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# The sweet side of venom: Glycosylated prothrombin activating metalloproteases from *Dispholidus typus* (boomslang) and *Thelotornis mossambicanus* (twig snake)

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ABSTRACT

*Dispholidus typus* and *Thelotornis mossambicanus* are closely related rear-fanged colubrid snakes that both possess strongly procoagulant venoms. However, despite similarities in overall venom biochemistry and resulting clinical manifestations, the underlying venom composition differs significantly between the two species. As a result, the only available antivenom—which is a monovalent antivenom for *D. typus*—has minimal cross reactivity with *T. mossambicanus* and is not a clinically viable option. It was hypothesised that this lack of cross reactivity is due to the additional large metalloprotease protein within *T. mossambicanus* venom, which may also be responsible for faster coagulation times. In this study, we found that *T. mossambicanus* venom is a more powerful activator of prothrombin than that of *D. typus* and that the SVMP transcripts from *T. mossambicanus* form a clade with those from *D. typus*. The sequences from *D. typus* and *T. mossambicanus* were highly similar in length, with the calculated molecular weights of the *T. mossambicanus* transcripts being significantly less than the molecular weights of some isoforms on the 1D SDS-PAGE gels. Analyses utilising degylcosylating enzymes revealed that *T. mossambicanus* SVMPs are glycosylated during post-translational modification, but that this does not lead to the different molecular weight bands observed in 1D SDS-PAGE gels. However, differences in glycosylation patterns may still explain some of the difference between the enzymatic activities and neutralization by antivenom that have been observed in these venoms. The results of this study provide new information regarding the treatment options for patients envenomated by *T. mossambicanus* as well as the evolution of these dangerous snakes.

## 1. Introduction

Procoagulant species of venomous snakes are responsible for devastating effects upon the coagulation systems of envenomed patients. These venoms activate clotting factor zymogens (Factor X or prothrombin) to generate endogenous thrombin, resulting in the production of stable fibrin clots (Lister et al., 2017; Oulion et al., 2018; Rogalski et al., 2017; Sousa et al., 2018; Zdenek et al., 2019). Venoms which activate prothrombin are divided into functional groups (A, B, C, or D) based upon their requirement for the cofactors FVa and calcium (Joseph and Kini, 2002; Kini, 2005; Kini and Koh, 2016; McCleary and Kini, 2013; Rosing and Tans, 1991; Rosing and Tans, 1992). Group A and B are metalloproteases while C and D are serine proteases. Group A prothrombin activators do not require the aid of any cofactors and are found in several viper venoms, most notably *Echis carinatus* 'ecarin', while B requires the aid of calcium, such as 'carinactivase-1', also found in *Echis carinatus* (Joseph and Kini, 2002; Kini, 2005; Kini and Koh, 2016; McCleary and Kini, 2013; Rosing and Tans, 1991; Rosing and Tans, 1992; Yamada et al., 1996, 1997). It must be noted, however, that these convenient functional divisions are artificial distinctions based upon studies of purified toxins. However, the total venom composition is such that the venoms may be quite variable in their cofactor requirements. Thus relative calcium dependence is best viewed a sliding scale, rather than discrete divisions, as has been shown for venoms including vipers such as *Bothrops* and *Echis* and elapids including *Hoplocephalus*, *Notechis*, *Oxyuranus*, *Paroplocephalus*, *Pseudonaja*, and *Tropidechis* (Lister et al., 2017; Oulion et al., 2018; Rogalski et al., 2017; Sousa et al., 2018; Zdenek et al., 2019). For example, *Echis carinatus* varies from populations activity for which activity is decreased by only 40% in the absence of calcium but with activity decreased over 400% for other populations (Rogalski et al., 2017). Thus the simplistic division into Type A and Type B venoms does not reflect the more complex

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biological reality. This is a critically important when making conclusions based upon coagulation patterns, for if calcium is not included in the assay conditions there may be a radical shift in the venom patterns and thus evolutionary or clinical interpretations.

In addition to being central to the clinical picture of front-fanged snakes, procoagulation has also been shown to be responsible for the lethal effects of rear-fanged genera such as the closely related rear-fanged colubrid genera *Dispholidus* and *Thelotornis*, with this action being calcium and phospholipid independent in these species (Bradlow et al., 1980; Debono et al., 2017; Grasset and Schaafsma, 1940; Guillin et al., 1978; Hiestand and Hiestand, 1979; Kamiguti et al., 2000). While *D. typus* has been shown to be a true procoagulant species through prothrombin activation, closely related members of the *Thelotornis* genus have had far less investigation performed, despite their equal medical importance (Atkinson et al., 1980; Chapman, 1968; Debono et al., 2017; Du Toit, 1980; FitzSimons, 1909, 1919; FitzSimons, 1962; Weinstein et al., 2011; Weinstein et al., 2013).

