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Utilising venom activity to infer dietary composition of the Kenyan horned viper (*Bitis worthingtoni*)

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ABSTRACT

Bitis are well known for being some of the most commonly encountered and medically important snake species in all of Africa. While the majority of species possess potently anticoagulant venom, only *B. worthingtoni* is known to possess procoagulant venom. Although known to be the basal species within the genus, *B. worthingtoni* is an almost completely unstudied species with even basic dietary information lacking. This study investigated various aspects of the unique procoagulant effects of *B. worthingtoni* venom. Coagulation assays determined the primary procoagulant effect to be driven by Factor X activating snake venom metalloprotease toxins. In addition to acting upon the mammalian blood clotting cascade, *B. worthingtoni* venom was also shown to clot amphibian plasma. As previous studies have shown differences in clotting factors between amphibian and mammalian plasmas, individual enzymes in snake venoms acting on plasma clotting factors can be taxon-selective. As venoms evolve under purifying selection pressures, this suggests that the procoagulant snake venom metalloprotease toxins present in *B. worthingtoni* have likely been retained from a recent common ancestor shared with the related amphibian-feeding *Proatheris superciliaris*, and that both amphibians and mammals represent a substantial proportion of *B. worthingtoni* current diet. Thus, taxon-specific actions of venoms may have utility in inferring dietary composition for rare or difficult to study species. An important caveat is that to validate this hypothesis field studies investigating the dietary ecology of *B. worthingtoni* must be conducted, as well as further investigations of its venom composition to reconstruct the molecular evolutionary history of the toxins present.

1. Introduction

Comprised of eighteen species, the *Bitis* genus is the most geographically widespread and taxonomically diverse viperid genus in Africa (Barlow et al., 2019). The Kenyan horned viper (*Bitis worthingtoni*) is one of the rarest and most elusive species of *Bitis* (Parker, 1932; Phelps, 2010). Unlike many *Bitis* species, *B. worthingtoni* has not been considered of medical importance due to its rarity and remote distribution (Mallow et al., 2003). It is found only in a restricted region of the central rift valley in Kenya, inhabiting high altitude montane grassland (Barlow et al., 2019; Parker, 1932). Genetic and morphological analyses have also placed *B. worthingtoni* as basal, sister to all other *Bitis* (Alencar et al., 2016; Barlow et al., 2019; Lenk et al., 1999). No detailed research has been conducted on its venom or dietary ecology, however it is noted to be a predominantly nocturnal, ambush predator (Mallow and Ludwig, 2003; Phelps, 2010; Spawls and Branch, 2020) with procoagulant venom (Youngman et al., 2019a).

Similar to the majority of the species within its genus, *B. worthingtoni* has retained the basal dwarf morphology condition reaching a total average length of only 20 to 40 cm (Mallow and Ludwig, 2003; Phelps, 2010; Spawls and Branch, 2020). *Proatheris superciliaris* which is phylogenetically sister to *Bitis* shares this dwarf morphology and is an amphibian feeding specialist (Phelps, 2010) with coagulotoxic venom effects (Keyler, 2008; Valenta et al., 2008). While the majority of dwarf *Bitis* are members of the subgenus *Calechidna*, *B. worthingtoni* is placed within the subgenus *Keniabitis* (Alencar et al., 2016; Barlow et al., 2019; Lenk et al., 1999). The Angolan adder (*B. heraldica*) has historically been placed within *Calechidna* but this placement was based upon size rather than genetics. Recent genetic testing shows that it is not with other dwarf species in the subgenus *Calechidna*, but instead it is basal to the

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lineage of giant species in the subgenus *Macrocerastes* (Ceríaco et al., 2020). The placement of *B. heraldica* as basal among the *Macrocerastes* clade further supports that the dwarf morphology is the basal condition for the *Bitis* genus while also highlighting that splitting a genus into subgenera based upon morphological characters is an artificial construct of limited usefulness.

In addition to being the most basal species, *B. worthingtoni* also has the most northern distribution of all dwarf *Bitis*, with all other species of dwarf *Bitis* restricted to Southern Africa (Barlow et al., 2019). The high altitude montane grassland habitat of *B. worthingtoni* is also distinctive compared to all other dwarf *Bitis*, which are found in arenicolous (desert) or rupicolous (rock) habitat (Barlow et al., 2019; Phelps, 2010). This distinctiveness ecologically, geographically and taxonomically has direct implications towards its venom evolution, despite the lingering belief that the venom of all dwarf *Bitis* is to be considered of a similar nature.

