., 2016), so the two cofactors are mechanistically linked. There were, however, significant differences between the two genera regarding the influence of calcium and phospholipid. Further, our investigation demonstrated that cofactor dependence is far more variable in *Pseudonaja* than previous work suggests (Chester and Crawford, 1982; Rosing and Tans, 1992).

Calcium and phospholipid dependence patterns across the venoms remained largely the same between both venom concentrations (5 μg/mL vs. 20 μg/mL) (Figure S1 and S2), with all venoms shifting toward more cofactor dependence at the lower venom concentration. Only slight variations in the most dependent venoms were observed, and the least dependent venoms remained so at both venom concentrations. *Pseudonaja* venoms that accelerated clotting time in the absence of phospholipid did not do so at 5 μg/mL, indicating that, when the venom is more concentrated (e.g. in prey items), it is able to overwhelm its target without dependence on cofactors; whereas at a lower venom concentration, greater cofactor dependence becomes more evident. Given that cofactor effects on venom are even more pronounced at lower venom concentrations, it is fundamentally important to include cofactors in *in vitro* venom studies that involve evolutionary and pathophysiological interpretations.

While the FXa:FVa prothrombinase complex in *Oxyuranus* and *Pseudonaja* venoms can function in the absence of calcium and phospholipid (Bos et al., 2009; Kini et al., 2002), all venoms in these genera functioned faster in the presence of calcium (Fig. 2; Table 1). In agreement with Vargas et al. (2011), our five *O. scutellatus* venoms showed higher *in vitro* coagulant activity in the presence of calcium. In contrast, the *Pseudonaja* venoms were in general much less dependent (up to 15-times less dependent relative to *Oxyuranus*), with some approaching neutral level of dependence in that the reactions were only 0.25-times faster with calcium than without.

Counterintuitively, in *Pseudonaja* species we found evidence for accelerated venom-induced clotting in the absence of phospholipid (negative phospholipid-dependence values) at 20 μg/mL relative to the clotting times displayed in the presence of phospholipid (although these venoms were still faster than *Oxyuranus* even in the presence of phospholipid) (Fig. 3). The weaponised version of the prothrombinase complex in *P. textilis* has been shown to have 116 mutations, several of which lie within the likely prothrombin binding site and thus may result in improved activity in the absence of membranes (Lechtenberg et al., 2013), such as a phospholipid membrane surface.

The *Pseudonaja* condition where certain venoms exhibit accelerated action in the absence of phospholipid poses an interesting evolutionary question, as phospholipid (and calcium) is present in the plasma of all prey items (and humans). However, since the membranes of activated platelets and activated/apoptotic endothelial cells are the physiological source of phospholipids, and since the venom coagulotoxins probably circulate in the bloodstream without causing vascular damage, it is reasonable to presume there are naturally low phospholipid concentrations (estimated 3%) in the bloodstream (Bevers and Williamson, 2016). While some venom factors may be shown to activate platelets and produce phospholipid, the decreased reliance upon such secondary biochemical steps would facilitate faster clotting times. It is important to note that these *Pseudonaja* venoms were the fastest, regardless of the relative presence of phospholipid. Their acceleration in the absence of phospholipid (despite acting the fastest in the presence of phospholipid) indicates a remarkable level of functional flexibility and suggests that these enzymes may utilise alternative biochemical pathways depending on relative abundance of phospholipid.

Our demonstration of accelerated venom effects in low levels of phospholipid is consistent with the rapid acceleration of venom effects in *Pseudonaja* envenomations, in which non-anaphylactic sudden collapse occurs as the venom spreads through the blood stream (Sutherland and Tibballs, 2001). The more rapid onset of these effects in *Pseudonaja* envenomations than in *Oxyuranus* envenomations may reflect the fact that factor V in *P. textilis* (and possibly other *Pseudonaja* venoms) can function not only via membrane binding (like endogenous factors and *Oxyuranus* venoms) (Bos et al., 2009; Gilbert et al., 2012) but also via alternate pathways that do not require membrane binding. In prey items with much smaller blood volumes than in humans, the formation of stroke-inducing blood clots by low-phospholipid-dependent coagulotoxins would accelerate after localised depletion of phospholipid, yielding a selective advantage for this venom trait. Moreover, determining the mechanism of this accelerated venom action in the absence of phospholipid has immediate implications for understanding the pathology of produced in

sudden coagulopathy-induced collapse and death in humans. Since circulating levels of phospholipid are usually low in the absence of systemic platelet activation, the ability to work in a low phospholipid environment would be evolutionarily advantageous. Similarly, eliminating one more step in the biochemical coagulation cascade would speed up the reaction and subsequent venom effects.

4.3 Antivenom efficacy

Antivenom is the most effective and only specific treatment for snakebite (Maduwage and Isbister, 2014; Winkel et al., 2006) and thus critical for minimising morbidity and mortality in victims, but it can be expensive leading to poor availability across many high-risk regions (Harrison et al., 2009). The snakebite mortality rate in Papua New Guinea is about 100-fold higher than in Australia, with *O. scutellatus* being the leading reported cause (Lalloo et al., 1995a). The ICP Taipan antivenom used in this study is a newly developed product which has financial advantages over the CSL taipan antivenom and may help to improve antivenom accessibility when it goes into production (Herrera et al., 2014).

It has been suggested that CSL antivenom may be less effective in neutralising *O. scutellatus* venom (from PNG) than venom from the Australian nominal subspecies (Vargas et al., 2011). Likewise, stronger immunorecognition of *O. scutellatus* venom from Australia was recognised for CSL Taipan antivenom compared to ICP Taipan antivenom (Herrera et al., 2014). In contrast, we found that the ICP Taipan antivenom performed equally well against Australian *O. scutellatus* and Saibai Island *O. scutellatus* (Figure 5; Table 1). This cross-reactivity of taipan antivenom against both Australian and PNG *O. scutellatus* populations is consistent with previous studies (Crachi et al., 1999; Herrera et al., 2014; Vargas et al., 2011), which is unsurprising given the minimal genetic divergence between these populations (Doughty et al., 2007; Wüster et al., 2005). Similarly, the neutralisation of *O. microlepidotus* is consistent with its close genetic relationship to *O. scutellatus* (Wüster et al., 2005).

Our demonstration of the broad efficacy of ICP Taipan antivenom across all coagulotoxic members suggests that the ICP antivenom would be appropriate for treating coagulotoxic envenomations by any *Oxyuranus* species. This of course should be clinically validated, but the *in vitro* results in this study are extremely promising and may be of use to zoological collections and private exotic animal collections across the world which house these iconic snakes and require affordable, effective antivenom.

Despite a high similarity in toxin sequences between *Oxyuranus* and *Pseudonaja* venoms (93% for FXa subunits and 97% for FVa subunits), the *Pseudonaja* venoms were comparatively less neutralised by the ICP Taipan antivenom. These results are consistent

with previous studies showing poor cross-reactivity of antibodies against taipan venoms used against the venoms of brown snakes (Isbister et al., 2010a) and agree with the relative amounts of prothrombinase complex in these venoms (discussed further below) (Lavin and Masci, 2009; Rao and Kini, 2002). The most neutralised *Pseudonaja* species (*P. aspidorhyncha*) was still neutralised less than that of the least neutralised *Oxyuranus* species (*O. microlepidotus* from Boulia, QLD). Clearly, the small difference in toxin structure due to independent modification of the surface chemistry confers important structure-function immunological relationships of these venoms and likely has major implications for the clinical options in managing *Pseudonaja* bites (particularly *P. textilis*, as this species is also found in PNG).

One explanation for the difference in neutralisation capacity by antivenom between these venoms would be varying venom potency, whereby a more potent procoagulant venom would be less well neutralised than a less potent procoagulant venom simply because of stoichiometric differences, in that venoms with lower relative levels of procoagulant toxins would be better neutralised by the same antivenom level than venoms with higher relative toxin levels. However, our examination of the well-neutralised *Oxyuranus* clade revealed no correlation between antivenom efficacy and clotting time (PGLS: $df = 1$, $t = -0.0670$, $p = 0.9492$).

Hererra et al. (2012) found the coagulotoxicity of the PNG *O. scutellatus* to be greater than the Australian *O. scutellatus* population (and the latter was thus better neutralised by ICP antivenom in that study), but with a larger dataset there was no direct correlation between clotting time and antivenom efficacy. In addition to the larger *Oxyuranus* dataset in this study, Hererra et al. (2012) and others (Vargas et al., 2011) used a manual observation method (MCD: Minimal Coagulant Dose (Theakston and Reid, 1983)) to determine clotting time 'by eye' which resulted in large error bars, whereas this study used a robot-operated machine to measure clotting times, which resulted in remarkably small error bars (smaller than the line symbols in most cases). In addition, the MCD tests assume a linearity between data points when estimating the 60 second clotting dose. However, the clotting time relative to venom concentration varies in a curved rather than linear manner. Thus, our newly developed protocol affords a more accurate determination of the dose and the response and has been successfully used by us for other venoms (Debono et al., 2017; Lister et al., 2017; Oulion et al., 2018; Rogalski et al., 2017).