In a previous study we investigated the variation in venom composition, activity, and antivenom neutralization between *D. typus* and *T. mossambicanus*. Despite both genera presenting virtually identical clinical symptoms, we found that *T. mossambicanus* venom was far more complex in composition and activity than that of *D. typus*. This was particularly noteworthy among the SVMP toxin family which is largely responsible for the potentially lethal procoagulant action of these venoms. As a result of these differences, *Dispholidus* antivenom had little to no effect on *T. mossambicanus* venom (Debono et al., 2017). Because of this, replacement therapy is the only treatment available for bite victims (Atkinson et al., 1980; Du Toit, 1980; Grasset and Schaafsma, 1940; Visser and Chapman, 1978; Weinstein et al., 2011). Here we investigated the molecular variation between both *Dispholidus typus* and *Thelotornis mossambicanus* by exploring their venom gland transcriptomes. We focused on variation within the SVMP toxin family, which could lead to a variation in prothrombin activating capabilities. In addition, we investigated variations in glycosylation within both venoms as this may also contribute to a lack of antivenom neutralization. This is the first time that factor X or prothrombin activation and transcriptomics have been investigated for *Thelotornis mossambicanus*. This research reinforces the high rates of venom diversification even between closely related snake species and how this can have direct implications for the treatment of envenomed patients.

## 2. Methods

### 2.1. Venom supplies

Pooled venoms from *Dispholidus typus* (South African origin) and *Thelotornis mossambicanus* (Mozambique) were supplied by Latoxan (Portes-lès-Valence, France).

### 2.2. Venom gland RNA extraction and mRNA purification: reconstruction of SVMP phylogeny

Venom gland tissue (20 mg) is homogenized using a rotor homogenizer and total ribonucleic acid (RNA) extracted using the standard TRIzol Plus methodology (Invitrogen). RNA quality is assessed using a Nanodrop (Nanodrop 2000, Spectrophotometer, Thermo Scientific). mRNA is extracted and isolated using standard Dynabeads mRNA DIRECT Kit (Life Technologies Ambion, 1443431). Dynabeads work by hybridising poly-thymine (polyT) sequences, covalently bound to the Dynabead surface, to the mRNA poly-adenine (polyA) tail. This ensures that mRNA is selected for and not lost in centrifuge and washing stages, while other RNA forms (which do not have a polyA tail) are removed. This therefore facilitates a higher concentration of mRNA yield for sequencing purposes.

#### 2.2.1. Sequencing

Venom gland transcriptomics were conducted by the IMB Sequencing Facility (Institute for Molecular Bioscience, The University of Queensland, St Lucia, Qld, Australia). Libraries were prepared with the TruSeq Stranded mRNA kit (Illumina, San Diego, CA, USA), and were sequenced on the Illumina NextSeq (Illumina) 500 using $2 \times 150$ bp reads and V2 chemistry.

The transcriptome described in this paper was one of several species that were part of a multiplexed run. Throughout our workflow GNU parallel 20170822 was used to run similar tasks in simultaneously, CD-HIT 4.8.1 was used to remove duplicate sequences, and Seqtk 1.2 was used to obtain subsets of sequence files (Fu et al., 2012; Li, 2012; Li and Godzik, 2006; Tange, 2011). Also, as a result of multiplexing, we experienced the common problem of low, but meaningful amounts of cross contamination between our multiplexed samples (Kircher et al., 2011). We attempted to mitigate this issue at several points in our methodology.

#### 2.2.2. Assembly

Illumina reads that were likely to be cross contamination between multiplexed samples were removed from our read files by identifying 57 nucleotide k-mers in our focal read set that were present in another read set from the same lane at a 1000-fold or higher level. Reads with 25% or more of their sequence represented by such k-mers were filtered from the data set. This was accomplished using Jellyfish 2.2.6 (Marçais and Kingsford, 2011) and K-mer Analysis Toolkit (KAT) 2.3.4 (Mapleson et al., 2016). We then removed adaptors and low-quality bases from the reads and removed any reads shorter than 75 base pairs using Trim Galore version 0.4.3 (Krueger, 2015). We then used PEAR 0.9.10 (Zhang et al., 2013) to combine pairs of reads whose ends overlapped into one, longer, merged read. We then carried out several independent *de novo* assemblies of these reads using the programs Extender version 1.04 (Rokyta et al., 2012), Trinity version 2.4.0 (Grabherr et al., 2011), and SOAPdenovo version 2.04 (Xie et al., 2014). SOAPdenovo was run repeatedly with k-mer sizes of 31, 75, 97, and 127.

