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Mud in the blood: Novel potent anticoagulant coagulotoxicity in the venoms of the Australian elapid snake genus *Denisonia* (mud adders) and relative antivenom efficacy

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ABSTRACT

Due to their potent coagulotoxicity, Australian elapid venoms are unique relative to non-Australian members of the Elapidae snake family. The majority of Australian elapids possess potent procoagulant venom, while only a few species have been identified as possessing anticoagulant venoms. The majority of research to-date has concentrated on large species with range distributions overlapping major city centres, such as brown snakes (*Pseudonaja* spp.) and taipans (*Oxyuranus* spp.). We investigated the venom from the poorly studied genus *Denisonia* and documented anticoagulant activities that were differentially potent on amphibian, avian, and human plasmas. Both species were potently anticoagulant upon amphibian plasma, consistent with these snakes preying upon frogs as their primary food source. While *D. devisi* was only relatively weakly active on avian and human plasma, *D. maculata* was potently anticoagulant to amphibian, avian, and human plasma. The mechanism of anticoagulant action was determined to be the inhibition of prothrombin activation by Factor Xa by blocking the formation of the prothrombinase complex. Fractionation of *D. maculata* venom followed by MS sequencing revealed that the toxins responsible were Group I phospholipase A_2. As no antivenom is produced for this species or its near relatives, we examined the ability of Seqirus Australian snake polyvalent antivenom to neutralise the anticoagulant effects, with this antivenom shown to be effective. These results contribute to the body of knowledge regarding adaptive evolution of venom, revealing a unique taxon-specific anticoagulant effect for *D. devisi venom*. These results also reveal the potential effects and mechanisms behind envenomation by the potently acting *D. maculata* venom on human plasma, while the discovery of the efficacy of an available antivenom provides information crucial to the design of snakebite management strategies.

1. Introduction

Australia is inhabited by more venomous than non-venomous species of snakes (Wilson and Swan, 2013). While the high abundance of venomous snakes present in Australia has led to vigorous research into Australian snake venoms, past research has primarily focused on species from a select few of the most medically important and commonly encountered genera: *Pseudonaja* (brown snakes), *Notechis* (tiger snakes), *Pseudechis* (black snakes), *Acanthophis* (death adders), and *Oxyuranus* (taipans). Thus, despite the wide diversity of ecologically unique species present in Australia, due to their infrequent contact with humans most have been understudied and therefore data deficient in regards to potential clinical effects. However, as urban sprawl continues and new housing states are established in rural areas, these species may be encountered more frequently.

The most common toxin classes within Australian elapid snake venoms are: coagulotoxins, including anticoagulants which inhibit platelet aggregation (phospholipase A_2s) and procoagulants which generate endogenous thrombin (mutated forms of the blood coagulation factors Xa and Va); myotoxins which cause rhabdomyolysis; and

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neurotoxins which impede neuromuscular function by acting either pre-synaptically (PLA_2s) or post-synaptically (three finger toxins [3FTx]) (Fry, 1999; Jackson et al., 2016). The most clinically relevant symptoms are procoagulant coagulotoxicity and neurotoxicity (Fry, 2015; Sutherland and Tibballs, 2001). Most notably, Australian elapids are known for the presence of potent coagulotoxic venoms which target the blood clotting cascade (Isbister, 2009).

In vertebrates, to prevent spontaneous bleeding or blood loss in the event of an injury, a number of blood components are activated in a series, called a clotting cascade, to form a blood clot where required. The blood clotting cascade can be activated by one of two separate pathways (intrinsic and extrinsic) which both act upon the common pathway (Kini and Koh, 2016). The primary components of the common pathway are Factor X (and the activated form FXa), Factor V (and the activated form FVa), prothrombin (and the activated form thrombin) and fibrinogen (and the activated form fibrin) (Jin and Gopinath, 2016). Coagulotoxic venoms can affect blood hemostasis *via* coagulant, anticoagulant, and/or fibrinolytic actions. While procoagulant venoms activate zymogens of clotting factors which lead to the fibrin clot, anticoagulant venoms inhibit the activation of these zymogens, thereby inhibiting blood clotting. Australian elapids commonly have potent procoagulant venoms which generate endogenous thrombin leading to the formation of fibrin clots, with this well-documented for genera such as *Hoplocephalus, Notechis, Paroplocephalus,* and *Tropidechis* genera (Lister et al., 2017). However relatively few Australian species have anticoagulant venoms, with *Austrelaps* and *Pseudechis* the best known in this regard, inhibiting platelet aggregation *via* PLA_2 toxins (Du et al., 2016; Kamiguti et al., 1994; Lane et al., 2011; Sutherland and Tibballs, 2001).