The lower cost of the ICP Taipan antivenom (less than 25% of that of the Seqirus Taipan antivenom) renders it very appealing and should substantially improve the availability of safe, clinically effective antivenom for treating taipan envenoming in PNG. While it would be advantageous to use taipan antivenom to treat envenomation by

Pseudonaja, particularly because no monovalent antivenom for *Pseudonaja* species is stocked in PNG, our results indicate that taipan antivenom is not likely to effectively neutralise the coagulotoxins that cause the primary pathophysiology in *Pseudonaja* envenomations. While strongly positive results such as those obtained here for the neutralisation of *Oxyuranus* venoms do not necessarily translate directly to the clinical setting, the corresponding lack of efficacy against *Pseudonaja* venoms suggests that there would also be lack of clinical efficacy, even at very high antivenom doses.

4.4 Factor Xa substrate activity

We observed a significant correlation between clotting times and FXa activity (Fig. 6). The differences in the order of potency between these two parameters may be explained by the experimental set-up, as the clotting assays were conducted on whole plasma, while Factor Xa activity was assessed in an artificial construct acting as a proxy for the clotting system. The substrate tests did not use native proteins—the substrates are artificial—and also did not incorporate the various thrombin-mediated feedback loops that characterise endogenous fibrin clot formation; consequently, the results must be interpreted with caution, as they are far from reflecting the biological reality. This illustrates the importance of bioactivity tests using either plasma or at least native proteins such as purpose-built clotting reactions.

4.5 Study limitations

There are several potential limitations to this study. Firstly, given this study investigated only procoagulant effects, the antivenom efficacy seen here cannot be extended to venom effects such as neurotoxicity and myotoxicity that were not assessed. Therefore, considerations regarding the broader clinical usefulness of the ICP antivenom for all coagulotoxic *Oxyuranus* envenomations must consider this caveat. Secondly, while we included a diverse range of venoms, covering the entire clade, the number of individual replicates per locality was limited due to the danger, difficulty, and expense in acquiring such an extensive venom collection. In an attempt to overcome this limitation, multiple individuals per species were included where possible to account for intraspecific venom variation (Chippaux et al., 1991). Table 1 shows that there ere no substantial differences between individuals within a species. Overall, despite this sampling limitation, this work is the most diverse and comprehensive study ever undertaken for either genus.

A minor limitation of this study is that, while we rigorously investigated the relative dependence of snake venoms on calcium and phospholipid at two different venom concentrations, and while the plasma used in our assays was depleted of both cofactors,

there may have been trace amounts of either in the plasma, thus potentially slightly skewing the results. Future work investigating cofactor dependence of venoms should be undertaken in a plasma-free assay that utilises purified prothrombin and fibrinogen instead of plasma.

Lastly, being *in vitro*, our tests do not simulate the complex pathophysiology that occurs when snake venoms interact with multiple systems in the human body and thus could under- or over-estimate precise antivenom efficacy. However, *relative* antivenom efficacy across species reported here is still likely to be clinically informative. In particular, if an antivenom does not perform well in such ideal conditions as our functional assays, it is unlikely that it will perform well in a complex circulatory system with a much greater plasma-to-antivenom ratio. Thus, it is reasonable to expect that the ICP antivenom would perform poorly against *Pseudonaja* envenomations in a clinical setting but may offer a genus-wide option for *Oxyuranus* envenomations, at least in the context of coagulopathy.

5. *Conclusion*

Multiple conclusions can be made from this large dataset. This study found that cofactor dependence is far more variable (particularly in *Pseudonaja*) than previously considered, with some venoms accelerating in the absence of phospholipid (ie. exhibiting negative phospholipid-dependence values) at high venom concentrations, which would be expected in prey items. While the precise mechanisms and evolutionary origins of this variation remain enigmatic, and elucidating them is beyond the scope of this study, the authenticity of this result is supported by the phylogenetic signal whereby all three species within a single clade of *Pseudonaja* did not exhibit this effect and instead exhibited the secondary reversal to the ancestral state of greater phospholipid-dependence.

Furthermore, our results indicate that, while ICP Taipan antivenom was developed using *O. scutellatus* venoms from PNG, it effectively neutralises the coagulotoxins of all Australian *Oxyuranus* venoms. As such, this antivenom may be suitable for treating envenomation by *Oxyuranus* in Australia or in zoological or private collections overseas, with an important caveat that neurotoxicity and myotoxicity were not tested here and also that clinical trials are ideal first for confirmation.

Our results suggest that this new ICP Taipan antivenom (and probably the CSL Taipan antivenom as well) is unlikely to be suitable for treating *Pseudonaja* envenomations without requiring very large antivenom doses to achieve even moderate neutralisation of the best neutralised species. Thus, *P. textilis* bites in PNG, which are of unknown frequency, remain without a readily available and affordable treatment. However, the intriguing parallels between cofactor dependence and antivenom efficacy demonstrated by

the *Pseudonaja* clade of *P. affinis, P. aspidorhyncha* and *P. inframacula* point the way for next-generation antivenom research efforts involving rational design of epitope targeting.

Future work should ensure the inclusion of cofactors calcium and phospholipid in bioactivity tests of venoms to avoid under– or over–estimating venom potency levels. In addition, the strength of the resulting clots formed by *Oxyuranus* and *Pseudonaja* venoms should be assessed by thromboelastography to determine if weak or strong clots are formed. These future studies will provide a better understanding of the selection pressures operating upon these venoms as well as the clinical effects by these extremely medically important snakes.

The results of this study are of clinical importance for understanding the relative coagulotoxicity across all species of *Oxyuranus* and *Pseudonaja*. The also provide a platform for future research investigate the efficacy of different antivenoms and unveil the structure-function relationships of the potent FXa:FVa prothrombinase complex across these venoms.

Acknowledgments: CNZ, BODB, and JD were recipients of PhD scholarships administered by the University of Queensland. BGF was funded by a University of Queensland Infrastructure and Equipment grant and Australian Research Council Discovery Project DP190100304. We are grateful for the comments from two anonymous reviewers who improved this manuscript.

Author Contributions: B.G.F conceived and designed the experiments; C.N.Z. performed the experiments; C.N.Z., B.G.F., and K.A. analysed the data; C.H., J.D., L.A., N.D., T.M., M.H., J.G, and D.W. contributed reagents and materials; C.N.Z. B.G.F, C.H., K.A., B.O.D.B., M.B., T.N.W.J., J.G, and D.W. wrote the paper.

Conflicts of Interest: María Herrera and José M. Gutiérrez work at Instituto Clodomiro Picado, where the antivenom tested in this study was manufactured.

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Figure 1. Ancestral state reconstruction of clotting times (sec) (left side) compared area under the clotting curves (AUC) (right side) of venoms, where warmer colours represent faster clotting times. Maximal clotting time was shown to be a strong predictor of clotting AUC (PGLS $df = 1$, $t = 11.4880$, $p = 1.1 \times 10^{-11}$). Bars indicate 95% confidence intervals for the estimate at each node. Note: due to the high dynamicity of venom evolution, the ranges quickly become broad as one moves down the tree. Lineages in grey are non-coagulotoxic. ND = Not detectable due to non-procoagulant venom.

Figure 2. Ancestral state reconstruction of clotting times (sec) at 20 μg/mL maximal concentration tested (left side) compared to calcium dependence (the shift in clotting time between conditions in the presence vs. absence of calcium) (right side) of venoms, where warmer colours represent faster clotting times or higher dependence on calcium. Calcium dependence showed very strong correlation with clotting time (PGLS $df = 1$, $t = 4.3042$, $p = 2.11 \times 10^{-04}$) and was a good predictor of the latter parameter. Bars indicate 95% confidence intervals for the estimate at each node. Note: due to the high dynamicity of venom evolution, the ranges quickly become broad as one moves down the tree. Lineages in grey are non-coagulotoxic. ND = Not detectable due to non-procoagulant venom. A value of zero would indicate no change with or without calcium.

Figure 3. Ancestral state reconstruction of clotting times (sec) at 20 μg/mL maximal concentration tested (left side) compared to phospholipid dependence (right side) (the shift in clotting time between conditions in the presence vs. absence of phospholipid) of venoms, where warmer colours represent faster clotting times or higher dependence on phospholipid. Phospholipid dependence showed a significant correlation with clotting time (PGLS $df = 1$, $t = 1.9902$, $p = 0.05718$) and was a good predictor of the latter parameter. Error bars at the nodes indicate 95% confidence intervals for the estimate at each node. Note: due to the high dynamicity of venom evolution, the ranges quickly become broad as one moves down the tree. Lineages in grey are non-coagulotoxic. ND = Not detectable due to non-procoagulant venom. Negative values indicate that clotting times were quicker in the absence of phospholipid. A value of zero would indicate no change with or without phospholipid.