#### 2.2.3. Annotation

The *de novo* assemblies were concatenated and TransDecoder 5.2.0 was used to predict open reading frames within the contigs (Haas et al., 2013). We removed any ORFs that did not include both a start and stop codon. The remaining ORFs were then compared against reference toxin sequences obtained from UniProt using ProteinOrtho 6.01 (Lechner et al., 2011; The UniProt Consortium, 2016). The resulting ORFs with similarity to known toxins were further quality controlled by manual inspection and BLAST searches against the Reptilia subsection of the NCBI Nucleotide database (Altschul et al., 1990; Altschul et al., 1997; Madden et al., 1996). The Burrows–Wheeler Aligner (BWA) 0.7.16a and SAMtools 1.5 were used to align the original reads from each species to the total list of annotated ORFs from every assembly (Li and Durbin, 2009; Li et al., 2009). These results were visualized using Tablet 1.15 to screen for signs of chimerical assembly (Milne et al., 2012). Those that showed sharp discontinuities in coverage maps across all species were removed from further analysis. A combination of read coverage, assembly quality, and BLAST search results were used to confirm the species of origin for each remain ORF. Given the evolutionary distance between the species we sequenced and the rate at which toxin genes mutate, it is extremely unlikely that any two of these species would express identical toxins. Because of this, we interpret reads from multiple species aligning to a single contig as instances of contamination and tentatively assign the contig to the species with significantly higher expression levels. This assignation can be further reinforced if our BLAST results indicate that the sequence in question is more similar to the high-expression taxon than the lower one. The distribution of reads mapped to the sequence can also be informative if the pattern matches the norm for that toxin family in one species but

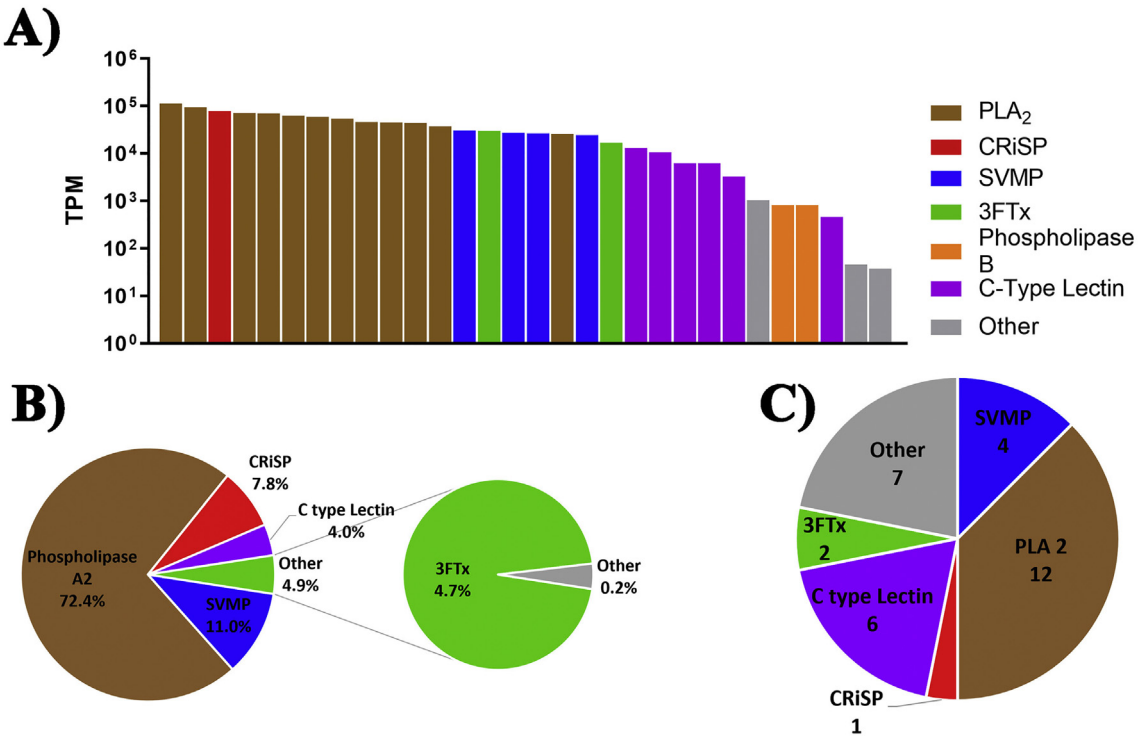


**Fig. 1.** A) Transcripts Per Million (Y axis = TPM) for each toxin family from *Thelotornis mossambicanus* venom gland transcriptome, B) percentage of TPM from toxin families recovered from *T. mossambicanus* transcriptome, C) total number of transcripts from toxin families recovered from *T. mossambicanus* transcriptome. Other = Kallikrein, Waprin, Kunitz, VEGF, hyaluronidase, SVSPs, PLB.

not the others. For most sequences, all three indicators were unambiguous and in agreement while in a small minority of cases two other indicators could be used to decide between somewhat equivocal results of a third. No sequences that had made it to this point of the quality control process could not be confidently assigned using these methods.