The venom of many viperid species contains toxins which disturb haemostasis by targeting the coagulation cascade, either activating coagulation factors (procoagulant toxins) to induce clotting or by inhibiting and destroying coagulation factors (anticoagulant toxins) to prevent clotting (Jin and Gopinath, 2016; Kini and Koh, 2016). Significant research has focussed on the characteristics of procoagulant toxins from medically important species such as those from the genera *Echis* and *Daboia* (Morita and Iwanaga, 1978; Rogalski et al., 2017; Yamada et al., 1996). Procoagulant toxins in viper venoms are primarily characterised within the snake venom metalloproteases (SVMPs) toxin class, although some kallikrein-type snake venom serine proteases (SVSPs) have been shown to activate clotting factors (Latinović et al., 2020). These toxins target factors within the coagulation cascade, primarily Factor X (FX) or prothrombin, converting them into their active form to potentiate the clotting cascade (Kini and Koh, 2016). The final stages of the clotting cascade are thrombin converting both fibrinogen to fibrin as well as Factor XIII (FXIII) to FXIIIa, resulting in fibrin crosslinking with FXIIIa to form a strong fibrin clot (Jesty and Beltrami, 2005). A third category of coagulotoxins are pseudo-procoagulant toxins, which are kallikrein-type serine proteases that directly cleave fibrinogen to fibrin, although causing weak and aberrant clots which readily break down, thereby depleting fibrinogen levels and having a net anticoagulant effect (Debono et al., 2019a; Debono et al., 2019b; Oulion et al., 2018).

Recent investigation into the coagulotoxic venom activity of species within the *Bitis* genus has indicated that *B. worthingtoni* is the sole member of the genus to possess an overall potently procoagulant venom effect on plasma, while also possessing pseudo-procoagulant toxins that evolved at the base of the viperid radiation (Youngman et al., 2019a). All other *Bitis* possess venom that is anticoagulant (either through factor inhibition or destructive cleavage of fibrinogen), pseudo-procoagulant or has a neutral effect upon the clotting time of plasma (Morné et al., 2016; Paixão-Cavalcante et al., 2015; Sánchez et al., 2011; Youngman et al., 2019a). Although the anticoagulant mechanisms of action for many of *Bitis* venoms have been investigated in detail (Brink and Steytler, 1974; Fernandez et al., 2014; Morné et al., 2016; Paixão-Cavalcante et al., 2015; Sánchez et al., 2011; Youngman et al., 2019a), it is unknown what coagulation factors *B. worthingtoni* venom targets to exert its procoagulant effect, what toxin types are responsible for this action, and therefore the evolutionary implications of these characteristics in relation to other closely related procoagulant viperid species such as *P. superciliaris*.

This study aimed to investigate the coagulotoxic activity of *B. worthingtoni* venom to fill this knowledge gap. Herein we determine the mechanism of action behind the venoms procoagulant effects, identify the toxin types responsible for both the procoagulant and pseudo-procoagulant effects of the venom and discuss the significance of these findings.

2. Materials and methods

2.1. Venoms and reagents

A pooled venom sample was used from the venom of four captive *B. worthingtoni* individuals. The individual *B. worthingtoni* samples were supplied by Serpentarium Calden, Calden, Germany and Universeum, Gothenburg, Sweden. *Proatheris superciliaris* venom was supplied by Serpentarium Calden, Calden, Germany. *Daboia russelii* venom was sourced from a long-term cryogenic research collection and came from captive specimens of Pakistan founding stock. Original venom extractions were flash-frozen, lyophilized and stored at −80 °C. Using a NanoDrop2000 to determine venom concentrations, venom working stock solutions were made at a concentration of 1 mg/ml (50% glycerol and 50% double-deionised water) and stored at −20 °C until required to preserve enzymatic activity. Calcium chloride (Stago catalog #00367), phospholipid (Stago catalog #00597) and Owren-Koller buffer (Stago catalog #00360) were supplied by Stago (Stago, Paris, France). Pooled human plasma was supplied by the Australian Red Cross Blood Service and stored at −80 °C. All venom and plasma work was undertaken under University of Queensland Biosafety Committee Approval #IBC134BSBS2015 and Ethics Committee Approval #2016000256.