Figure 4. Ancestral state reconstruction of calcium dependence (left side) (the shift in clotting time between conditions in the presence vs. absence of calcium) compared to phospholipid dependence (right side) (the shift in clotting time between conditions in the presence vs. absence of phospholipid) of venoms, where warmer colours represent less dependence on the cofactor. Calcium dependence showed an extremely strong correlation with phospholipid dependence (PGLS $df = 1$, $t = 7.7003$, $p = 3.592 \times 10^{-08}$) and was a good predictor of the latter parameter. Error bars at the nodes indicate 95% confidence intervals for the estimate at each node. Note: due to the high dynamicity of venom evolution, the ranges quickly become broad as one moves down the tree. Lineages in grey are non-

coagulotoxic. ND = Not detectable due to non-procoagulant venom. Negative values indicate that clotting times were quicker in the absence of phospholipid.

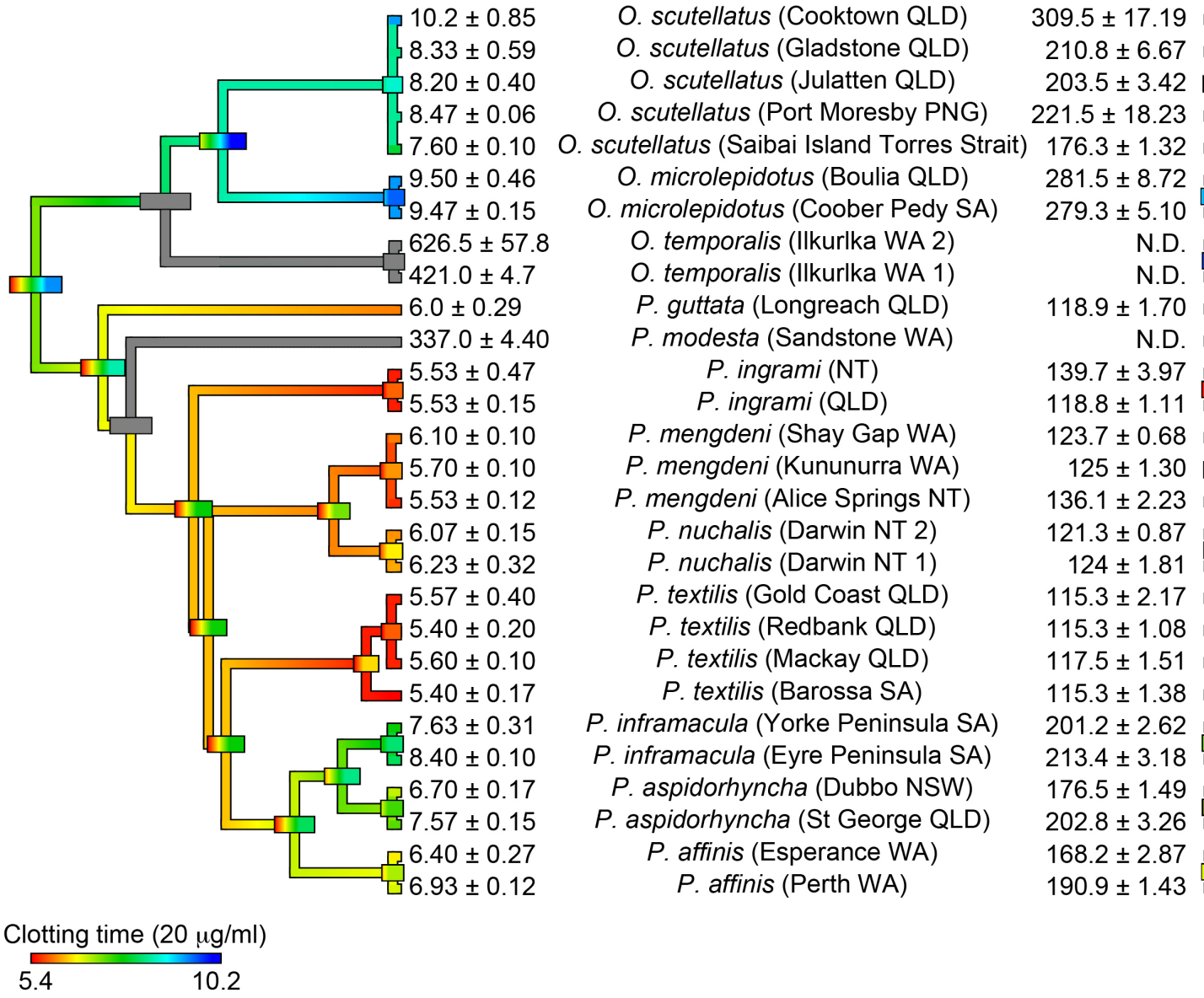
Figure 5. Ancestral state reconstruction of antivenom efficacy as determined by (AUC for runs with venom preincubated with antivenom)/(AUC with venom only), where warmer colours represent greater neutralisation of venoms by the antivenom. The selectivity of Taipan ICP antivenom for *Oxyuranus* venoms compared to *Pseudonaja* venoms was highly significant (PGLS $df = 1$, $t = 4.9364$, $p = 3.974 \times 10^{-5}$). Error bars at the nodes indicate 95% confidence intervals for the estimate at each node. Note: due to the high dynamicity of venom evolution, the ranges quickly become broad as one moves down the tree. Lineages in grey are non-coagulotoxic. ND = Not detectable due to non-procoagulant venom.

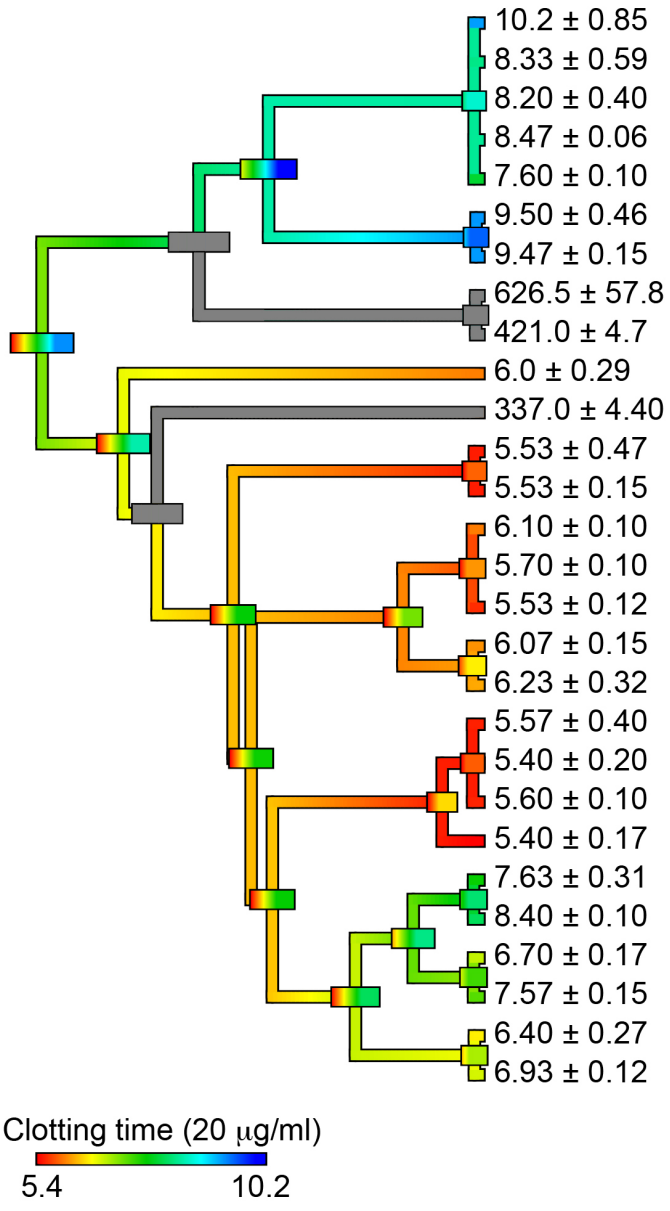
Figure 6. Ancestral state reconstruction of clotting times (s) (left side) compared to substrate cleavage (right side) of venoms, where warmer colours represent faster clotting times or higher substrate cleavage activity (FXa activity). Factor Xa activity was found to have only a moderately non-significant correlation as a predictor of clotting time (PGLS $df = 1$, $t = -1.9682$, $p = 0.0598$) (note: the negative t-value is due to the two variables being inversely related). Error bars at the nodes indicate 95% confidence intervals for the estimate at each node. Lineages in grey are non-coagulotoxic. ND = Not detectable due to non-procoagulant venom. Note: due to the high dynamicity of venom evolution, the ranges quickly become broad as one moves down the tree.

