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Getting stoned: Characterisation of the coagulotoxic and neurotoxic effects of reef stonefish (*Synanceia verrucosa*) venom

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HIGHLIGHTS

- Venom of *Synanceia verrucosa* binds to α1 nicotinic acetylcholine receptor mimotopes.
- Cardiotoxicity of the venom is in part due to binding to DIV Ca_V channel mimotopes.
- Anticoagulant venom activity is likely caused by the degradation of phospholipids.
- Lyophilised venom altered some pathophysiological functions.

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ABSTRACT

The reef stonefish (*Synanceia verrucosa*) is a venomous fish which causes excruciatingly painful envenomations. While some research on the pathophysiology and functions of the venom have been conducted, there are still some gaps in the understanding of the venom effects due to the extreme lability of fish venom toxins and the lack of available testing platforms. Here we set out to assess new functions of the venom whilst also attempting to address some unclear pathophysiological effects from previous literature. Utilising a biolayer interferometry assay, our results highlight that the venom binds to the orthosteric site of the α-1 nicotinic acetylcholine receptor as well as the domain IV of voltage-gated Ca^{2+} ($Ca_V1.2$) channel mimotopes. Both these results add some clarity to the previously ambiguous literature. We further assessed the coagulotoxic effects of the venom using thromboelastography and Stago STA-R Max coagulation analyser assays. We reveal that the venom produced anticoagulant activity and significantly delayed time until clot formation of recalcified human plasma which is likely through the degradation of phospholipids. There was a difference between fresh and lyophilised venom activity toward the nicotinic acetylcholine receptor mimotopes and coagulation assays, whilst no difference was observed in the activity toward the domain IV of $Ca_V1.2$ mimotopes. This research adds further insights into the neglected area of fish venom whilst also highlighting the extreme labile nature of fish venom toxins.

1. Introduction

Stonefish (*Synanceia* spp.) have arguably one of the most painful defensive venoms causing excruciating pain (pain perception has only been tested on humans and rats), and are aptly named for their stone-like camouflage, blending into reefs and shallow rocky shores of tropical and sub-tropical Indo-pacific regions (Ferrari and Ferrari, 2002). Species in this genera are equipped with ~13 mobile dorsal spines that are each associated with a distinct venom gland (Endean, 1961; Smith et al., 2016). Clinical symptoms of envenomations from the genus *Synanceia* are fairly consistent across the species and include intense localised pain, inflammation, muscle paralysis, hypotension and in severe cases, cardiac collapse leading to death (Church and Hodgson, 2002; Khoo, 2002; Ziegman and Alewood, 2015).

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The reef stonefish (*S. verrucosa*) has been a key study species in understanding the composition and functional activities of fish venom. The venom of *S. verrucosa* has an intravenous (i.v.) LD_{50} in mice of 0.36 mg/kg, which is comparable to some snake venoms (Shiomi et al., 1989). The venom is highly cytotoxic, causing localised inflammation and necrosis as well as having highly specific haemolytic activity toward rabbit erythrocytes (Shiomi et al., 1989). The haemolytic activity was later attributed to an isolated pore forming toxin, verrucotoxin (VTx), which also caused a hypotensive response and was lethal in mice (Garnier et al., 1995). However, no further studies have attempted to attribute the other cytotoxic activities to this toxin, despite the likelihood that VTx produces numerous cytotoxic activities given the known promiscuity of perforin-like toxins to multiple cell types (Ellisdon et al., 2015). Nephrotoxicity has also been observed (Wahsha et al., 2019), which causes haemorrhaging and degeneration of tubules with cellular proliferations. Further, another pore forming toxin was also discovered, neo-verrucotoxin (neo-VTx), which had similar haemolytic activities to VTx, but possessed some differences in its structure (only 87–95 % sequence similarities) that may result in differential cytotoxic activities (Ueda et al., 2006).