2.2. Coagulation assays

Coagulation assays were carried out on a Stago STA-R Max coagulation analysing robot (Stago, France) and adapted from previously validated coagulation assay protocols (Bittenbinder et al., 2018; Youngman et al., 2019a; Youngman et al., 2020; Zdenek et al., 2019a; Zdenek et al., 2019b). Pooled frozen human plasma aliquots were thawed and warmed to 37 °C for 5 min in a water bath before being placed in the Stago STA-R Max machine. Coagulotoxic effects upon plasma were measured by taking venom from a 1 mg/ml glycerol stock solution and manually diluting in Owren-Koller buffer (isotonic saline) to make a 100 μg/ml working solution. A total of 50 μl of 100 μg/ml venom was added by the STA-R Max to 50 μl calcium, 50 μl phospholipid and 25 μl Owren Koller Buffer, then incubated for 120 s before the robot added 75 μl human plasma. Directly after the addition of the human plasma time until clot formation was measured. Calcium and phospholipid were included in all coagulation assays to replicate the *in vivo* conditions present in the human body. Experiments were carried out in triplicates at each concentration point. Assays in which a clot had not formed after 999 s were automatically stopped by the robot as this is the machine maximum measurement time. Negative controls were conducted with stocks of 50% Owren-Koller buffer and 50% glycerol used in replacement of venom, to represent the time healthy plasma clots spontaneously. To determine the inhibitory ability of prinomastat, a total of 50 μl of 100 μg/ml venom was added by the STA-R Max to 50 μl calcium, 50 μl phospholipid and 25 μl 2 mM prinomastat diluted in Owren Koller Buffer, then incubated at 37 °C for 120 s before the robot added 75 μl human plasma.

The direct coagulotoxic effects of the venom upon fibrinogen were measured by taking venom from a 1 mg/ml glycerol stock solution and manually diluting in Owren-Koller buffer (isotonic saline) to make a 100 μg/ml working solution. A total of 50 μl of 100 μg/ml venom was added by the STA-R Max to 50 μl calcium, 50 μl phospholipid and 25 μl Owren Koller Buffer, then incubated for 120 s before the robot added 75 μl 4 mg/ml fibrinogen. To determine the inhibitory ability of AEBSF, 25 μl of 10 mM AEBSF diluted in Owren Koller Buffer replaced buffer in the previous assay. For both assays the time until clot formation was measured directly after the addition of fibrinogen.

2.3. Fluoroskan ascent assays

The activation of coagulation factors VII, IX, X, XI, XII and prothrombin by *B. worthingtoni* venom were investigated using a Fluoroskan

Ascent™ (Thermo Scientific, Vantaa, Finland) following previously validated protocols (Oulion et al., 2018). In a 384-well plate (black, Lot#1171125, nunc™ Thermo Scientific, Rochester, NY, USA), reagents indicated in Table 1 were plated manually, and 70 µl of buffer containing (5 mM CaCl2,150 mM NaCl, and 50 mM Tri-HCl (pH 7.3)) and Fluorogenic Peptide Substrate, (ES011Boc-Val-Pro-Arg-AMC. Boc: t-Butyloxycarbonyl; 7-Amino-4-methylcoumarin; R & D systems, Cat# ES011, Minneapolis, Minnesota) in 500: 1 ratio was dispensed automatically at the start of the reaction. The plate was warmed to 37 °C, and a plate was set to shake for 3 s before each measurement. Fluorescence generated by the cleavage of the substrate was measured every 10 s for 300 min at 390/460 nm (excitation/emission) by Ascent® Software v2.6 (Thermo Scientific, Vantaa, Finland). Blank responses (Table 1) were subtracted from all measurements. Then, to exclude the venom activity upon the substrate directly (rather than upon the zymogen), "venom without zymogen" results were subtracted from "venom with zymogen" results. This output was taken as the final result. As a positive control for representative comparison, *D. russelii* venom was run against factors (FX), which indicated significant activation by *B. worthingtoni* venom. Assays replacing either calcium or phospholipid with buffer were also run for *B. worthingtoni* and *D. russelii* venom to determine calcium or phospholipid dependency for FX activation.