Table 1. Mean clotting times, antivenom efficacy, and calcium and phospholipid dependence (presented by mean ± SD), at 20 μg/mL venom concentration of *Oxyuranus* and *Pseudonaja* venoms

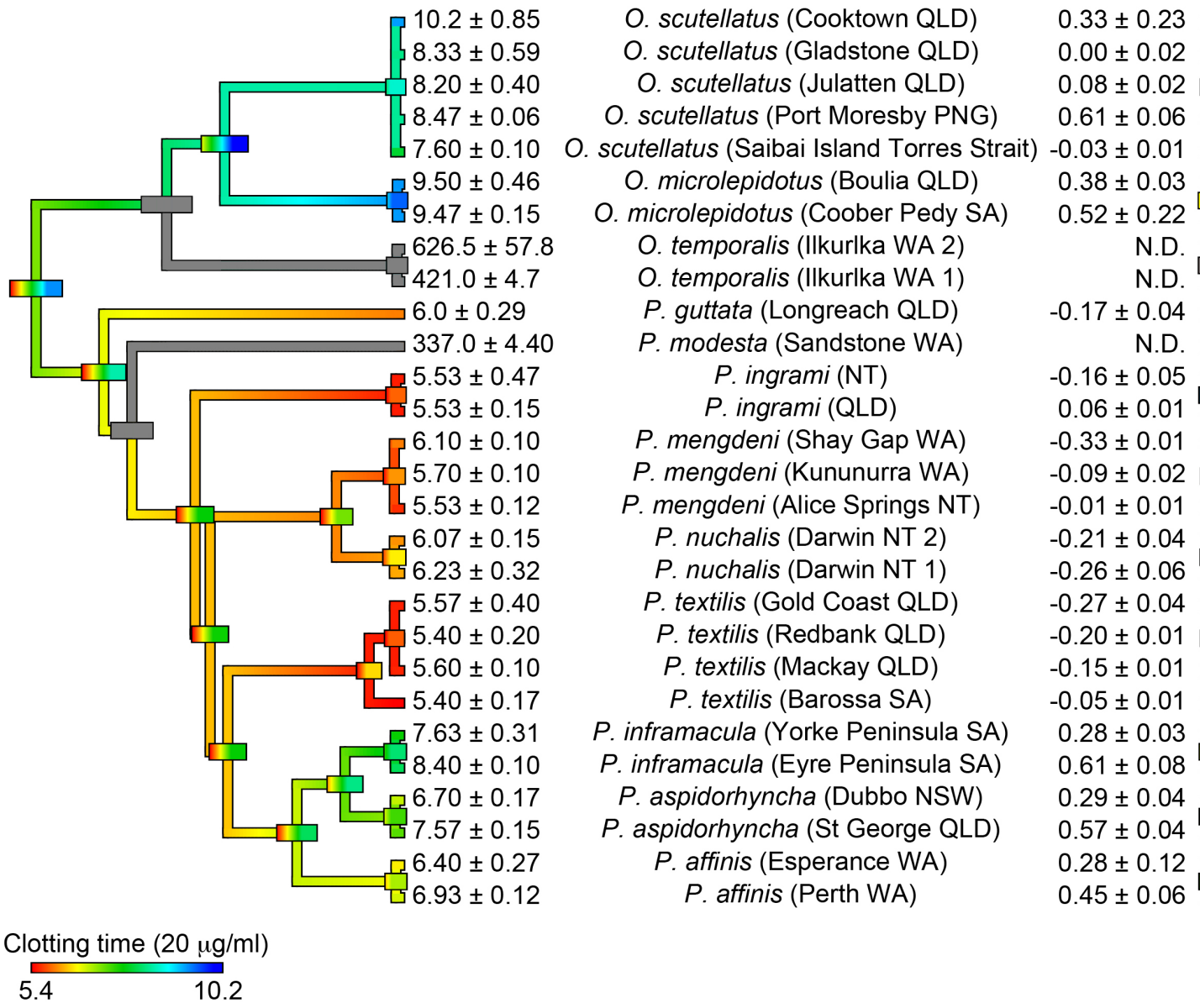
Species (locality), sex	**Clotting time (s)**	**AV efficacy***	**Calcium dependence****	**Phospholipid dependence ****
O. microlepidotus (Boulia, QLD), m	9.5 ± 0.46	1.21 ± 0.01	3.61 ± 0.11	0.38 ± 0.03
O. microlepidotus (Coober Pedy, SA), unk.	9.47 ± 0.15	1.84 ± 0.05	3.8 ± 0.54	0.52 ± 0.22
O. scutellatus (Port Moresby, PNG), unk.	8.47 ± 0.06	1.82 ± 0.07	3.56 ± 0.39	0.61 ± 0.06
O. scutellatus (Saibai Island, PNG), unk.	7.6 ± 0.1	1.87 ± 0.04	1.53 ± 0.26	-0.03 ± 0.01
O. scutellatus (Cooktown, QLD), f	10.2 ± 0.85	2.22 ± 0.04	2.79 ± 0.05	0.33 ± 0.23
O. scutellatus (Gladstone, QLD), m	8.33 ± 0.59	1.84 ± 0.01	2.25 ± 0.21	0 ± 0.02
O. scutellatus (Julatten, QLD), m	8.2 ± 0.4	2.28 ± 0.24	2.25 ± 0.17	0.08 ± 0.02
O. temporalis (Ilkurka, WA), f	421.0 ± 4.7	—	—	—
O. temporalis (Ilkurka, WA 2), m	626.5 ± 57.8	—	—	—
P. affinis (Esperance, WA), m	6.4 ± 0.27	0.93 ± 0.02	2.17 ± 0.39	0.28 ± 0.12
P. affinis (Perth, WA), unk.	6.93 ± 0.12	0.85 ± 0.01	2.62 ± 0.11	0.45 ± 0.06
P. aspidorhyncha (Dubbo, NSW), m	6.7 ± 0.17	0.89 ± 0.09	1.13 ± 0.08	0.29 ± 0.04
P. aspidorhyncha (St. George, QLD), unk.	7.57 ± 0.15	0.83 ± 0.01	3.03 ± 0.11	0.57 ± 0.04
P. guttata (Longreach, QLD), pooled	6.0 ± 0.29	0.42 ± 0.01	0.45 ± 0.07	-0.17 ± 0.04
P. inframacula (Eyre Peninsula, SA), unk.	8.4 ± 0.1	0.86 ± 0.01	2.76 ± 0.2	0.61 ± 0.08
P. inframacula (York Peninsula, SA), unk.	7.63 ± 0.31	0.88 ± 0.14	2.53 ± 0.24	0.28 ± 0.03
P. ingrami (NT), unk.	5.53 ± 0.47	0.58 ± 0.02	1.88 ± 0.53	-0.16 ± 0.05
P. ingrami (QLD), unk.	5.53 ± 0.15	0.81 ± 0.03	1.65 ± 0.03	0.06 ± 0.01
P. mengdeni (Alice Springs, NT), m	5.53 ± 0.12	0.39 ± 0.12	0.96 ± 0.06	-0.01 ± 0.01
P. mengdeni (Kununurra, WA), m	5.7 ± 0.1	0.92 ± 0.00	1.04 ± 0.02	-0.09 ± 0.02
P. mengdeni (Shay Gap, WA), unk.	6.1 ± 0.1	0.09 ± 0.02	0.88 ± 0.09	-0.33 ± 0.01
P. modesta (Sandstone, WA), unk.	337.0 ± 4.4	—	—	—
P. nuchalis (Darwin, NT), m	6.23 ± 0.32	0.60 ± 0.00	0.24 ± 0.01	-0.26 ± 0.06
P. nuchalis (Darwin, NT 2), m	6.07 ± 0.15	0.63 ± 0.03	0.38 ± 0.03	-0.21 ± 0.04
P. textilis (Barossa, SA), unk.	5.4 ± 0.17	0.47 ± 0.00	0.7 ± 0.03	-0.05 ± 0.01
P. textilis (Gold Coast, QLD), unk.	5.57 ± 0.4	0.55 ± 0.00	0.35 ± 0.16	-0.27 ± 0.04
P. textilis (Mackay, QLD), unk.	5.6 ± 0.1	0.46 ± 0.01	0.56 ± 0.25	-0.15 ± 0.01
P. textilis (Redbank, QLD), unk.	5.4 ± 0.2	0.47 ± 0.01	0.49 ± 0.05	-0.2 ± 0.01