Cardiotoxic activities are also a major clinical symptom of envenomations, leading to hypotension and cardiac arrythmia (Abe et al., 1996; Garnier et al., 1996, 1997). The exact mechanism of action causing these cardiac effects was initially suggested as voltage-gated Ca^{2+} (Ca_V) channel binding by a toxin named cardioleputin (Abe et al., 1996). However, it was later suggested that the altered Ca_V channel activity might also be caused by VTx, which may be promiscuous in further activating ATP-sensitive K_V channels (Garnier et al., 1997). Yet, further investigations into the cardiac effects of the crude venom suggested the overall activity was the activation of β-adrenoreceptors by catecholamines (Garnier et al., 1996; Sauviat et al., 1995) and/or VTx (Yazawa et al., 2007). This could then cause Ca_V channel activity seen in other studies, since activation of β-adrenoreceptors increases the stimulation of L-type Ca_V channels (van der Heyden et al., 2005). Thus, it is evident that the exact cardiotoxic mechanisms appear relatively unresolved and it is uncertain if both these activities occur independently or if the activity of β-adrenoreceptors is the sole cause of cardiotoxicity.

In addition to cardiotoxicity, neurotoxic effects, namely partial or complete limb paralysis in mice, have been observed with crude venom of *S. verrucosa* (Breton et al., 1999); however, no further studies have been conducted. Venom from the closely related *S. horrida* has been investigated for neurotoxic activity using a chick biventer cervicis nerve muscle preparation assay (CBCNM). Fresh crude venom of *S. horrida* markedly inhibited both nerve-evoked and directly evoked twitches, increasing basal tension and abolishing the activity of agonists acetylcholine (ACh) and carbachol (CCh), which is indicative of postsynaptic nicotinic acetylcholine receptor (nAChR) blocking. However, the authors suggested this activity might be due to myotoxic effects of the venom since the response to potassium chloride (KCl) decreased (Church and Hodgson, 2000). Despite this, the authors noted that they could not completely rule out that the increase in basal tension was due to direct action of the venom at the nAChRs. Given the similarities in venom activities between *S. horrida* and *S. verrucosa*, it is possible that these same effects may occur; however, the neurotoxic activity of *S. verrucosa* has never been fully investigated.

Despite the research already conducted on the venom activities of *Synanceia* species, some fundamental gaps in the understanding of the venom composition and mechanisms still require addressing. There has been a lapse in research in recent years, not only on *Synanceia* spp. but on fish venom in general (Harris and Jenner, 2019). This is likely in part due to the extreme labile nature of fish venoms (Barnett et al., 2017; Carlson et al., 1971; Malacarne et al., 2018; Shiomi et al., 1989). The venom toxins can be extremely sensitive to denaturing with slight changes in pH, temperature, lyophilisation and even fractionation processes causing the crude venom/toxins to decrease or even abolish toxic activities. This lability has caused many problems in functional testing and characterising of toxins, which is due to denaturing of the large enzymatic toxins which cause the majority of the pathophysiological effects (Church and Hodgson, 2002). These issues have been well documented for both *S. horrida* (Barnett et al., 2017; Church and Hodgson, 2000) and *S. verrucosa* (Abe et al., 1996; Garnier et al., 1995, 1997) venoms.

As there is a need to examine the clinically relevant *Synanceia* species, we set out to investigate new functional activities of *S. verrucosa* crude venom and to unravel ambigous biological activities from previous studies. We utilised a validated biolayer interferometry (BLI) assay in which mimotopes (a small part of the epitope) were designed upon specification of the amino acid sequence from whole channel receptor types. We assessed the postsynaptic binding activity toward the orthosteric site