Information regarding the transcriptome, the short reads, and the final toxin sequences that were determined to originate from the transcriptome of *Thelotornis mossambicanus* have been deposited in GenBank under the BioProject accession code PRJNA561062.

#### 2.2.4. Phylogenetic reconstruction

Protein sequences for SVMP sequences from representative taxa were downloaded from the UniProt database (The UniProt Consortium, 2016). These were then combined with the translated SVMP sequences from our *T. mossambicanus* transcriptome and all available full-length high quality *Dispholidus typus* SVMP sequences (Pla et al., 2017). The sequences were aligned using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm implemented in AliView 1.18 (Edgar, 2004; Larsson, 2014). We reconstructed the phylogeny of these sequences using MrBayes 3.2 for 15,000,000 generations and 1,000,000 generations of burnin with lset rates = invgamma (allows rate to vary with some sites invariant and other drawn from a γ distribution) and prset aamodelpr = mixed (allows MrBayes to generate an appropriate amino acid substitution model by sampling from 10 predefined models) (Ronquist et al., 2012). The runs were stopped when convergence values reached 0.01.

### 2.3. De-glycosylation of venom proteins

For protein de-glycosylation under denaturing conditions, 20 µg of crude venom samples from *Dispholidus typus* and *Thelotornis mossambicanus* were subjected to analysis following the manufacturer's instructions from New England Bio Labs 'De-glycosylation' (Lot# 10034949, USA). Total reaction volumes of 10 µl were incubated with 1 µl Glycoprotein denaturing buffer (10×) (Bio Labs, Lot# 10017111) for 10 mins at 100 °C. Following denaturing, 2 µl 10× Glycoprotein 2 (Lot# 10029303), 2 µl 10% NP-40 (Lot# 10021820) and 1 µl (454 U) PNGase F (Lot# 10018057) was added to the mixture and incubated for 1 h at 37 °C. Post incubation, samples were submitted to 1D SDS PAGE under reducing and non-reducing conditions (by addition of DTT or not) following methods previously described (Debono et al., 2017).

### 2.4. Factor X activation and prothrombin activation

To determine the relative rate of Factor X activation or prothrombin activation by venom samples, a colorimetric assay was performed previously validated by us (Debono et al., 2019). A working stock solution (at 1 mg/ml in 50% deionized $H_2O$ + 50% glycerol) was manually diluted with OK buffer (STA Owren Koller Buffer) (50 µl at 20 µg/ml) to a cuvette. A total of 50 µl of $CaCl_2$ (25 mM), 50 µl phospholipid (cephalin prepared from rabbit cerebral tissue from STA C. K Prest standard kit, solubilized in OK buffer) and 25 µl of OK buffer was added to cuvette and incubated for 120 s at 37 °C before adding 75 µl of a solution containing the colorimetric substrate (Liquid Anti-Xa substrate, Stago LOT 253047) and either 0.01 µg/µl FX (Human Factor X, Lot #HH0315, HTI) or 0.1 µg/µl prothrombin (Human Prothrombin, Lot # GG1026, HTI) (total volume 250 µl/cuvette). A change in the optical density (OD) was measured each second for 300 s using a STA-R Max® automated analyzer (Stago, Asnières sur Seine, France). All tests were performed in triplicate. The rate of FX activation or prothrombin activation, by the samples, was calculated in comparison to the variation in the optical density – corresponding to the cleavage of the substrate by FXa or thrombin, after the activation of the zymogen by the samples, in relation to direct cleavage of substrate by the samples in the absence of the zymogen. Human FXa (Liquid Anti-Xa FXa LOT 253047) or thrombin (Liquid Fib, Stago, LOT 253337) was used as positive control to ensure quality of the substrate. The samples analyzed, in same conditions, without the presence of the zymogen were used as a negative control to establish and venom-induced baseline substrate cleavage in