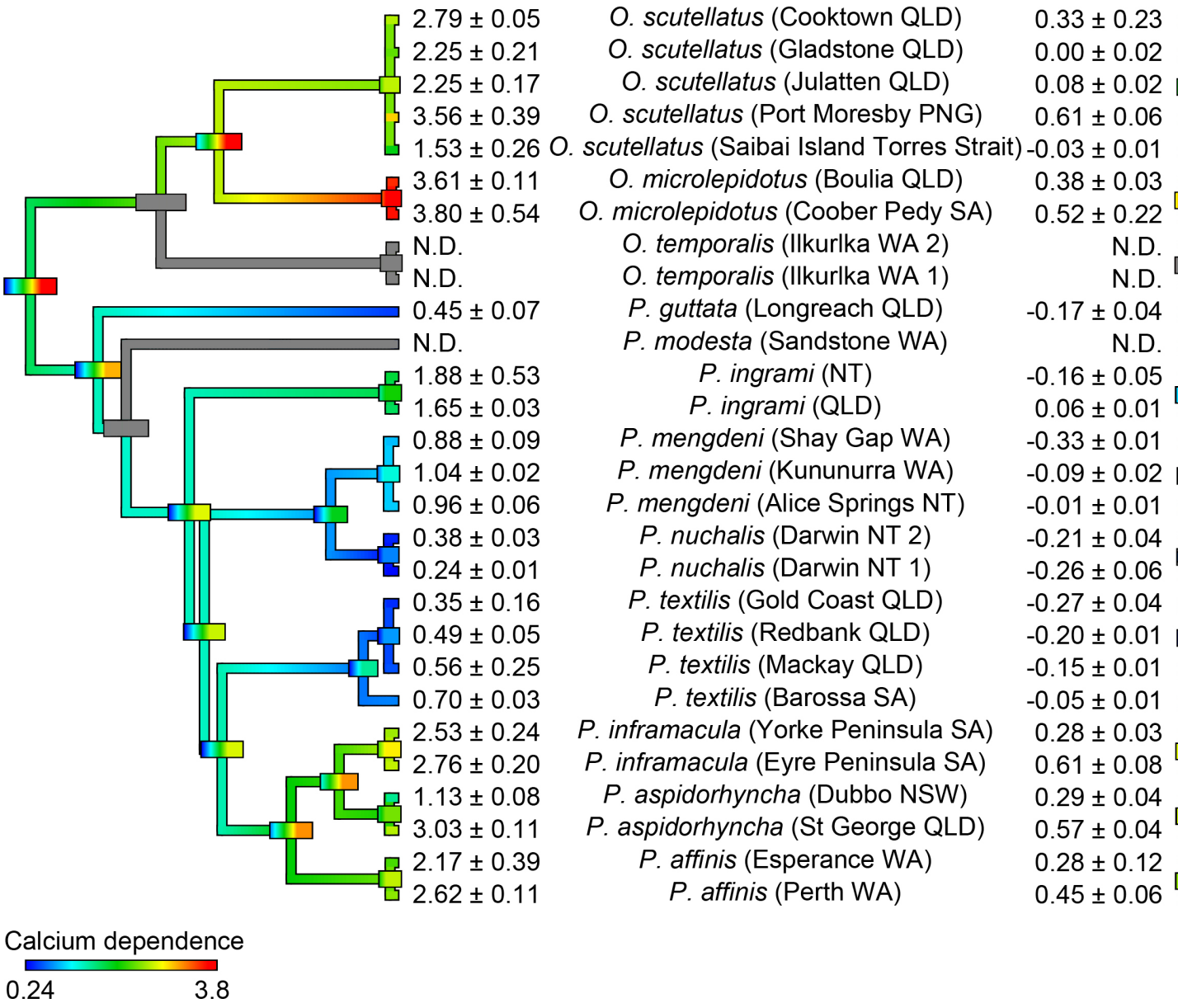
* Antivenom efficacy, as described in Materials and Methods section 2.6, was calculated using the relationship (AUC for venom + antivenom ÷ AUC for venom alone), then subtracted by 1 (so that no shift in AUC values (a value of 1 divided by 1) would have a value of 0 instead of 1). ** Calcium and phospholipid dependence are indicated as the x-fold shift in clotting time between conditions in the presence vs. absence of calcium or phospholipid, as described in Materials and Methods section 2.5. Abbreviations: O.= Oxyuranus; P.= Pseudonaja; unk. = unknown; f = female; m = male; NT = Northern Territory; QLD = Queensland; SA = South Australia; NSW = New South Wales; WA = Western Australia; — = not assessed because of non-procoagulant venom.