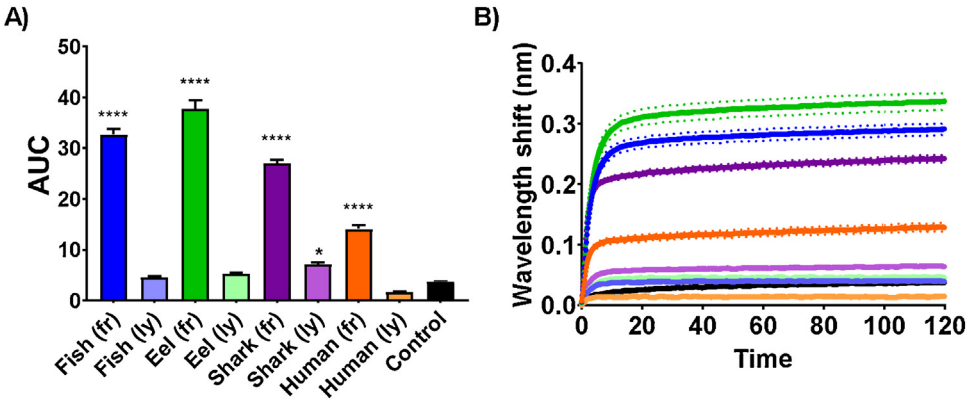

Fig. 1. The effects of fresh (fr) and lyophilised (ly) venom from *S. verrucosa* against fish, eel, shark and human representative mimotope sequences. A) Bar graphs represent the mean area under the curve (AUC) values of the adjacent curve graphs. B) Curve graphs show the mean wavelength (nm) shift in light with increased binding of venoms over a 120 s association phase. The venom was tested in triplicate (n = 3). The control was venom from *Crotalus horridus* which has been used previously as a control for nAChR binding (Harris et al., 2020a, b; Youngman et al., 2021) since there is no nAChR binding toxins within the venom. Error bars on all graphs represent the SEM (error bars on graph B are represented by dots surrounding the curves). AUC values were statistically analysed using a one-way ANOVA with a Dunnett's multiple comparisons post-hoc test comparing to the control. A statistical significance is annotated by * ($p < 0.05$) and **** ($p < 0.0001$) above the corresponding bar. All raw data and statistical analyses outputs can be found in Supplementary data 1.

(acetylcholine binding region) of nAChRs which has been previously validated (Harris et al., 2020a, b; Harris et al., 2020c; Zdenek et al., 2019). This allows us to determine if there is in fact nAChR activity of *Synanceia* venom or if the activity observed by Church and Hodgson (2000) was solely due to myotoxicity. The BLI was also utilised to assess venom interference with the function of one or more of the four domains of L-type $Ca_V1.2$ ion channels (Wu et al., 2015). $Ca_V1.2$ is widely expressed in smooth muscle, particularly in the heart (Shaw and Colecraft, 2013) and is associated with excitation and muscle contraction (Tsien, 1983). $Ca_V1.2$ is a prime target by some animal toxins and causes current inhibition (Klint et al., 2014; Vieira et al., 2005) which likely leads to cardiac arrhythmias and, in certain cases, cardiac collapse. Assessing if the venom targets this channel will indicate whether the Ca_V channel activity determined by Abe et al. (1996) was genuine, or if the β-adrenoreceptor action observed by Sauviat et al. (1996) was the cause of the Ca_V channel activity. Furthermore, we assessed the effects of *S. verrucosa* crude venom upon the blood coagulation cascade using a thromboelastography (TEG) and Stago STA-R Max coagulation analyser assay since blood coagulation activity is found in other fish venom, including the closely related *S. horrida* (Khoo et al., 1992; Memar et al., 2016; Sommeng et al., 2020). Additionally, given the extreme lability of fish venom, we examined each of these activities under both fresh and lyophilised venom conditions to determine if there is any loss or decrease in activity.

2. Results and discussion

We found differences in our results when screening both fresh and lyophilised venom binding on mimotopes designed to mimic the orthosteric site of taxa representative nAChRs from potential predators such as fish, eel, shark, and human. The fresh crude venom bound to the mimotopes whereas the activity of the lyophilised venom was significantly reduced/inactivated (Fig. 1). There was a statistical significance between all mimotopes tested with the fresh venom compared to the lyophilised venom and the control ($p < 0.0001$), whilst there was no significance found between the lyophilised venom and the control.

These data suggest that there are some toxins within the crude venom of *S. verrucosa* that bind to the orthosteric site of postsynaptic nAChRs and this is the first confirmed instance for this species. Research conducted on the biological effects of the closely related *S. horrida* venom in a chick biventer cervicis nerve preparation assay reported that the venom inhibited both nerve-evoked and directly-evoked twitches also increasing basal tension and abolishing the action of agonists ACh and CCh which is indicative of nAChR blocking (Church and Hodgson, 2000). However, due to an increase in KCl response the authors suggested that the results may be caused by myotoxic effects but stated the possibility of nAChR binding toxins could not be disregarded. Given the similarities between *S. horrida* and *S. verrucosa* venom (Church and Hodgson, 2002; Ziegman and Alewood, 2015), in combination with the data we present here, it is possible that the activity seen by Church and Hodgson (2000) was nAChR binding and that due to a combination of known cytotoxic venom components the data was somewhat obscured from fully concluding any nAChR activity. Since cytotoxicity would not affect the mimotopes, these data are strongly suggestive that nAChR binding toxins are present within the venom of *S. verrucosa*, although further data are needed to fully conclude on this. Additionally, since the lyophilised venom did not have the same activity as the fresh venom, the toxin(s) responsible for the nAChR targeting are likely large and labile.