2.4. Thromboelastography assays

Thromboelastography was utilised to test the viscoelastic clotting properties of amphibian plasma in the presence of *B. worthingtoni* venom. Thromboelastography assays conducted were adapted from previously published protocols utilising thromboelastography techniques (Grashof et al., 2020; Youngman et al., 2019a). In this assay, 7 µl of venom (1 mg/ml in 50% glycerol) was added to 72 µl calcium, 72 µl phospholipid, 20 µl Owren Koller buffer and 189 µl amphibian (*Rhinella marina*) plasma. *P. superciliaris* venom was additionally tested as a comparison to *B. worthingtoni* venom. A positive control was conducted using 7 µl of Kaolin in replacement of venom for this assay, which represents a fully generated clot.

2.5. Data analysis

All data in this study was analysed using Prism 7.0 software (GraphPad Software Inc., La Jolla, Ca, USA). Student *t*-tests were used to determine if mean values between treatments being compared were significantly different. For all statistical tests p-values ≤ 0.05 were considered statistically significant.

3. Results

B. worthingtoni venom was potently procoagulant, and comparison of the area under the curve (AUC) showed this procoagulant effect was neutralised by incubation with the inhibitor prinomastat ($t(4) = 28.18$, $p \leq 0.0001$), ascertaining that it was metalloprotease driven (Fig. 1). Zymogen activation tests determined that the procoagulant action was due to FX activation, with no substantial activation of factors VII, IX, XI, XII or prothrombin observed (Supplementary Data). The activation of FX was strongly calcium dependent, with activity significantly lower in the absence of calcium ($t(4) = 102.8$, $p \leq 0.0001$) (Fig. 2). In addition to the potent procoagulant activity when tested on human plasma, in tests using just fibrinogen the venom was shown to retain some of the viperid-ancestral activity of direct pseudo-procoagulant clotting abilities on fibrinogen, rapidly forming weak, transient fibrin clots (Fig. 3). The pseudo-procoagulant activity of the venom was significantly neutralised by the inhibitor AEBSF ($t(4) = 28.56$, $p = 0.0012$), revealing it to be driven by kallikrein-type serine proteases (Fig. 3). Thromboelastography results indicated that *B. worthingtoni* venom acted upon amphibian plasma, and the rate of clotting was significantly faster than that of the positive kaolin control both in regards to SP ($t(4) = 4.696$, $p = 0.0093$) and R ($t(4) = 6.957$, $p = 0.0022$) measurements (Fig. 4). Strong clots were also formed however these were not stronger than the positive kaolin control.

Table 1
Manually plated reagents for fluoroskan ascent assays.

Treatment	Reagents
Blank	20 µl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tri-HCl (pH 7.3) + 10 µl PPL)
Positive control with activated zymogen	10 µl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tri-HCl (pH 7.3) + 10 µl PPL + 10 µl activated zymogen (10 µg/ml for FVIIa, FIXa, FXa, FXIa, FXIIa, 1 µg/ml for thrombin))
Negative control with zymogen	10 µl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tri-HCl (pH 7.3) + 10 µl PPL + 10 µl zymogen (1 µg/ml for FVII, FIX, FX, FXI, FXII, 0.1 µg/ml for prothrombin))
Venom without zymogen	10 µl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tri-HCl (pH 7.3) + 10 µl PPL + 10 µl venom)
Venom with zymogen	10 µl zymogen (1 µg/ml for FVII, FIX, FX, FXI, FXII, 0.1 µg/ml for prothrombin) + 10 µl PPL + 10 µl venom

4. Discussion

The evolution and activity of *B. worthingtoni* venom has remained neglected despite the unique taxonomical position of this species within the *Bitis* genus. Previous research on its venom has been limited to a single study showing it has procoagulant effects upon plasma, as well as direct pseudo-procoagulant effects upon fibrinogen (Youngman et al., 2019a). This is a stark difference in venom activity compared to the remainder of the species within the genus, which predominantly possess potently anticoagulant venom. The closely related species *P. superciliaris*, similarly possess procoagulant venom suggesting that the last common ancestor of *Proatheris* and *Bitis* possessed procoagulant venom. Assays incubating the known SVMP inhibitor prinomastat significantly inhibited the procoagulant effects of *B. worthingtoni* venom, identifying the toxins responsible as SVMP toxins (Fig. 1). This result is consistent with viperid venoms being SVMP rich and exerting coagulotoxic effects functionally (Brust et al., 2013; Casewell et al., 2009; Wagstaff et al., 2009).