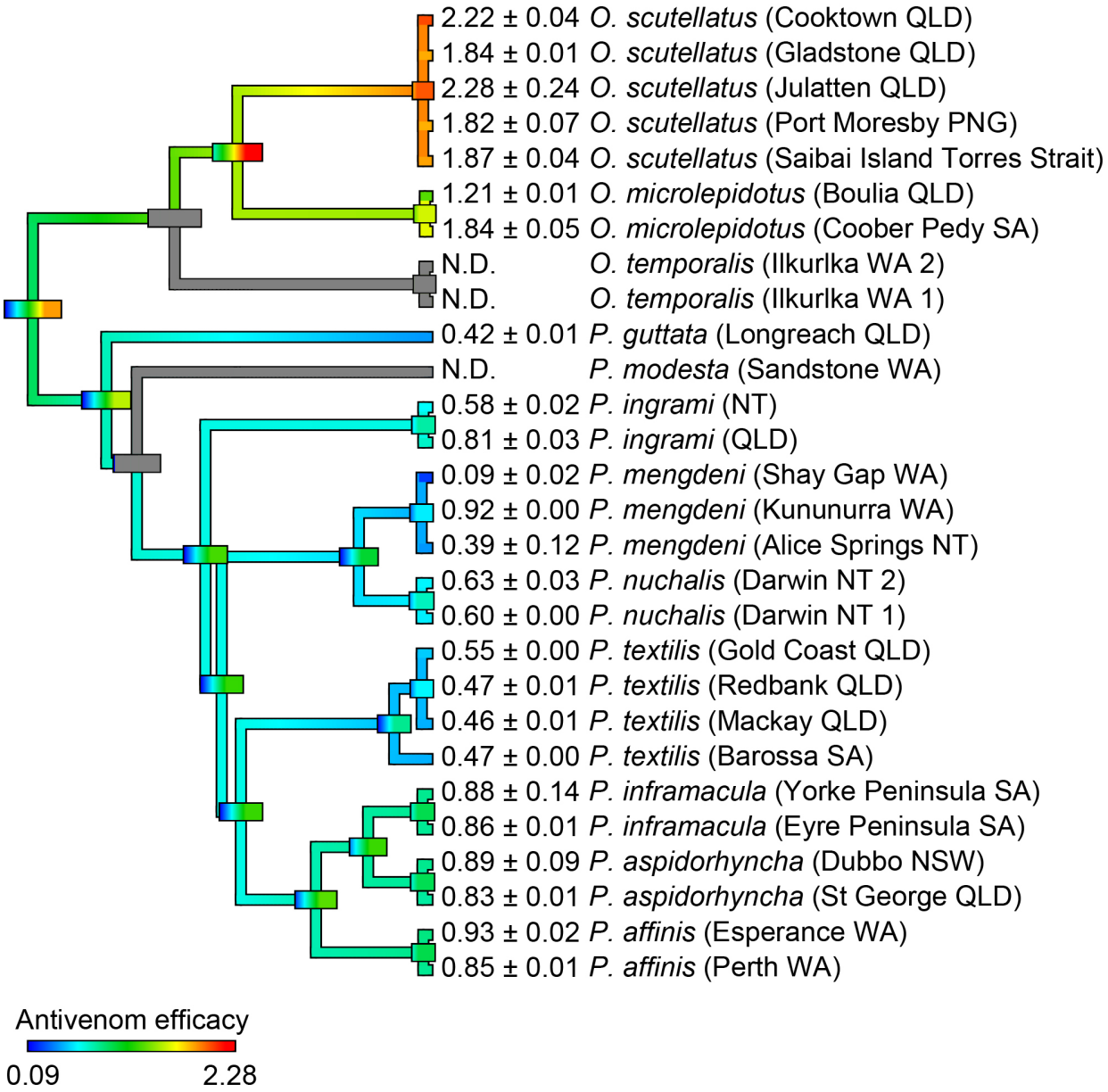

10.2 ± 0.85
8.33 ± 0.59
8.20 ± 0.40
8.47 ± 0.06
7.60 ± 0.10
9.50 ± 0.46
9.47 ± 0.15
626.5 ± 57.8
421.0 ± 4.7
6.0 ± 0.29
337.0 ± 4.40
5.53 ± 0.47
5.53 ± 0.15
6.10 ± 0.10
5.70 ± 0.10
5.53 ± 0.12
6.07 ± 0.15
6.23 ± 0.32
5.57 ± 0.40
5.40 ± 0.20
5.60 ± 0.10
5.40 ± 0.17
7.63 ± 0.31
8.40 ± 0.10
6.70 ± 0.17
7.57 ± 0.15
6.40 ± 0.27
6.93 ± 0.12
O. scutellatus (Cooktown QLD) 309.5 ± 17.19
O. scutellatus (Gladstone QLD) 210.8 ± 6.67
O. scutellatus (Julatten QLD) 203.5 ± 3.42
O. scutellatus (Port Moresby PNG) 221.5 ± 18.23
O. scutellatus (Saibai Island Torres Strait) 176.3 ± 1.32
O. microlepidotus (Boulia QLD) 281.5 ± 8.72
O. microlepidotus (Coober Pedy SA) 279.3 ± 5.10
O. temporalis (Ilkurlka WA 2) N.D.
O. temporalis (Ilkurlka WA 1) N.D.
P. guttata (Longreach QLD) 118.9 ± 1.70
P. modesta (Sandstone WA) N.D.
P. ingrami (NT) 139.7 ± 3.97
P. ingrami (QLD) 118.8 ± 1.11
P. mengdeni (Shay Gap WA) 123.7 ± 0.68
P. mengdeni (Kununurra WA) 125 ± 1.30
P. mengdeni (Alice Springs NT) 136.1 ± 2.23
P. nuchalis (Darwin NT 2) 121.3 ± 0.87
P. nuchalis (Darwin NT 1) 124 ± 1.81
P. textilis (Gold Coast QLD) 115.3 ± 2.17
P. textilis (Redbank QLD) 115.3 ± 1.08
P. textilis (Mackay QLD) 117.5 ± 1.51
P. textilis (Barossa SA) 115.3 ± 1.38
P. inframacula (Yorke Peninsula SA) 201.2 ± 2.62
P. inframacula (Eyre Peninsula SA) 213.4 ± 3.18
P. aspidorhyncha (Dubbo NSW) 176.5 ± 1.49
P. aspidorhyncha (St George QLD) 202.8 ± 3.26
P. affinis (Esperance WA) 168.2 ± 2.87
P. affinis (Perth WA) 190.9 ± 1.43
Clotting time (20 μg/ml)
5.4
10.2

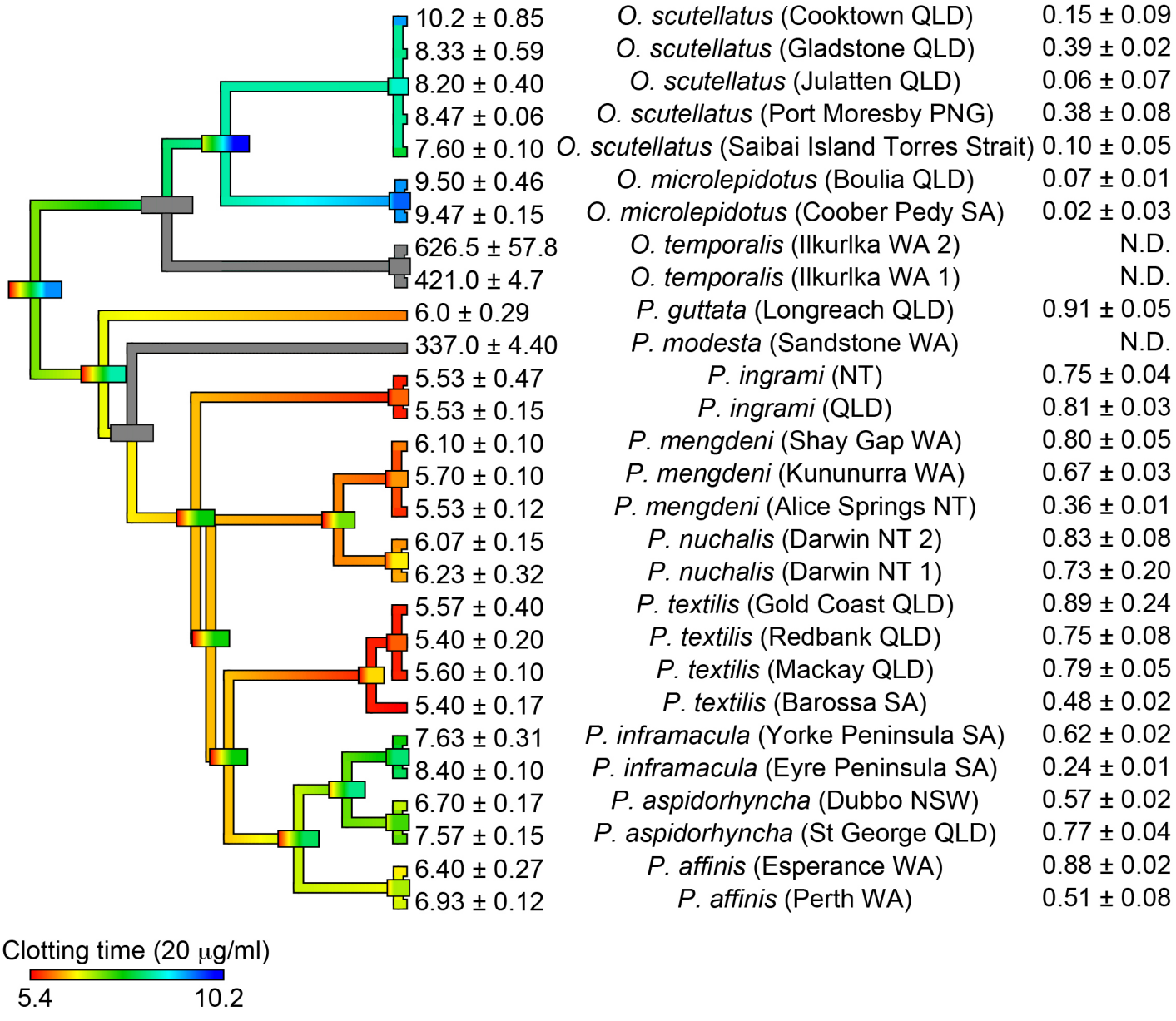


10.2 ± 0.85	*O. scutellatus* (Cooktown QLD)	2.79 ± 0.05
8.33 ± 0.59	*O. scutellatus* (Gladstone QLD)	2.25 ± 0.21
8.20 ± 0.40	*O. scutellatus* (Julatten QLD)	2.25 ± 0.17
8.47 ± 0.06	*O. scutellatus* (Port Moresby PNG)	3.56 ± 0.39
7.60 ± 0.10	*O. scutellatus* (Saibai Island Torres Strait)	1.53 ± 0.26
9.50 ± 0.46	*O. microlepidotus* (Boulia QLD)	3.61 ± 0.11
9.47 ± 0.15	*O. microlepidotus* (Coober Pedy SA)	3.80 ± 0.54
626.5 ± 57.8	*O. temporalis* (Ilkurlka WA 2)	N.D.
421.0 ± 4.7	*O. temporalis* (Ilkurlka WA 1)	N.D.
6.0 ± 0.29	*P. guttata* (Longreach QLD)	0.45 ± 0.07
337.0 ± 4.40	*P. modesta* (Sandstone WA)	N.D.
5.53 ± 0.47	*P. ingrami* (NT)	1.88 ± 0.53
5.53 ± 0.15	*P. ingrami* (QLD)	1.65 ± 0.03
6.10 ± 0.10	*P. mengdeni* (Shay Gap WA)	0.88 ± 0.09
5.70 ± 0.10	*P. mengdeni* (Kununurra WA)	1.04 ± 0.02
5.53 ± 0.12	*P. mengdeni* (Alice Springs NT)	0.96 ± 0.06
6.07 ± 0.15	*P. nuchalis* (Darwin NT 2)	0.38 ± 0.03
6.23 ± 0.32	*P. nuchalis* (Darwin NT 1)	0.24 ± 0.01
5.57 ± 0.40	*P. textilis* (Gold Coast QLD)	0.35 ± 0.16
5.40 ± 0.20	*P. textilis* (Redbank QLD)	0.49 ± 0.05
5.60 ± 0.10	*P. textilis* (Mackay QLD)	0.56 ± 0.25
5.40 ± 0.17	*P. textilis* (Barossa SA)	0.70 ± 0.03
7.63 ± 0.31	*P. inframacula* (Yorke Peninsula SA)	2.53 ± 0.24
8.40 ± 0.10	*P. inframacula* (Eyre Peninsula SA)	2.76 ± 0.20
6.70 ± 0.17	*P. aspidorhyncha* (Dubbo NSW)	1.13 ± 0.08
7.57 ± 0.15	*P. aspidorhyncha* (St George QLD)	3.03 ± 0.11
6.40 ± 0.27	*P. affinis* (Esperance WA)	2.17 ± 0.39
6.93 ± 0.12	*P. affinis* (Perth WA)	2.62 ± 0.11