Toxins that target nAChRs are predominantly predatory since the clinical manifestation is flaccid paralysis (e.g. α-neurotoxins from elapids and cone snails (Barber et al., 2013)). However, given

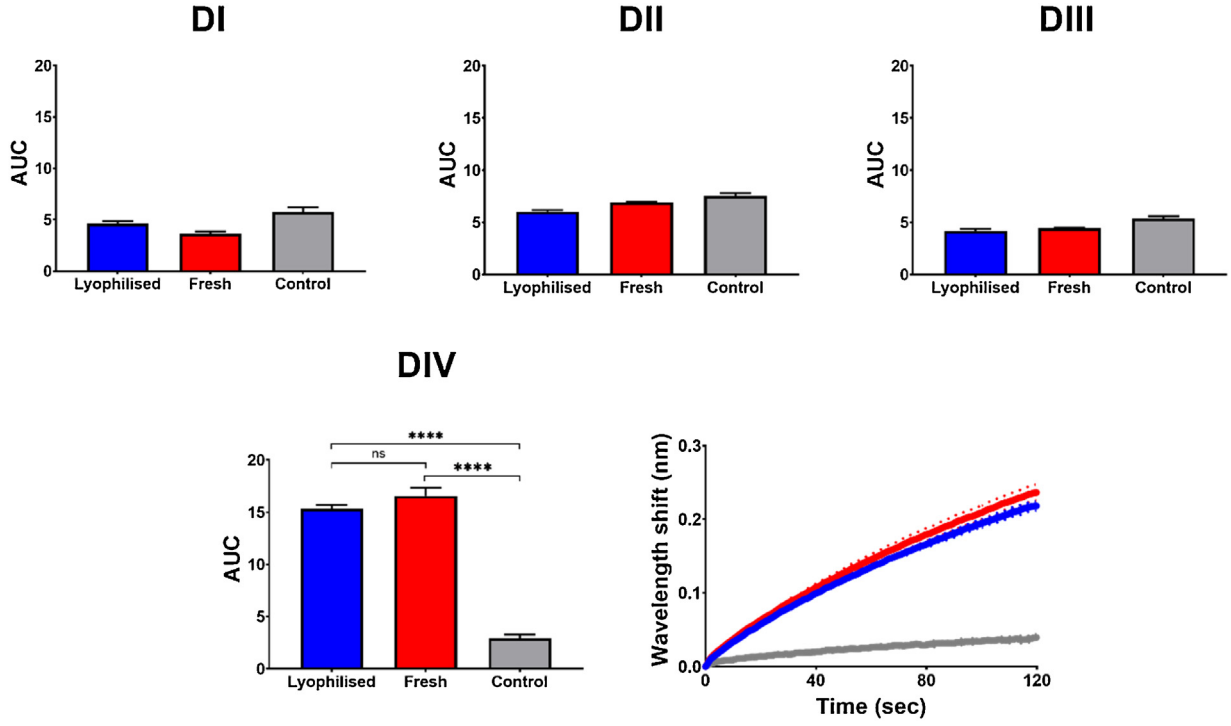

Fig. 2. The effects of fresh and lyophilised venom from *S. verrucosa* against L-type $Ca_V1.2$ DI-IV mimotopes. Bar graphs represent the mean area under the curve (AUC) values. The negative control was venom from the cobra *Naja kaouthia*, which was representative of a venom devoid of significant activity upon Ca_V channels and thus any binding observed would be non-specific. The curve graph shows the mean wavelength (nm) shift in light with increased binding of venoms over a 120 s association phase of DIV. The venom was tested in triplicate (n = 3). Error bars on all graphs represent the SEM. AUC values were statistically analysed using a one-way ANOVA with a Tukey's multiple comparisons post-hoc test. A statistical significance is annotated by **** above the corresponding bar with a significance threshold of $p < 0.0001$, whilst no significance is denoted as ns. All raw data and statistical analyses outputs can be found in Supplementary data 1.

that *S. verrucosa* utilises its venom for defense, this finding does not seem to fit the classical use of these toxins types. One explanation may be that these toxins cause numbing/paralysis of the mouth of their predators that handle prey with their mouths, such as sharks and rays. This would render the musculature of the mouth weakened through paralysis in combination with causing intense pain through other toxins, ultimately leading to a greater escape potential. The ecology of *S. verrucosa* and their predators is an area which is somewhat data deficient. Thus, the ecological inferences proposed here need to be fully tested in the future.