The main mechanism of action for the procoagulant activity of *B. worthingtoni* venom was identified as being FX activation (Fig. 2). The venom activated FX at approximately the same percentage relative to the FXa control both in the presence and absence of phospholipid. In

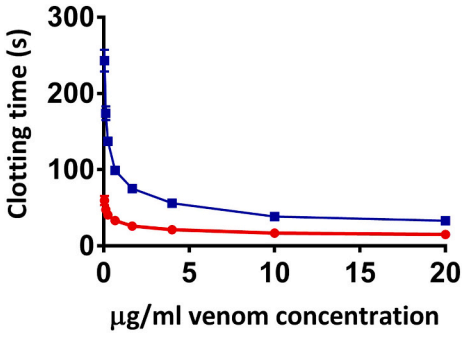

Fig. 1. Concentration-response curve demonstrating the procoagulant toxic effects of *B. worthingtoni* venom and relative neutralisation by prinomastat. Concentration response curves show the venom only assay conditions (red line) and venom incubated with 0.2 mM prinomastat assay (blue line). Negative control values were 437.9 ± 22.7 s, representing the mean spontaneous clotting time of recalcified plasma. Data points are N = 3 mean and standard error of the mean. Note: for most data points the error bars are smaller than the representative icon.

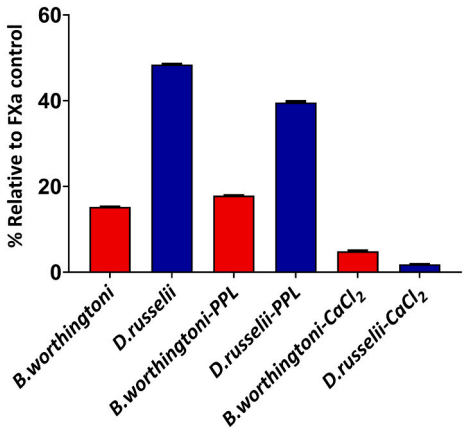

Fig. 2. Relative effect of FX activation by *B. worthingtoni* venom in comparison to that of a known FX activating venom from *D. russelli*. Assays were conducted in the presence of both phospholipid and calcium, as well as in the absence of each co-factor independently. Data points are N = 3 mean and standard error of the mean.

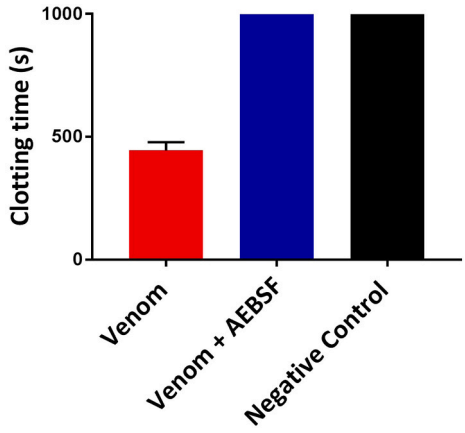

Fig. 3. Pseudo-procoagulant effects of *B. worthingtoni* venom upon fibrinogen. Incubation of 20 μg/ml venom with fibrinogen showed direct clotting ability of the venom upon fibrinogen (red bar), while incubation of 20 μg/ml venom +2 mM of AEBSF with fibrinogen neutralised the activity of the venom to clot fibrinogen (blue bar). The negative control assay represents the natural state of fibrinogen to remain uncleaved, with no clot formation (black bar). Data points are N = 3 mean and standard error of the mean. Note: 999 s represents the maximum machine reading time.

contrast, *D. russelli* venom had a substantial shift of FX activation in the absence of phospholipid. This reinforces that even venoms which are still active in the absence of phospholipid may be skewed in relative potency. Even more dramatic was the change in potency when calcium was removed, with both *B. worthingtoni* and *D. russelii* being dramatically less, revealing that for both venoms the toxins responsible for FX activation have a high degree of calcium dependency (Fig. 2). However similar to phospholipid, the relative change was unequal with *Daboia* being proportionally less active in the absence of calcium than the observed change for *B. worthingtoni*.