10.2 ± 0.85
8.33 ± 0.59
8.20 ± 0.40
8.47 ± 0.06
7.60 ± 0.10
9.50 ± 0.46
9.47 ± 0.15
626.5 ± 57.8
421.0 ± 4.7
6.0 ± 0.29
337.0 ± 4.40
5.53 ± 0.47
5.53 ± 0.15
6.10 ± 0.10
5.70 ± 0.10
5.53 ± 0.12
6.07 ± 0.15
6.23 ± 0.32
5.57 ± 0.40
5.40 ± 0.20
5.60 ± 0.10
5.40 ± 0.17
7.63 ± 0.31
8.40 ± 0.10
6.70 ± 0.17
7.57 ± 0.15
6.40 ± 0.27
6.93 ± 0.12
O. scutellatus (Cooktown QLD)
O. scutellatus (Gladstone QLD)
O. scutellatus (Julatten QLD)
O. scutellatus (Port Moresby PNG)
O. scutellatus (Saibai Island Torres Strait)
O. microlepidotus (Boulia QLD)
O. microlepidotus (Coober Pedy SA)
O. temporalis (Ilkurlka WA 2)
O. temporalis (Ilkurlka WA 1)
P. guttata (Longreach QLD)
P. modesta (Sandstone WA)
P. ingrami (NT)
P. ingrami (QLD)
P. mengdeni (Shay Gap WA)
P. mengdeni (Kununurra WA)
P. mengdeni (Alice Springs NT)
P. nuchalis (Darwin NT 2)
P. nuchalis (Darwin NT 1)
P. textilis (Gold Coast QLD)
P. textilis (Redbank QLD)
P. textilis (Mackay QLD)
P. textilis (Barossa SA)
P. inframacula (Yorke Peninsula SA)
P. inframacula (Eyre Peninsula SA)
P. aspidorhyncha (Dubbo NSW)
P. aspidorhyncha (St George QLD)
P. affinis (Esperance WA)
P. affinis (Perth WA)
0.33 ± 0.23
0.00 ± 0.02
0.08 ± 0.02
0.61 ± 0.06
-0.03 ± 0.01
0.38 ± 0.03
0.52 ± 0.22
N.D.
N.D.
-0.17 ± 0.04
N.D.
-0.16 ± 0.05
0.06 ± 0.01
-0.33 ± 0.01
-0.09 ± 0.02
-0.01 ± 0.01
-0.21 ± 0.04
-0.26 ± 0.06
-0.27 ± 0.04
-0.20 ± 0.01
-0.15 ± 0.01
-0.05 ± 0.01
0.28 ± 0.03
0.61 ± 0.08
0.29 ± 0.04
0.57 ± 0.04
0.28 ± 0.12
0.45 ± 0.06
Clotting time (20 μg/ml)
5.4
10.2


2.79 ± 0.05
2.25 ± 0.21
2.25 ± 0.17
3.56 ± 0.39
1.53 ± 0.26
3.61 ± 0.11
3.80 ± 0.54
N.D.
N.D.
0.45 ± 0.07
N.D.
1.88 ± 0.53
1.65 ± 0.03
0.88 ± 0.09
1.04 ± 0.02
0.96 ± 0.06
0.38 ± 0.03
0.24 ± 0.01
0.35 ± 0.16
0.49 ± 0.05
0.56 ± 0.25
0.70 ± 0.03
2.53 ± 0.24
2.76 ± 0.20
1.13 ± 0.08
3.03 ± 0.11
2.17 ± 0.39
2.62 ± 0.11
O. scutellatus (Cooktown QLD)
O. scutellatus (Gladstone QLD)
O. scutellatus (Julatten QLD)
O. scutellatus (Port Moresby PNG)
O. scutellatus (Saibai Island Torres Strait)
O. microlepidotus (Boulia QLD)
O. microlepidotus (Coober Pedy SA)
O. temporalis (Ilkurlka WA 2)
O. temporalis (Ilkurlka WA 1)
P. guttata (Longreach QLD)
P. modesta (Sandstone WA)
P. ingrami (NT)
P. ingrami (QLD)
P. mengdeni (Shay Gap WA)
P. mengdeni (Kununurra WA)
P. mengdeni (Alice Springs NT)
P. nuchalis (Darwin NT 2)
P. nuchalis (Darwin NT 1)
P. textilis (Gold Coast QLD)
P. textilis (Redbank QLD)
P. textilis (Mackay QLD)
P. textilis (Barossa SA)
P. inframacula (Yorke Peninsula SA)
P. inframacula (Eyre Peninsula SA)
P. aspidorhyncha (Dubbo NSW)
P. aspidorhyncha (St George QLD)
P. affinis (Esperance WA)
P. affinis (Perth WA)
0.33 ± 0.23
0.00 ± 0.02
0.08 ± 0.02
0.61 ± 0.06
-0.03 ± 0.01
0.38 ± 0.03
0.52 ± 0.22
N.D.
N.D.
-0.17 ± 0.04
N.D.
-0.16 ± 0.05
0.06 ± 0.01
-0.33 ± 0.01
-0.09 ± 0.02
-0.01 ± 0.01
-0.21 ± 0.04
-0.26 ± 0.06
-0.27 ± 0.04
-0.20 ± 0.01
-0.15 ± 0.01
-0.05 ± 0.01
0.28 ± 0.03
0.61 ± 0.08
0.29 ± 0.04
0.57 ± 0.04
0.28 ± 0.12
0.45 ± 0.06
Calcium dependence
0.24
3.8


2.22 ± 0.04 O. scutellatus (Cooktown QLD)
1.84 ± 0.01 O. scutellatus (Gladstone QLD)
2.28 ± 0.24 O. scutellatus (Julatten QLD)
1.82 ± 0.07 O. scutellatus (Port Moresby PNG)
1.87 ± 0.04 O. scutellatus (Saibai Island Torres Strait)
1.21 ± 0.01 O. microlepidotus (Boulia QLD)
1.84 ± 0.05 O. microlepidotus (Coober Pedy SA)
N.D. O. temporalis (Ilkurlka WA 2)
N.D. O. temporalis (Ilkurlka WA 1)
0.42 ± 0.01 P. guttata (Longreach QLD)
N.D. P. modesta (Sandstone WA)
0.58 ± 0.02 P. ingrami (NT)
0.81 ± 0.03 P. ingrami (QLD)
0.09 ± 0.02 P. mengdeni (Shay Gap WA)
0.92 ± 0.00 P. mengdeni (Kununurra WA)
0.39 ± 0.12 P. mengdeni (Alice Springs NT)
0.63 ± 0.03 P. nuchalis (Darwin NT 2)
0.60 ± 0.00 P. nuchalis (Darwin NT 1)
0.55 ± 0.00 P. textilis (Gold Coast QLD)
0.47 ± 0.01 P. textilis (Redbank QLD)
0.46 ± 0.01 P. textilis (Mackay QLD)
0.47 ± 0.00 P. textilis (Barossa SA)
0.88 ± 0.14 P. inframacula (Yorke Peninsula SA)
0.86 ± 0.01 P. inframacula (Eyre Peninsula SA)
0.89 ± 0.09 P. aspidorhyncha (Dubbo NSW)
0.83 ± 0.01 P. aspidorhyncha (St George QLD)
0.93 ± 0.02 P. affinis (Esperance WA)
0.85 ± 0.01 P. affinis (Perth WA)
Antivenom efficacy
0.09
2.28


10.2 ± 0.85
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421.0 ± 4.7
6.0 ± 0.29
337.0 ± 4.40
5.53 ± 0.47
5.53 ± 0.15
6.10 ± 0.10
5.70 ± 0.10
5.53 ± 0.12
6.07 ± 0.15
6.23 ± 0.32
5.57 ± 0.40
5.40 ± 0.20
5.60 ± 0.10
5.40 ± 0.17
7.63 ± 0.31
8.40 ± 0.10
6.70 ± 0.17
7.57 ± 0.15
6.40 ± 0.27
6.93 ± 0.12
O. scutellatus (Cooktown QLD) 0.15 ± 0.09
O. scutellatus (Gladstone QLD) 0.39 ± 0.02
O. scutellatus (Julatten QLD) 0.06 ± 0.07
O. scutellatus (Port Moresby PNG) 0.38 ± 0.08
O. scutellatus (Saibai Island Torres Strait) 0.10 ± 0.05
O. microlepidotus (Boulia QLD) 0.07 ± 0.01
O. microlepidotus (Coober Pedy SA) 0.02 ± 0.03
O. temporalis (Ilkurlka WA 2) N.D.
O. temporalis (Ilkurlka WA 1) N.D.
P. guttata (Longreach QLD) 0.91 ± 0.05
P. modesta (Sandstone WA) N.D.
P. ingrami (NT) 0.75 ± 0.04
P. ingrami (QLD) 0.81 ± 0.03
P. mengdeni (Shay Gap WA) 0.80 ± 0.05
P. mengdeni (Kununurra WA) 0.67 ± 0.03
P. mengdeni (Alice Springs NT) 0.36 ± 0.01
P. nuchalis (Darwin NT 2) 0.83 ± 0.08
P. nuchalis (Darwin NT 1) 0.73 ± 0.20
P. textilis (Gold Coast QLD) 0.89 ± 0.24
P. textilis (Redbank QLD) 0.75 ± 0.08
P. textilis (Mackay QLD) 0.79 ± 0.05
P. textilis (Barossa SA) 0.48 ± 0.02
P. inframacula (Yorke Peninsula SA) 0.62 ± 0.02
P. inframacula (Eyre Peninsula SA) 0.24 ± 0.01
P. aspidorhyncha (Dubbo NSW) 0.57 ± 0.02
P. aspidorhyncha (St George QLD) 0.77 ± 0.04
P. affinis (Esperance WA) 0.88 ± 0.02
P. affinis (Perth WA) 0.51 ± 0.08
Clotting time (20 μg/ml)
5.4
10.2