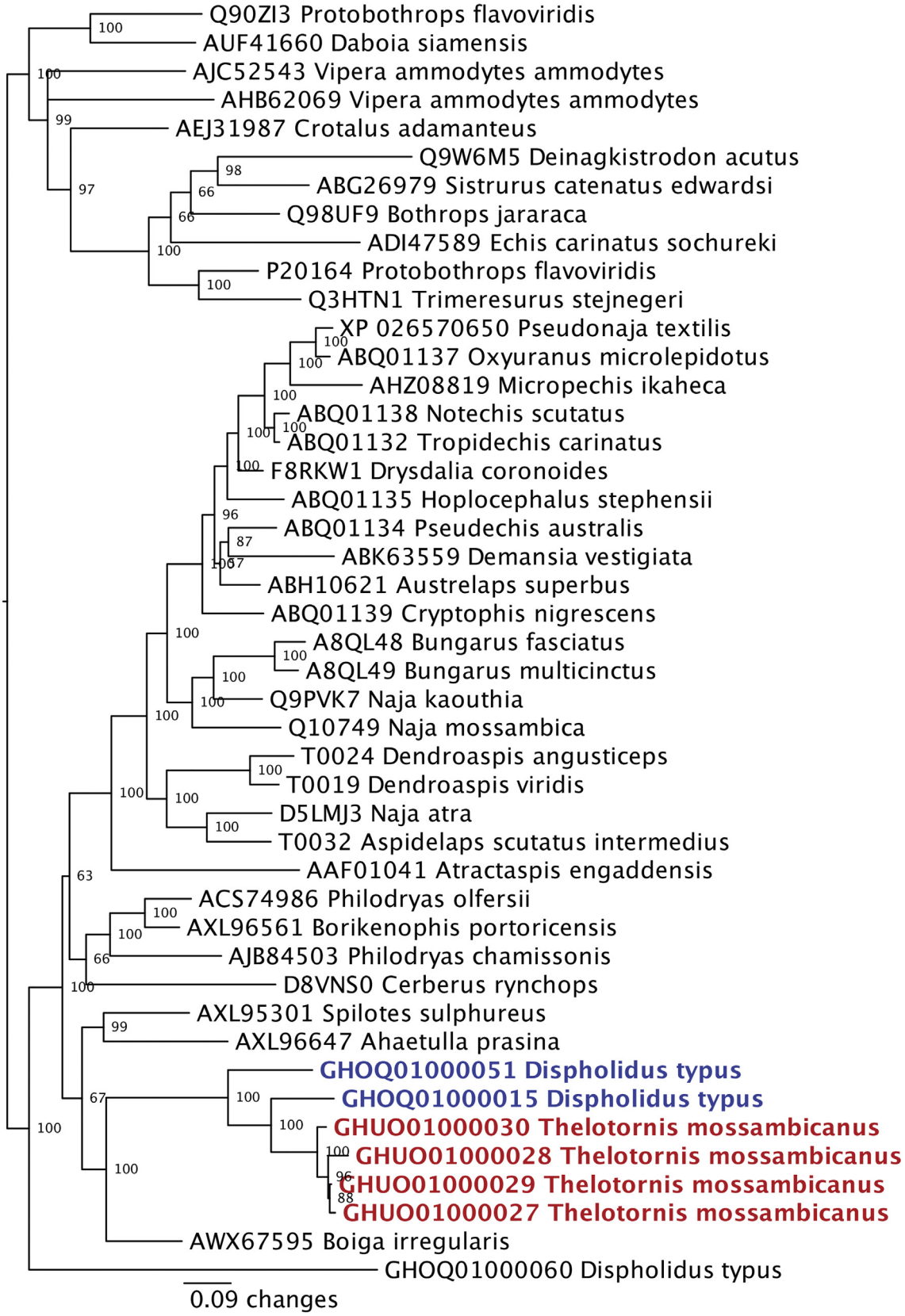


**Fig. 2.** Phylogeny of publicly available SVMPs amino acid sequences, with all recovered *Thelotornis mossambicanus* SVMP venom gland transcriptome sequences, with nodes showing posterior probability. Scale bar represents 0.5 substitutions per site.

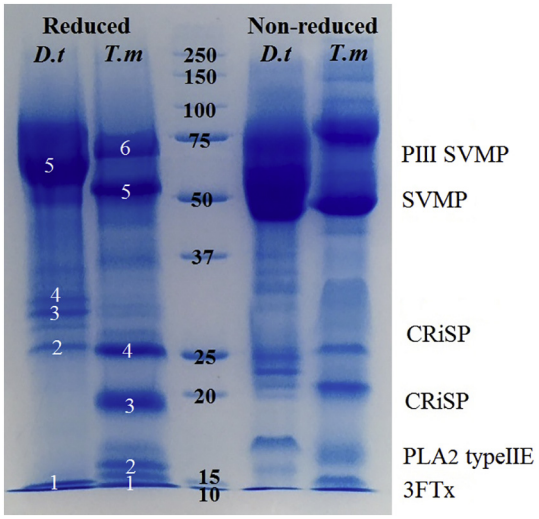


**Fig. 3.** 1D SDS page with identified groups of proteins (as previously published by us (Debono et al., 2017)). D.t = *Dispholidus typus*, T.m = *Thelotornis mossambicanus*. Reduced conditions on the left, Non-reduced conditions on the right. Molecular weight marker indicated by centered numbers (kDa). Annotations (right) refer to bands identified (white numbers) within the reduced column.

the absence of the zymogen. A solution containing deionized $H_2O$/glycerol at a 1:1 ratio was used as a second negative control. Multiple linear regression and ANOVA on the AUC values were used to test for differences between the three treatments and yielded similar results.