With the continued urbanisation of Australia leading to increased habitat encroachment, understanding the action of venoms from understudied species is increasingly important. The genus *Denisonia* includes only two species, *D. devisi* and *D. maculata*, both of which are small, robust elapids which range from 30 to 40 cm in length and whose diet consists almost exclusively of frogs (Shine, 1983). *D. devisi* has a wide distribution, found in Brigalow scrub habitat along the eastern coast and central semi-arid regions of New South Wales, Victoria and Queensland (Clemann et al., 2007; Shine, 1983). In comparison *D. maculata* has a considerably smaller range, being restricted to central eastern Queensland (Shine, 1983). *D. devisi* and *D. maculata* are known to be venomous, however few experimental studies have investigated the venom composition or functional properties of either species (Fry, 2015; Isbister et al., 2016; Jackson et al., 2013; Kellaway, 1934; Pycroft et al., 2012).

Recent research has identified significant interclass diversity in the toxin classes present in venoms from the genus *Denisonia* (Jackson et al., 2016, 2013). *D. devisi* has been shown to have neurotoxic effects in the chick biventer cervicis nerve muscle assay of potency comparable with other species of notable Australian elapids from genera such as *Notechis* and *Oxyuranus* (Pycroft et al., 2012), consistent with *in vivo* rabbit studies (Kellaway, 1934). Previous *in vitro* coagulation assays identified the venom of *D. devisi* as having weak or very mild procoagulant effects (Isbister et al., 2016; Pycroft et al., 2012), which was consistent with previous *in vivo* rabbit studies (Kellaway, 1934). Although relatively little is known about *D. devisi* venom, even less is understood about the venom of *D. maculata*, which is described from only one snakebite case (Isbister et al., 2016). In this case, the venom caused non-specific symptoms, such as a raised white blood cell count, elevated creatinine kinase levels, and a raised international normalised ratio coagulation parameter (Isbister et al., 2016). Localised pain and swelling was also documented from this case of snakebite (Isbister et al., 2016).

While *D. devisi* and *D. maculata* are not recognised as medically significant snakes due to the remoteness of their home ranges and concomitant scarcity of bite reports, they are still considered potentially dangerous. Therefore, the aim of this study was to investigate and characterise the coagulotoxic functional effects of *Denisonia* venoms and determine the mechanisms of action.

2. Methods

2.1. Venom

Denisonia devisi venom was collected by BGF at Glenmorgan, QLD. *Denisonia maculata* venom was sourced *via* Footprints Environmental Consultants and collected at Zamia Creek, QLD with additional venom from Venom Supplies, Tanunda, South Australia. Venom was flash-frozen and stored at -80 °C. Venom working stock solutions (50% glycerol and 50% water) were made at a concentration of 1 mg/ml and stored at −20 °C.

2.2. Plasma

Human plasma was obtained from the Australian Red Cross (Research agreement #18-03QLD-09 and University of Queensland Human Ethics Committee Approval #2016000256). All plasma was prepared as 3.2% citrated stock, aliquoted into 1 ml quantities, which were flash-frozen in liquid nitrogen, and stored in a -80 freezer until needed, at which time an aliquot was defrosted by placing into a 37 C water bath for 10 min. All venom and plasma work was undertaken under University of Queensland Biosafety Approval #IBC134BSBS2015.