Supplementary figures

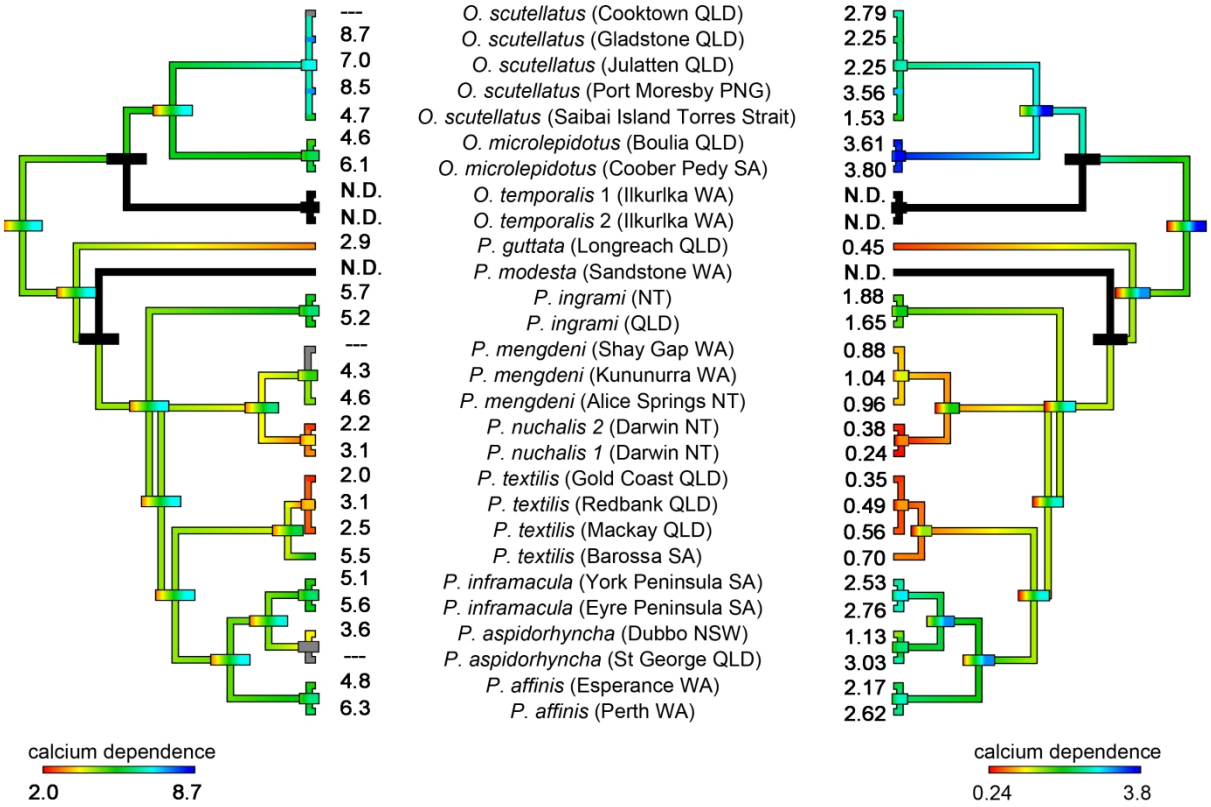

Figure S1. Ancestral state reconstruction of calcium dependency at 5ug/mL (left side) compared to 20ug/mL (right side) venom concentration. The shift in clotting time between conditions in the presence vs. absence of calcium is represented by warmer colours for lower dependence on calcium. Error bars at the nodes indicate 95% confidence intervals for the estimate at each node. Note: due to the high dynamicity of venom evolution, the ranges quickly become broad as one moves down the tree. Lineages in black are non-coagulotoxic. ND=Not Detectable, with clotting times >999 sec. Lineages in grey were not assessed due to lack of venom. Numbers at tips are calcium dependency means at the respective venom concentrations.

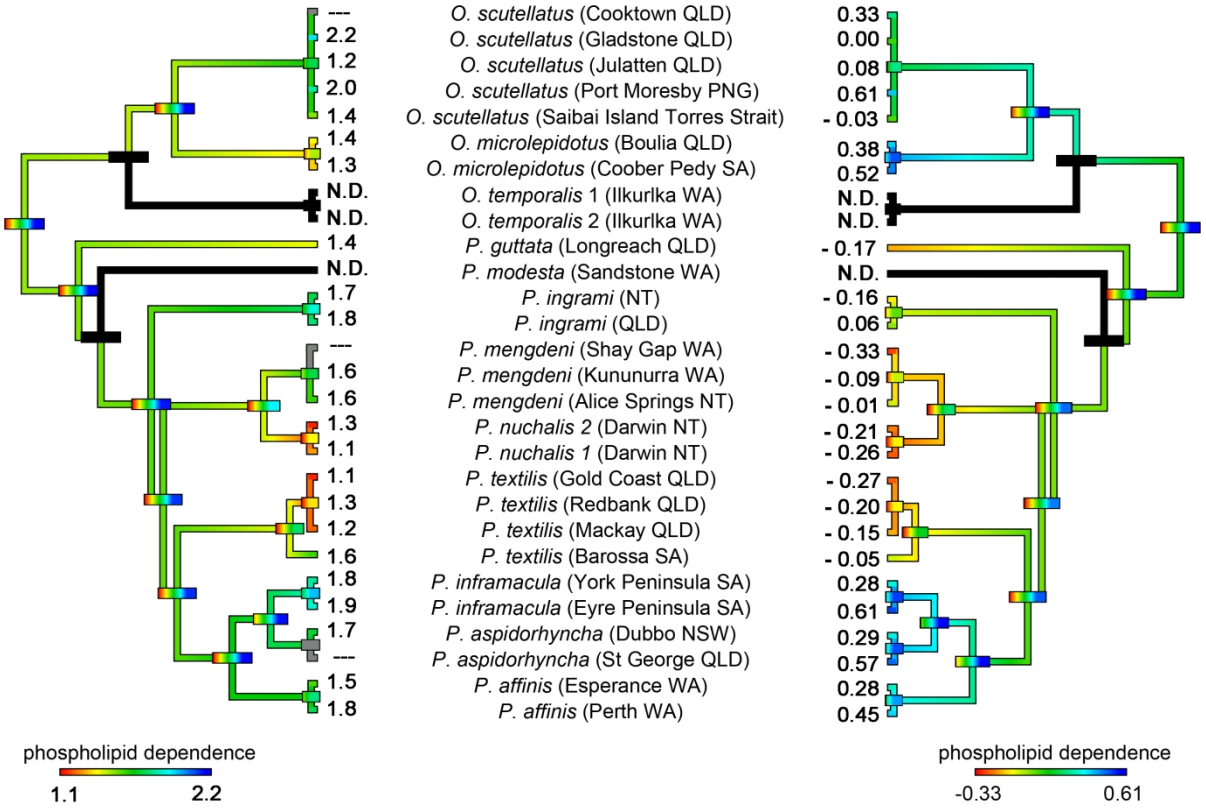

Figure S2. Ancestral state reconstruction of phospholipid dependence at 5ug/mL (left side) compared to 20ug/mL (right side) venom concentration. The shift in clotting time between conditions in the presence vs. absence of phospholipid is represented by warmer colours for lower dependence on phospholipid. Error bars at the nodes indicate 95% confidence intervals for the estimate at each node. Note: due to the high dynamicity of venom evolution, the ranges quickly become broad as one moves down the tree. Lineages in black are non-coagulotoxic. ND=Not Detectable, with clotting times >999 sec. Lineages in grey were not assessed due to lack of venom. Numbers at tips are phospholipid dependency means at the respective venom concentrations. Negative values indicate that clotting times were quicker in the absence of phospholipid.