## 3. Results

### 3.1. Transcriptomics

Transcriptomics recovered a diversity of toxin types including 3FTx, C-type lectin, CRiSP (cysteine rich secretory protein), $PLA_2$ (phospholipase $A_2$), PLB (phospholipase B), SVMP (snake venom metalloprotease). $PLA_2$ toxins were the most diverse toxin family and accounted for the greatest share of Transcripts Per Million (TPM) (72.4%, from 12 transcripts) while SVMPs contributed 11.0% of TPM from 4 transcripts (Fig. 1). We recovered 6 unique C-type lectins, yet these were expressed at relatively low levels (4% of total TPM). CRiSP and 3FTx were both responsible for greater shares of TPM despite lower numbers of total transcripts recovered (7.8% from 1 transcript and 4.7% from 2 transcripts, respectively). These results are consistent with our proteomics that showed abundant $PLA_2$s, but also significant quantities of SVMPs.

### 3.2. SVMP phylogeny

Four complete SVMP transcripts were recovered from the *T. mossambicanus* venom gland transcriptome. These transcripts were nested within complete, publicly available *D. typus* SVMPs transcripts (GHOQ01000060, GHOQ01000051, GHOQ01000015), distinct from neighbouring genera *Boiga* and *Ahaetulla* (Fig. 2). The SVMPs from *D. typus* and *T. mossambicanus* formed a tight, distinct clade within a larger rear-fanged and elapid clade separate from viper SVMPs, thus supporting *D. typus* and *T. mossambicanus* SVMP originating from a common molecular ancestor despite the poor cross-neutralization of *D. typus* antivenom (Debono et al., 2017).

### 3.3. Proteomics

As transcriptomes were not available at the time, previous proteomic work focused on protein ID (Debono et al., 2017) and did not investigate in-depth the structural differences of SVMP between *D. typus* and *T. mossambicanus* that result in the two distinct SVMP bands in *T. mossambicanus* when investigated on 1D SDS-PAGE (bands 5 and 6 in Fig. 3). Therefore, digestion with a deglycosylation enzyme was undertaken, with both *D. typus* and *T. mossambicanus* SVMP showing evidence of extensive glycosylation, as demonstrated by molecular weight changes between homologous bands in gel lanes with and without the deglycosylation enzyme (Fig. 4).

### 3.4. FX and prothrombin activation

To evaluate the comparative ability of *D. typus* and *T. mossambicanus* venoms to activate FX and prothrombin, assessment was carried out