2.3. Coagulation screening assays

Plasma coagulation assays were carried out on a Stago STA-R Max coagulation analyser machine (Stago, Asnières sur Seine, France). Pooled frozen human plasma, supplied by the Australian Red-Cross (research approval # 16-04QLD-10), was thawed and warmed to 37 ºC before being placed in the Stago STA-R Max machine. Experiments described below were carried out in triplicates at each concentration point. Assays in which a clot had not formed after a machine-maximum of 999 s were stopped. Calcium and phospholipid were added to the coagulation assays to imitate the *in vivo* conditions present in the human body. Controls were conducted with stocks of 50% de-ionised water and 50% glycerol used in replacement of venom (and, like the venom, diluted with Owren Koller (OK) Buffer (Stago Catalog # 00,360)), to indicate the time healthy plasma spontaneously clots. To identify the target in the clotting cascade which the venom was acting upon to result in the anticoagulant activity, either plasma or individual factors of the clotting cascade were incubated with the sample venom as the incubation step allows venom to bind and inhibit its target (Kini, 2006).

FXa inhibition assay	Step 1. 25 µl 0.2 µg/ml venom (1 mg/ml 50% glycerol stock diluted with OK buffer + 75 µl of [50 µl 0.025 M calcium (Stago catalog # 00367 + 25 µl OK buffer] + 50 µl phospholipid (Stago catalog #00597) + 50 µl FXa (Stago catalog # 00311). Step 2. 120 second incubation. Step 3. Addition of 75 µl plasma.
Prothombinase complex inhibition assay	Step 1. 25 µl 0.2 µg/ml venom (1 mg/ml 50% glycerol stock diluted with OK buffer + 75 µl of [50 µl 0.025 M calcium + 25 µl OK buffer] + 50 µl phospholipid + 75 µl plasma Step 2. 120 second incubation. Step 3. Addition of 50 µl Factor Xa
Prothombinase complex inhibition assay (antivenom assay conditions)	Step 1. 25 µl 0.2 µg/ml venom (1 mg/ml 50% glycerol stock diluted with OK buffer + 75 µl of [50 µl 0.025 M calcium + 25 µl of 5% concentration antivenom] + 50 µl phospholipid + 75 µl plasma Step 2. 120 second incubation. Step 3. Addition of 50 µl Factor Xa
Thrombin inhibition assay	Step 1. 25 µl 0.2 µg/ml venom (1 mg/ml 50% glycerol stock diluted with OK buffer + 75 µl of [50 µl 0.025 M calcium + 25 µl OK buffer] + 50 µl phospholipid + 50 µl thrombin (Stago catalog # 00611). Step 2. 120 second incubation. Step 3. Addition of 75 µl 4 mg/ml fibrinogen.

2.4. Venom fractionation and LC–MS identification

Reversed phase high pressure liquid chromatography was used to fractionate crude venoms by relative hydrophobicity of the toxins present. Pooled venoms of *D. devisi* (3 mg) and *D. maculata* (4.5 mg) were lypholized before being resuspended in 1.5 ml of 3% of buffer B (90% ACN/0.043% trifluoracetic acid). Each venom was then stored on ice before being loaded and run through a Shimazdu RP HPLC reader, utilising an acetonitrile gradient from 3% to 80%. The fractionation occurred across a time span of sixty minutes with dispensing of fractions into a new falcon tube every minute at a rate of 5 ml/minute. The fractions were prepared in the same manner as crude venom and were tested in the same manner as crude venom on the prothombinase complex assay. To identify the toxins present MS/MS was conducted on the active fractions of *D. maculata* and the respective corresponding fractions of *D. devisi* using protocols previously validated (Yang et al., 2016). Reduced, Alkylated (carbamidomethyl), and trypsin digested samples were analysed by LC–MS using a Thermo Ultimate 3000 RSLCnano HPLC coupled to a Bruker Amazon Speed ETD iontrap mass spectrometer. A gradient was established on a Thermo Pepswift PS-DVB 200um x 5 cm Column and 200um x 5 mm trap column comprising 5–40% B over 30 min, ramp to 99% B over 5 min, hold at 99% B for 2 min, drop to 5% over 0.5 min, and re-equilibrate at 5% B for the next run. Buffer A was 18.2 MOhm water with 0.2% Formic Acid and buffer B was 80% Acetonitrile with 0.2% Formic Acid, the trapping buffer was 18.2 MOhm water with 0.2% HBFA. A data dependant acquisition was used on the mass spectrometer, acquiring an MS scan (enhanced resolution (8100 *m/z*/s)) over 400–1400 *m/z* followed by the top 4 precursors for MS/MS (xtreme scan (52,000 *m/z*/s)). The acquired data for each individual sample was converted to an mgf file using Data Analysis 4.2 (Bruker) and searched against serpent database using Mascot 2.5.