Further, the human mimotope was the lowest binding of the different taxa representatives (Fig. 1). Since fish venoms are defensive and less likely to show target specificity, the most parsimonious explanation is that the human nAChR is less susceptible than the other orthosteric sites. This has previously been suggested since α-neurotoxins from snakes have a much lower binding affinity toward the human α-1 nAChR than native prey types (Dellisanti et al., 2007; Harris et al., 2020b; Ishikawa et al., 1985; Silva et al., 2018).

One drawback to these data is that the sequences for the eel representative is a freshwater species (*Gymnotus electricus*), thus might not be truly representative of a potential sympatric predator. Although this sequence seems conserved across freshwater eel lineages, there are no sequences for marine lineages to compare. Thus, it is uncertain if the freshwater eel sequence is conserved across other marine families to give an adequate representative of potential predators.

Testing of the crude venom on mammalian L-type $Ca_V1.2$ DI-IV mimotopes revealed moderate binding of both the fresh and lyophilised venom toward DIV but not to DI, II and III (Fig. 2). Both the fresh and lyophilised venom showed no significant difference suggesting that the binding activity of L-type $Ca_V1.2$ DIV is not affected by lyophilisation and the toxin(s) responsible for this activity are likely unaffected by freeze-thaw cycles. It is possible that these toxins are small peptides or possibly an amine type molecule which is more stable than the large enzymatic/proteinaceous molecules within the venom.

These data further support previous literature suggesting the presence of Ca_V channel activators within the crude venom (Abe et al., 1996; Garnier et al., 1997). Given that previous research also highlighted β-adrenoreceptor activity (Sauviat et al., 1995), which is linked to activation of L-type Ca_V channels (van der Heyden et al., 2005) and may have been causing the association of Ca_V channel activation in other studies, our data suggests the presence of toxins that bind to DIV of $Ca_V1.2$ channels. Thus, both β-adrenoreceptor and $Ca_V1.2$ activities are likely since defensive venoms are