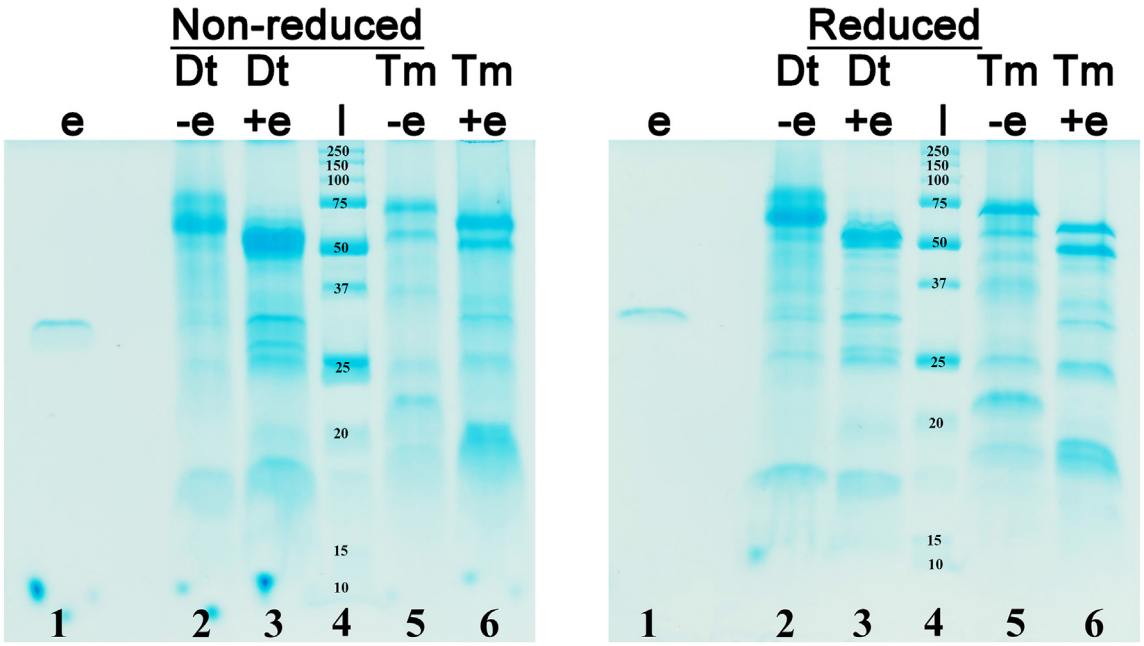


**Fig. 4.** Differential glycosylation of *Dispholidus typus* and *Thelotornis mossambicanus* venoms. e = ± deglycosylation enzyme, l = ladder. Lane 1 = control enzyme, lanes 2 and 3 = *D. typus* ± deglycosylation enzyme, lane 4 = ladder, lanes 5 and 6 = *T. mossambicanus* ± deglycosylation enzyme.

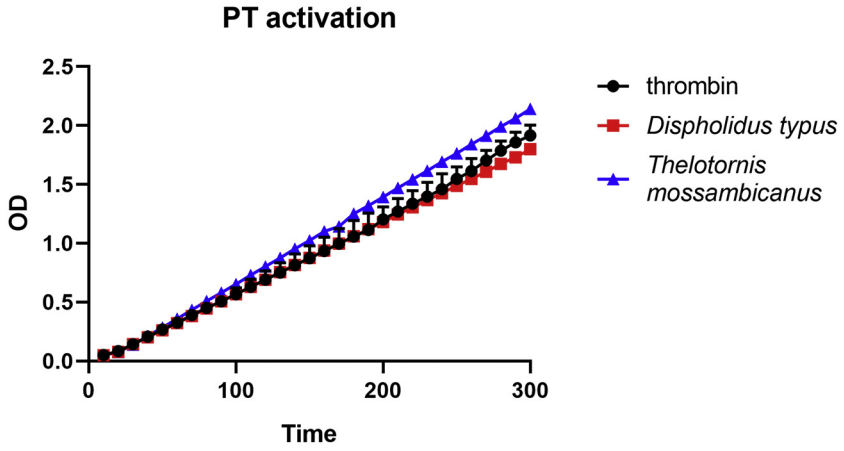


**Fig. 5.** Prothrombin activation of *Dispholidus typus* and *Thelotornis mossambicanus* at 10 μg/ml over 300 s. Blue line = *T. mossambicanus* activity, red line = *D. typus* activity, black line = positive control thrombin. Y axis = OD, optical density. X axis = time (s). $N = 3$, data points = average with standard deviation.

using a Stago STA R Max coagulation analyzer robot following the previously validated colorimetric assay (Debono et al., 2019). Zymogen testing revealed that neither species was able to activate FX (*data not shown*). While FX activation has also been shown from *Dispholidus typus* (Bradlow et al., 1980), this activity was recovered from ground up venom gland as opposed to venom milking which may have contributed to it being an artefact in activity due to the presence of non-venom proteins or activated blood factors. However, when testing for prothrombin activation, a starting concentration of 20 μg/ml exceeded the maximum reading capacity for both species indicating extremely high activation of prothrombin, so a lower level of venom concentration was tested. At a half dilution concentration of venom (10 μg/ml), *Thelotornis mossambicanus* produced a stronger prothrombin activation effect compared to the positive control of pure thrombin (Liquid Fib, Stago, LOT 253337) which was not diluted ($p < 0.0001$) (Fig. 5). *Dispholidus typus* also had a strong prothrombin activating response but weaker than that of *T. mossambicanus*, being just slightly less than that of the thrombin control ($p < 0.0001$). This variation was consistent with previously published differential clotting times for both species, indicating their extreme but divergent procoagulant effects (Debono et al., 2017).

## 4. Discussion

Our *Thelotornis mossambicanus* transcriptome revealed major differences in overall venom composition compared to that of *D. typus* (published by (Pla et al., 2017)). While *D. typus* venom is dominated by P-III SVMP (Fig. 1, (Debono et al., 2017), Fig. 3), *T. mossambicanus* has a higher percentage of smaller toxins such as $PLA_2$ and CRiSP than *D. typus* (Fig. 1). The SVMP of both venoms were shown for the first time to be heavily glycosylated (Fig. 4), as has been previously shown for pit viper venoms such as *Bothrops* species (Andrade-Silva et al., 2016; Oliveira et al., 2010; Zelanis et al., 2012; Zelanis et al., 2010). Despite the fact that *T. mossambicanus* venom contains proportionally less procoagulant SVMPs than *D. typus*, *T. mossambicanus* is much more powerfully procoagulant (Debono et al., 2017), due to stronger prothrombin activation compared to *Dispholidus typus* (Fig. 5).