2.5. Amphibian and avian plasma coagulation assays

To allow for representation across other major vertebrate clades, amphibian and avian plasmas were also screened against the venom of both *D. devisi* and *D. maculata* on the prothombinase complex inhibition assay as described above. Cane toad (*Rhinella marina*) and chicken (*Gallus gallus domesticus*) plasma were used as representative samples for amphibians and aves respectively. Plasmas were collected under University of Queensland Animal Ethics approval SBS/020/15/ARC as previously described (Lister et al., 2017). Each plasma sample was incubated with venom, calcium and phospholipid for 120 s before addition of Factor Xa using a Stago STA-R Max machine and all assays were conducted in triplicate.

3. Results and discussion

Our study aimed to characterise the function and underlying mechanisms of the venom for both species in the Australian elapid genus *Denisonia*. Addition of venom to recalcified human plasma extended the clotting time relative to that of the spontaneous clotting control of 424.5 ± 21.4 s, with *D. devisi* extending it to 681.5 ± 84.8, while *D. maculata* exceeded the machine maximum reading time of 999 s. Concentration curves to establish the site of action revealed that the incubation of plasma followed by the addition of Factor Xa trigger resulted in potent anticoagulation (Fig. 1). Conversely, incubation with FXa followed by the addition of plasma did not result in the inhibition of clotting, nor did the incubation with thrombin followed by the addition of plasma impede clotting. Thus the venom is impeding the formation of the prothrombinase complex by binding to either Factor Va or prothrombin. The Seqirus polyvalent snake antivenom was found to be effective in impeding the anticoagulant effects (Fig. 2).

The *D. maculata* venom was fractionated by reversed phase high performance liquid chromatography and all fractions tested. The two largest fractions (Fig. 3), had the same ability as the crude venom in

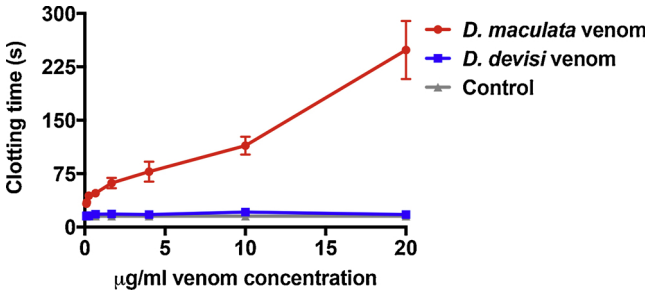

Fig. 1. Prothombinase complex inhibition assay, showing the relative inhibitory effects of *D. devisi* and *D. maculata* venom on preventing the activation of prothrombin to thrombin. Venom was incubated with human plasma for 2 min and then Factor Xa added, with clot time then immediately measured.

inhibiting the formation of the prothrombinase complex (Fig. 4). Subsequent MS/MS sequencing revealed these fractions to be Type I PLA_2 toxins (Supplementary Table 1). Anticoagulant $PLA2_2$ toxins have been described previously from elapid venoms, which inhibit clot formation by binding to Factor Xa either at the enzymatic site or at the Factor Va binding site (Kerns et al., 1999; Kini, 2005; Mukherjee et al., 2014; Sunagar et al., 2015). The interference of the prothrombinase complex formation by binding other than to Factor Xa has not been previously documented for elapid snakes, but has for the *Bothrops* genus of viperid snakes (Monteiro et al., 2001; Zingali et al., 2001). Thus this mechanism revealed in this study is a novel one for elapid snake venoms.