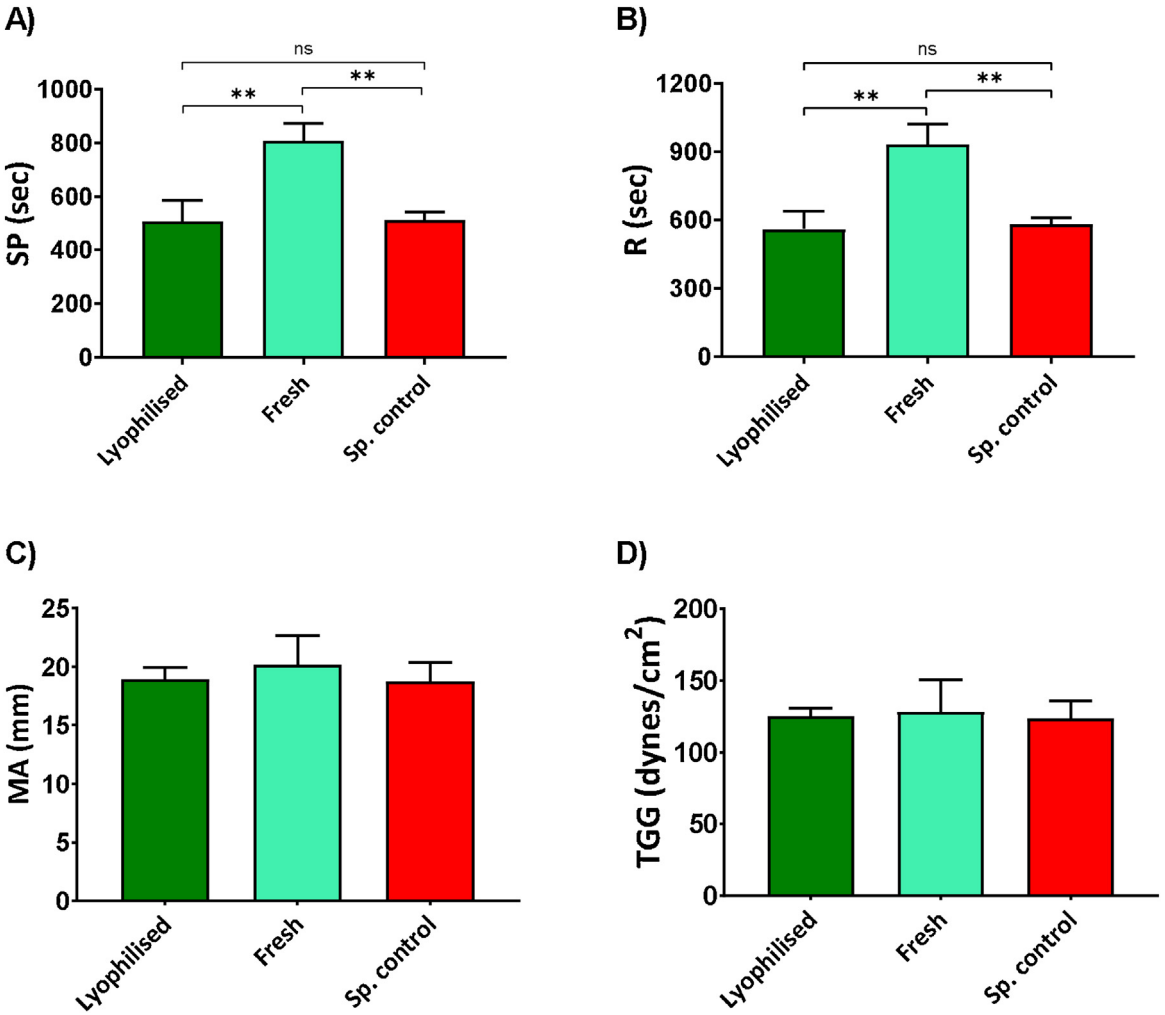

Fig. 3. Anticoagulant activity of *S. verrucosa* venom upon clotting of recalcified human plasma. Negative control represents spontaneous clotting of human plasma. A) SP (split point) = time taken until clot begins to form (sec). B) R = time to initial clot formation where formation is 2 mm+ (sec). C) MA (maximum amplitude) = maximum clot strength (mm). D) 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. Mean values were statistically analysed using a one-way ANOVA with a Tukey's multiple comparisons post-hoc test. A statistical significance is annotated by ** above the corresponding bar with a significance threshold of $p < 0.01$, whilst no significance is denoted as ns. All raw data and statistical analyses outputs can be found in Supplementary data 1.

intended to cause maximal pathophysiological stress in a short amount of time to enable escape, thus multiple toxins that target the same systems are likely to evolve within the venom.

Since the BLI only detects binding, it is unclear if the toxin that binds to $Ca_V1.2$ is an agonist or antagonist (activator or inhibitor). The mechanisms of Ca_V channels are not well described in comparison to Na_V and K_V channels and to date only some toxins that inhibit $Ca_V1.2$ channels have been described (Klint et al., 2014; Vieira et al., 2005), thereby underscoring the knowledge gap of these channel subtypes. It has been suggested that activation of $Ca_V1.2$ is achieved by DII and DIII whilst inactivation may be achieved by DIV (Pantazis et al., 2014). Given that binding to DIV was seen by *S. verrucosa* venom it is possible that inhibition is occurring. A toxin that inhibits $Ca_V1.2$ would likely shorten the cardiac action potential leading to arrhythmia, particularly in the presence of β-adrenoreceptor toxins. However, more extensive research is needed both on the physiological mechanisms of Ca_V channels and on toxins that target these receptors.

Previous studies on *S. verrucosa* venom have shown haemolytic activity, yet there has been no investigation into the effect of the venom upon coagulation cascade. We tested the venom on a thromboelastography assay which revealed that fresh *S. verrucosa* venom exhibited anticoagulant activity and significantly delayed time until clot formation of recalcified human plasma, extending the time until clot formation (SP) and the time until clot formation was greater than 2 mm (R) (Fig. 3A and B). Conversely, the lyophilised venom had no significant anticoagulant activity on time until clot formation (Fig. 3). Further, the fresh venom had no significant effect on the strength of the clot formed (Fig. 3C and D). Possessing toxins with anticoagulant activity may facilitate the spread of pain inducing toxins into the organism, as has been suggested for cobras (Bittenbinder et al., 2019, 2018).