The percentage of FX activation was also shown to be significantly less than that of the procoagulant viper *D. russelii* which was used as a positive control, however this does not negate the possibility that an envenomation from *B. worthingtoni* may be of medical importance. Furthermore, a number of other toxin types play a role in the overall effect of a venom, acting synergistically (Casewell et al., 2013). For example a secondary pseudo-procoagulant effect of the venom was further demonstrated in this study, supporting the findings of the previous study on this venom (Youngman et al., 2019a). In this study it is concluded that the direct pseudo-procoagulant action of *B. worthingtoni* venom upon fibrinogen is caused by SVSPs, which were neutralised by incubation with the inhibitor AEBSF (Fig. 3). Thus in a human envenomation, this background activity would contribute to the clinical picture by potentiating the depletion of fibrinogen resulting from the potential FX-activation venom-induced consumptive coagulopathy.

We also tested the procoagulant effects of the venom on amphibian plasma, as prey selectivity is a major factor which has been shown to cause venom variation, including in coagulotoxic venoms (Daltry et al., 1996; Sousa et al., 2018; Youngman et al., 2019b). Thromboelastography results show that *B. worthingtoni* venom is strongly procoagulant, both in regards to rate and strength of clot formation on amphibian plasma (Fig. 4). Rate of clot formation by *B. worthingtoni* venom was significantly greater than the kaolin positive control (Fig. 4). Additionally, a strong clot was formed by *B. worthingtoni* venom, although the clot formed by the kaolin control was stronger (Fig. 4). These results are noteworthy, as amphibian plasma is known to respond differently to the coagulotoxic effects of some snake venoms and the coagulation factors present in amphibian plasma vary to those of other vertebrates such as mammals (Doolittle, 2009; Ponczek et al., 2008; Sousa et al., 2018). No studies have investigated the diet of *B. worthingtoni* in the wild, however amphibians are known to be prevalent within their habitat and thus are a potential prey item (Parker, 1932). Amphibians are also known to represent a substantial proportion of the diet of *P. superciliaris* which share a common ancestor with *B. worthingtoni* (Mallow and Ludwig, 2003; Phelps, 2010), with *P. superciliaris* venom also potently clotting upon amphibian plasma (Fig. 4).

Therefore we propose that both amphibians and mammals potentially represent a substantial proportion of the diet of *B. worthingtoni*, leading to their venom possessing coagulotoxins which are selected to act upon both amphibian plasma as well as mammalian plasma. As venom toxins evolve under a strong 'use it or lose it' purifying selection pressure (Li et al., 2005a; Li et al., 2005b), these results are suggestive of both amphibians and mammals forming a proportion of the diet of *B. worthingtoni*. Due to the differences between amphibian and mammalian coagulation factors, toxins having potent effects upon mammal plasma can have little cross-reactivity with amphibian plasma and consequently the reverse is also true (Sousa et al., 2018; Youngman et al., 2019b). Therefore, in the absence of a benefit conferred by a diet that includes both amphibians and mammals as a proportion of their diet, toxins which are selective for either plasma would be under negative selection pressures. Ambush predators are also well known for taking a wide array of prey items and predominantly having a generalist diet (Glaudas et al., 2017; Shine et al., 2014). Thus, since venom is a complex mixture of numerous toxins, it follows that the venom of an ambush predator such as *B. worthingtoni* will be comprised of multiple toxins which are selective for different prey items. Diet breadth has also been proposed by previous research to mediate crude venom specificity, with species which possess broader diets tending to have broader venom activities (Harris et al., 2020; Lyons et al., 2020). The close morphological similarity and venom activity between *B. worthingtoni* and the confirmed amphibian-predator *P. superciliaris* along with *B. worthingtoni* ecological niche, is also further suggestive of amphibian predation by *B. worthingtoni*.

Thus, venom function may be a useful trait to infer diet for rare species located in remote regions, which are difficult to conduct field work upon. However, there is no substitute for natural history observations and extensive dietary studies. Therefore, future work should aim to investigate the dietary ecology of *B. worthingtoni* in the wild to elucidate any biases and if indeed amphibians or small mammals are a substantial prey for this species. If the inferences drawn by this study are confirmed by such observations, this would provide data supporting the use of venom activity to infer diet in difficult to study species.