As both *D. devisi* and *D. maculata* predate primarily upon amphibians, further testing was undertaken to investigate taxon-specific effects, which indicated that *D. devisi* is potent against only amphibians but *D. maculata* was potent against amphibians and avians (Fig. 5) in addition to the human toxicity described above. This revealed the first taxon-specific anticoagulant venom described to-date. Previous work on Australian procoagulant venoms showed that they were evolving under negative selection pressure due to the target itself being highly conserved (Lister et al., 2017). However, taxon-specific procoagulant effects have been recently described for the *Bothrops* genus of viperid snakes (Sousa et al., 2018). The scarcity of taxon-specific coagulotoxic effects described to-date is in contrast to the taxon-specific neurotoxic effects, which have been previously demonstrated more extensively in elapids and other venomous snake families (Hart et al., 2013; Heyborne and Mackessy, 2013; Pawlak et al., 2006, 2009).

While both *Denisonia* venoms were found to have a potent anticoagulant effect on amphibians, consistent with feeding primarily on anurans, *D. maculata* was found to be potent not only on avian plasma but also upon human plasma. The observed anticoagulant effect was found to be produced by inhibiting the formation of the prothrombinase complex. This mechanism of action is unique and novel for an Australian elapid, as no other species is known to target the common pathway by preventing the cleavage of prothrombin to thrombin during clot formation. The dichotomy in action upon human plasma is also supported, where bites from *D. maculata* are reported as being more severe compared to *D. devisi*. The lack of action on mammalian plasma by *D. devisi* is also consistent with previous *in vivo* studies which either noted no action upon coagulation (Kellaway, 1934) or only a very weak procoagulant activity at very high doses (Isbister et al., 2016; Pycroft et al., 2012). In contrast, a case study on a *D. maculata* envenomation noted a rise in the international normalised ratio (Isbister et al., 2016) which would be consistent with the anticoagulant effects described in this study. The Isbister et al. study reported on the bite by a *D. maculata* but conducted the laboratory venom studies on human using *D. devisi* venom and merged the two results into a general statement about *Denisonia* venom. However the results that the venoms are sharply different in their action upon human plasma, which explains the contrasting results in the Isbister et al study in which the *D. maculata* patient displayed effects of anticoagulation but the *D. devisi* venom did

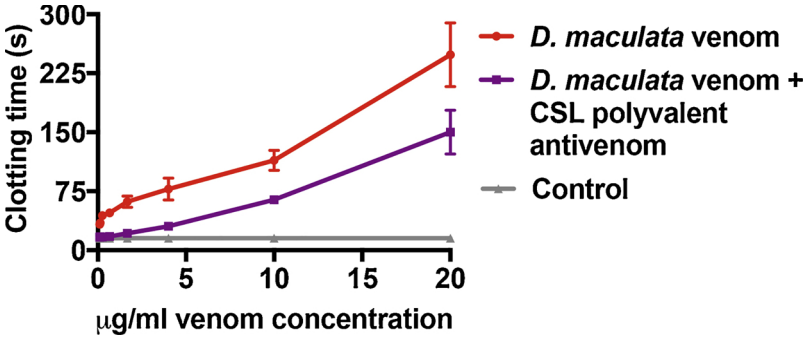

Fig. 2. Prothombinase complex inhibition concentration curves, showing the inhibitory effects of *D. maculata* venom on preventing the activation of prothrombin to thrombin and the effectiveness of CSL polyvalent antivenom at reducing the effects of *D. maculata* venom. Venom was incubated with human plasma and antivenom for 2 min, Factor Xa was then added and clot time immediately measured.