As the ultimate strength of the clot (which is reflective of fibrinogen levels) was not affected, this indicates that the anticoagulant activity was not due to destructive cleavage of fibrinogen but was instead impedance of an upstream clotting factor. Further investigation into the anticoagulant activity was unable to determine the mechanism of action, with assays testing the inhibitory effect of the venom upon the intrinsic pathway; thrombin, FXa, prothrombinase complex, FIXa and FXIa all showing no significant inhibition (see supplementary Fig. 1). This indicates that the toxins within *S. verrucosa* venom are likely acting on either the extrinsic pathway or interfering with coagulation cofactors such as phospholipids. The degradation and hydrolysis of phospholipids is a known anticoagulant mechanism from phospholipase class toxins (Dashevsky et al., 2021; Kini, 2005; Ouyang et al., 1981; Verheij et al., 1980). The closely related *S. horrida* venom has been revealed to also have similar anticoagulant activity (Khoo et al., 1992) whilst also possessing phospholipase A_2 (PLA2), phospholipase B and phospholipase D class toxins (Ziegman et al., 2019). It is uncertain if these phospholipase toxins are what cause the anticoagulant activity in *S. horrida*, however given that PLA_2 toxins can cause a wide array of enzymatic and non-enzymatic effects including in other venomous fish (*Pterois* spp.) inducing procoagulation (Memar et al., 2016; Sommeng et al., 2020), this is a likely scenario for *S. horrida* phospholipase toxins. Therefore, since the venom across *Synanceia* are very similar, it is possible that *S. verrucosa* may also possess phospholipase toxins which might be responsible for causing phospholipid degradation leading to the prolonged coagulation observed. However, further investigations into the activity of the venom upon phospholipid is merited with emphasis on the discovery and characterisation of the anticoagulant toxins.

Based on these data, it is clear that there is a current gap in knowledge regarding the venom composition of *S. verrucosa* venom. To bridge this gap and further understand which toxins are responsible for each pathophysiological effects, it is imperative that future research should endeavour to describe the composition of the crude venom.

Although this study provides solid evidence in bridging some fundamental knowledge gaps regarding the venom activities of *S. verrucosa,* there is a small limitation in that the venom was only used from a single individual. This is a limitation in that intraspecific venom variation may exist between individuals, although to the author's knowledge this has never been tested with any *Synanceia* species. However, given that the venom of *S. verrucosa* is defensive and the simplicity of fish venom, it is unlikely to vary greatly between individuals if at all. Regardless, future studies should address the potential for intraspecific venom variation.

In summary, this research set out to test some new functions of *S. verrucosa* venom and to assess some unclear functions described in the literature. For the first time we have shown venom activity to contain nAChR binding to the orthosteric site which supports previous studies which had suggested this mechanism but had ambiguous data. We also show that there are toxins binding to $Ca_V1.2$ DIV which was also unclear from previous literature since it was uncertain if Ca_V channel activity was due to β-adrenoreceptor activity of the venom. Further, we highlight for the first time the anticoagulant activity of *S. verrucosa* venom which is consistent with the closely related *S. horrida*. This study also highlighted the labile nature of fish venom and showed that some functional activities may be lost or significantly reduced with lyophilisation methods of venom.

3. Materials and methods

3.1. Venom collection and preparation

An adult female *S. verrucosa* (approx. 45 cm) was captured on reefs near Port Douglas, Queensland, Australia and held in aquarium facilities by Cairns Marine Pty Ltd, Cairns, Australia. She was then transported and kept in an aquarium (250 L) in the School of Biological Sciences, The University of Queensland. After two weeks of husbandry and care, the *S. verrucosa* was milked using a standard syringe fish venom extraction method. The venom was then placed on ice and the concentration of the fresh venom was immediately determined in triplicate using a NanoDrop 2000 UV–vis Spectrophotometer (Thermo Fisher, Sydney, Australia) at an absorbance wavelength of 280 nm and made into a working fresh stock of 1 mg/mL. The fresh working stock was then immediately tested on all assays mentioned. The fresh venom was then lyophilised and reconstituted in double deionised water (ddH2O) and then made into a working stock (1 mg/mL). The lyophilised stock was then tested on the same assays under the exact same conditions.