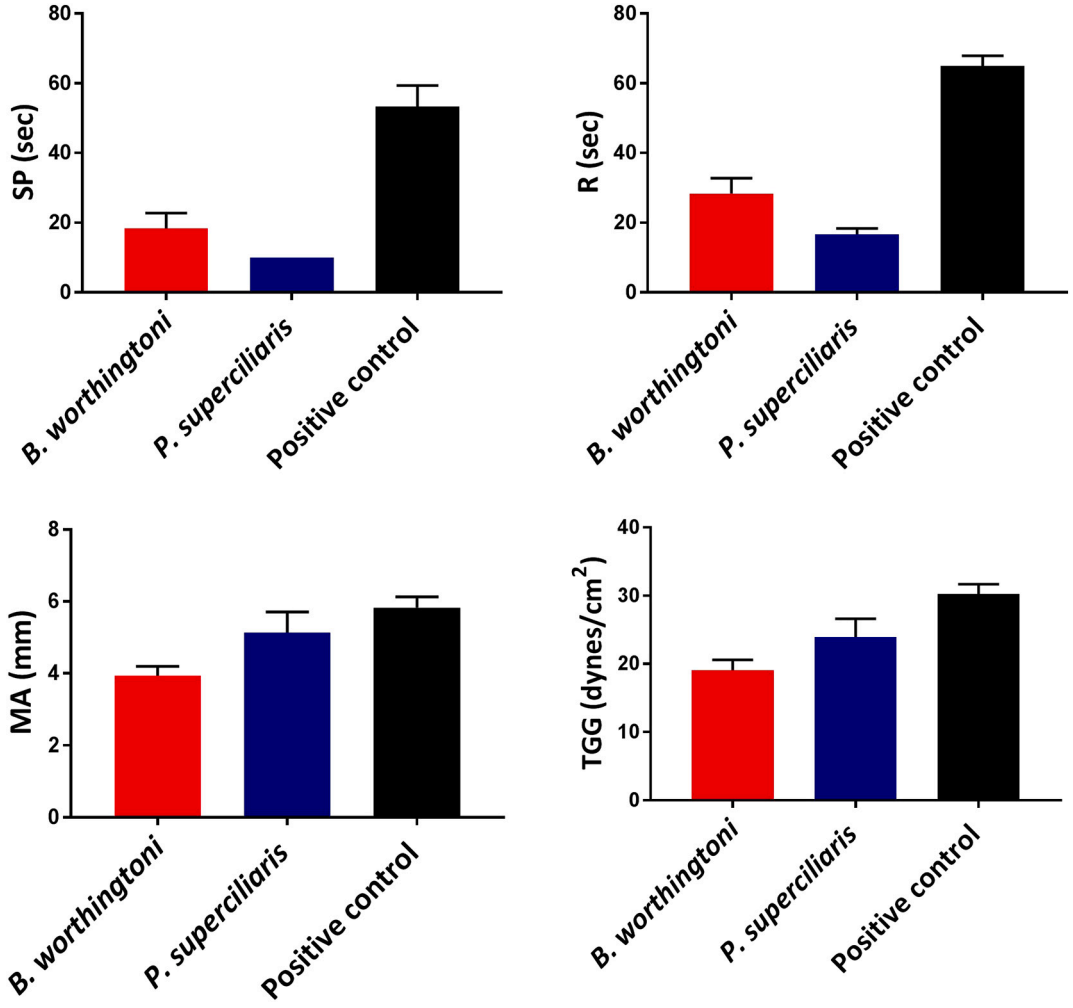

Fig. 4. Procoagulant effects of *B. worthingtoni* and *P. superciliaris* venom upon amphibian (*Rhinella marina*) plasma. The positive control represents a fully generated clot, utilising kaolin to active the intrinsic pathway of the clotting cascade. SP (split point) = time taken until clot begins to form (sec). R = time to initial clot formation where formation is 2 mm + (sec). MA (maximum amplitude) = maximum clot strength (mm). TGG = total thrombus generation as a measurement of clot strength (dynes/cm^2). Data points are N = 3 mean and standard error of the mean.

5. Conclusions

In conclusion this study investigated the procoagulant and pseudo-procoagulant activity of *B. worthingtoni* venom, identifying them as being driven by SVMP toxins and SVSP toxins respectively. The procoagulant SVMP toxins are also shown to be primarily FX activators. A main finding of this study is also the strong procoagulant effect *B. worthingtoni* venom has upon amphibian plasma, which leads to the hypothesis that either amphibians represent a proportion of their diet leading to selection for these toxins or that these toxins have been retained from a recent common ancestor. Future work should investigate the dietary preferences of this unique species, as well as look at venom composition as a whole to further unravel these questions.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2020.108921.

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