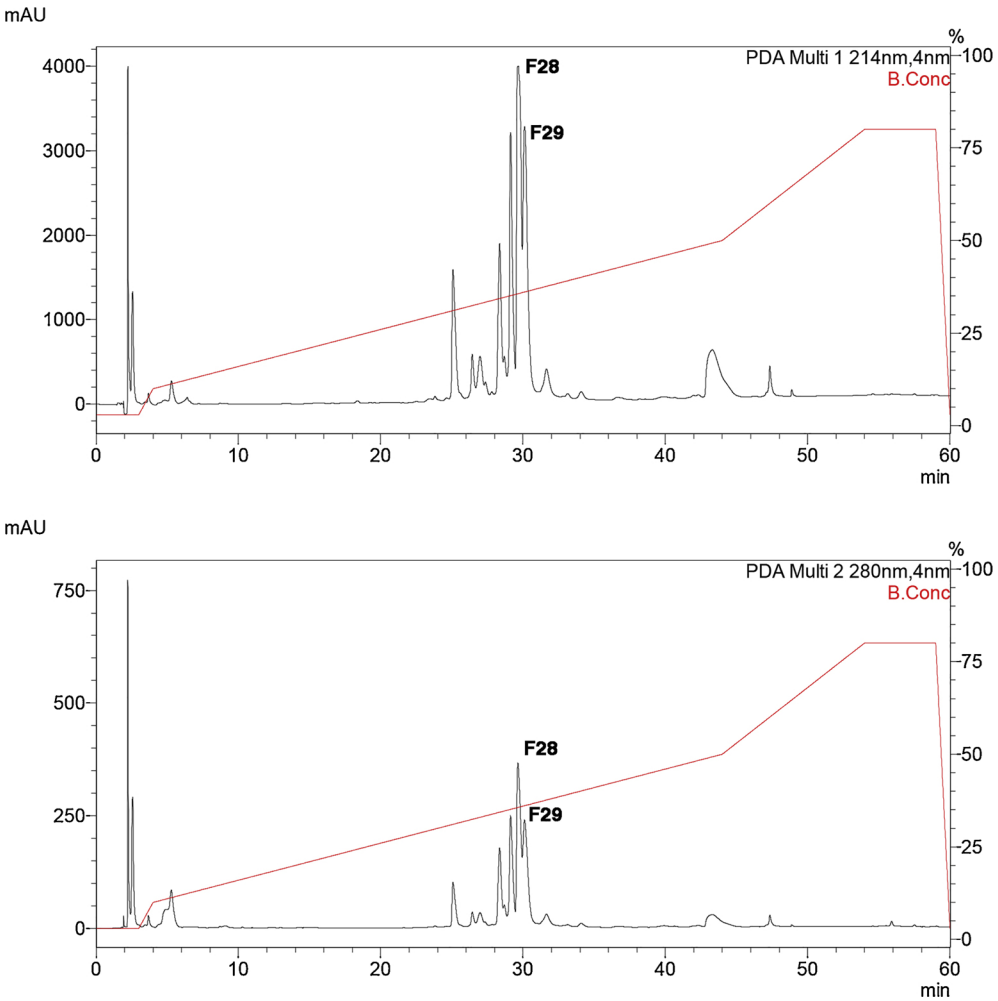

Fig. 3. Reversed phased high performance liquid chromatography fractionation of *D. maculata* venom. Fractions 28 and 29 were the active ones (Fig. 4) and MSMS sequencing revealed them to be Group I phosopholipase A_2 toxins (Supplementary material).

not display anticoagulant activity when tested *in vitro* on human plasma.

Thus while both venoms have similar biochemistry, and both species are amphibian-feeding specialists, evolutionary selection pressures have further refined *D. devisi* venom to be anuran-specific. Thus the taxon-differential action not only points to biochemical differences in the toxins used, but correspondingly reveals fundamental differences in the structure of the affected plasma proteins. Thus while the same part of the clotting cascade is being affected, evolutionary drift between the clotting proteins of the different toxin has resulted in a selection for specialisation by the *D. devisi* toxins relative to generalised action of the *D. maculata* forms. This is the first time that such taxon-specific diversification of anticoagulant toxins has been documented.