While *D. typus* and *T. mossambicanus* are lethally potent procoagulant colubrids, the currently available antivenom is effective for only one of the species – *D. typus* (Debono et al., 2017). Therefore, the only currently available treatment for *Thelotornis* bites is replacement therapy, strictly on a case-by-case basis (Weinstein et al., 2013). The potent procoagulant activity of *T. mossambicanus* venom is entirely independent of cofactors, meaning that neither calcium nor phospholipid is required for such fast clotting of plasma (Debono et al., 2017). This had previously been shown and reported for *D. typus* (Bradlow et al., 1980; Guillin et al., 1978; Hiestand and Hiestand, 1979; Weinstein et al., 2011; Weinstein et al., 2013; Yamada et al., 1997) however not for *T. mossambicanus*. SVMPs are the likely source of both species' procoagulant nature as these toxins are known prothrombin activators and both venoms express them at relatively high levels (Joseph and Kini, 2002; Kini, 2005; Kini and Koh, 2016; Rogalski et al., 2017; Rosing and Tans, 1991; Rosing and Tans, 1992; Rosing and Tans, 2010; Sousa et al., 2018). Furthermore, due to neither species requiring cofactors to cause such potent procoagulation, their potent prothrombin activating SVMPs can be categorised into Group A prothrombin activators (Joseph and Kini, 2002; Kini, 2005; Kini and Koh, 2016; Rosing and Tans, 1991; Rosing and Tans, 1992; Rosing and Tans, 2010) and are resistant to natural endogenous coagulation inhibitors such as serpins and antithrombin III (Kini, 2005; Kini and Koh, 2016; Yamada et al., 1996). To date, the best characterised Group A prothrombin activator is ecarin, isolated from *Echis carinatus* (Kini, 2005; Kini and Koh, 2016; Kornalik and Blombäck, 1975; Rogalski et al., 2017; Rosing and Tans, 1991; Rosing and Tans, 1992). SVMPs from vipers are well documented with many representatives displayed in Fig. 2. Interestingly, the SVMPs isolated from *T. mossambicanus* and *D. typus* form their own prothrombin activating clade, distinct from other Group A prothrombin activators such as from species within the *Echis* genus of vipers. This is evidence for the first time that the prothrombin activating SVMPs do not cluster with any of the other prothrombin activating SVMPs from other species. This also suggests that prothrombin activation as a neofunctionalisation of SVMP convergently evolved multiple independent occasions, spanning the full advanced snake taxonomical range from rear-fanged colubrids to viperid front-fanged snakes (Fig. 2).

Despite large similarities in P-III SVMPs from both species, four distinct transcripts were recovered from the venom gland transcriptome of *T. mossambicanus*. The amino acid sequence is identical for 73.9% of the total length of the mature peptide for each of the SVMPs. Because of this, the predicted molecular weights of these transcripts cannot fully explain the different SVMP band pattern between *D. typus* and *T. mossambicanus*. Post-translational modifications such as glycosylation can have significant effects on the weight of mature proteins and variation in glycosylation is a feature that has been shown in pit vipers to be linked to variations in activity and antivenom recognition (Andrade-Silva et al., 2016; Chen et al., 2008; Lin et al., 2010; Oliveira et al., 2010; Zelanis et al., 2012). However, our results revealed similar levels of N-glycosylation in *D. typus* SVMPs as well as both SVMP bands from *T. mossambicanus*. It is possible that each of these possesses different patterns of glycosylation along the length of the actual protein, but determining if that is the case and, if so, what effect that might have on the enzymatic activity of these toxins is a subject for future studies. The cause of the weight difference between the two bands of *T.*

*mossambicanus* SVMPs remains unclear, but it is likely the result of some other process of post-translational modification.

Irrespective of antivenom efficacy, these two species have been the centre of controversial discussion, with both genera causing infamous deaths to two imminent herpetologists as both species were deemed non-venomous (Atkinson et al., 1980; Bradlow et al., 1980; Chapman, 1968; Du Toit, 1980; FitzSimons, 1962; Grasset and Schaafsma, 1940). However, decades later, there is plenty of evidence to state otherwise, as both species are extremely procoagulant, with strong prothrombin activation capabilities (Bradlow et al., 1980; Debono et al., 2017; Du Toit, 1980; FitzSimons, 1909; Grasset and Schaafsma, 1940; Guillin et al., 1978; Hiestand and Hiestand, 1979; Visser and Chapman, 1978; Weinstein et al., 2011). Differences in venom composition, despite producing identical clinical symptoms, can be best attributed to recent evolutionary pressures from diet and prey escape potential linked to variations in ecology between the two lineages, leading to subtle structural and surfaces changes to key toxin families such as post translational modifications in the form of glycosylation, which should be the focus of future work. Research such as this can lead to improving alternative therapeutic designs and pave the way for further P-III SVMP research and Group A prothrombin activators.

## Transparency document

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

## Acknowledgements

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