Screening and subsequent MS/MS revealed that the active fractions responsible for the potent anticoagulant activity observed from *D. maculata* crude venom were PLA_2 toxins. Many novel therapeutic drugs have been designed from previous toxins discovered in snake venoms (Fry, 2015). Thus we propose that the PLA_2s responsible for the anticoagulant activity upon the activation of prothrombin found here in the venom of *D. maculata* is a potential candidate for designing a therapeutic drug for stroke management or venous thrombosis.

In conclusion, this study substantially increases the knowledge of the functional activity of the venom from the Australian elapid species *D. devisi* and *D. maculata*. Through the implementation of novel anticoagulant assays, the venom of *D. maculata* has been shown to possess a potent anticoagulant PLA_2 toxin which inhibits the formation of the

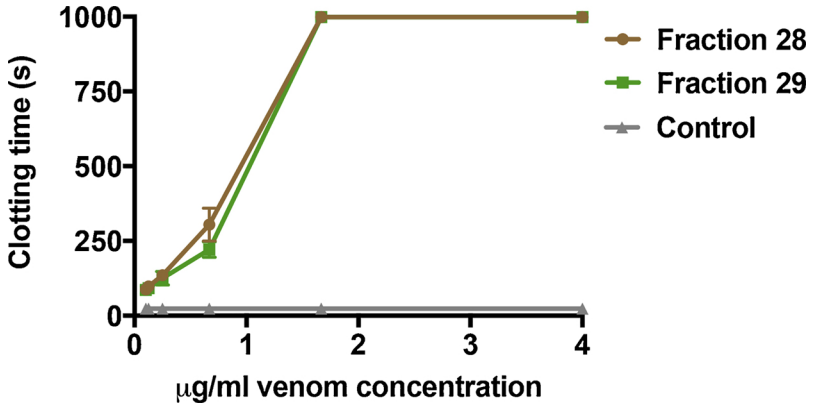

Fig. 4. Prothombinase complex inhibition concentration curves, showing the inhibitory effects of purified Group I phospholipase A_2 fractions from *D. maculata* crude venom.

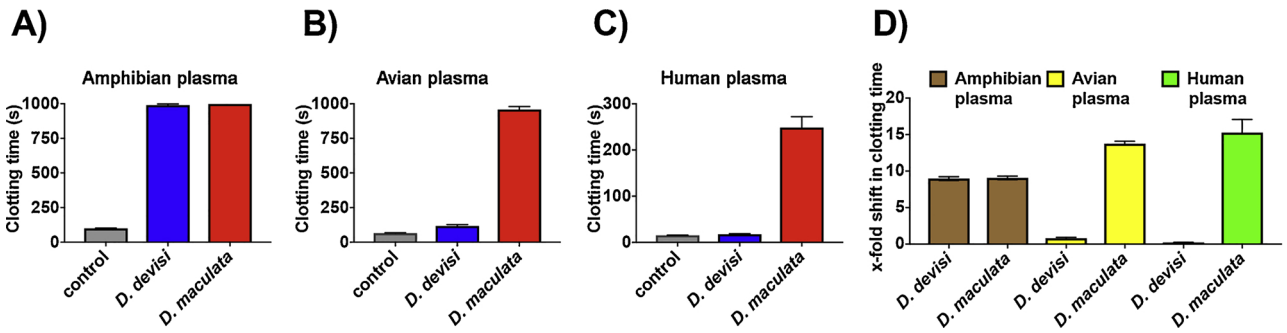

Fig. 5. Prothombinase complex inhibition assay conducted with venom incubated with **A)** toad (*Rhinella marina*) plasma, **B)** chicken (*Gallus gallus domesticus*), or **C)** human plasma. **D)** Shows the x-fold shift in clotting times relative to the control, where a value of 0 would indicate no shift. Venoms were incubated with plasma for 2 min, Factor Xa was then added and clot time immediately measured. Values are N = 3 means and standard deviation.

prothrombinase complex. The anticoagulant PLA2 toxin from the venom of *D. maculata* also has the potential to act as a model for therapeutic drugs which further studies should take into consideration and explore. Our results reinforce the need to investigate understudied venomous species not only to evaluate the risk to humans but also as potential sources of biodiscovery.

Declaration of interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2018.11.015.

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