All animal housing and experimental procedures were conducted in accordance with local and international regulations. The housing of the animal was approved by the University of Queensland Animal Ethics Committee (SBS/260/20). The milking of the fish and attainment of the venom was also conducted in accordance with the Animal Ethics Committee (SBS/260/20) and the Biosafety Committee (IBC/134B/SBS/2015) under approved permits.

3.2. Mimotope production and preparation

Following methods from a previously developed assay (Harris et al., 2020a, b; Harris et al., 2020c; Zdenek et al., 2019), a 13–14 amino acid mimotope of the vertebrate α-1 nAChR orthosteric site was synthesised by GenicBio Ltd. (Shanghai, China), and designed upon specification obtained from chrna1 sequences from UniProt

and GenBank databases. Accession codes for the amino acid sequences of the α-1 nAChR orthosteric site for each taxa were as follows: fish α-1 (uniprot P02710), eel α-1 (uniprot E1BT92 and uniprot P09688), shark α-1 (GenBank CM012962.1 46493573 to 46493839), human α-1 (uniprot P02708). Further details on mimotope preparation and specifications can be found in previous literature utilising this assay (Harris et al., 2020a, b; Harris et al., 2020c, d; Zdenek et al., 2019).

The L-type $Ca_V1.2$ domain I-IV mimotopes were designed based on specifications of the human CACNA1C sequence (uniprot Q13936) and further literature (Pantazis et al., 2014).

Mimotope dried stocks were solubilised in 100 % dimethyl sulfoxide (DMSO) and diluted in ddH2O at 1:10 dilution to obtain a stock concentration of 50 μg/mL in 10 % DMSO. Stocks were stored at −80 °C until required.

3.3. Biolayer interferometry (BLI)

Full details of the developed assay, including a full methodology and data analysis, can be found in the validated protocol (Zdenek et al., 2019). In summary, the BLI assay was performed on the Octet HTX system (ForteBio, Fremont, CA, USA). Venom samples were diluted at 1:20 (50 μg/mL per well). Mimotope aliquots were diluted at 1:50 (1 μg/mL per well). The assay running buffer was 1X DPBS w/ 0.1 % BSA and 0.05 % Tween-20. Streptavidin biosensors were hydrated in the running buffer for 30 min whilst on a shaker at 2.0 revolutions per minute (RPM). The dissociation of analytes occurred using a standard acidic solution glycine buffer (10 mM glycine (pH 1.5–1.7) in ddH2O). Raw data are provided in Supplementary File 1. All data obtained from BLI on Octet HTX system (ForteBio) were processed in accordance with the validation of this assay (Zdenek et al., 2019) and then imported into Prism8.0 software (GraphPad Software Inc., La Jolla, CA, USA) and graphs were created.

3.4. Plasma collection and preparation

Human plasma was collected from healthy donors and donated by the Australian Red Cross. Pooled human plasma (Label #540020731976, AB +, citrate 3.2 %) was aliquoted, then flash-frozen in liquid nitrogen and immediately stored at – 80 °C until required. When required for experiments, plasma was thawed at 37 °C in water bath and used immediately. All venom and plasma work was undertaken under University of Queensland Biosafety Committee Approval (IBC/134B/SBS/2015) and Ethics Committee Approval (#2016000256).

3.5. Thromboelastography

Thromboelastography testing was utilised to investigate the coagulotoxic activity of both lyophilised and fresh *S. verrucosa* venom on human plasma. Assays conducted were adapted from previously published protocols which have utilised thromboelastography assays to measure the effect of the venom on the clotting time and clotting strength of recalcified human plasma (Grashof et al., 2020; Youngman et al., 2019a). For each 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 human plasma. Pooled frozen human plasma was thawed and warmed to 37 °C for 5 min in a water bath before use. Clotting was measured over a 30 min period and the parameters measured included; time taken for a clot to begin to form (SP), time to initial clot formation where formation is 2 mm+ (R), maximum amplitude (MA) and total thrombus generation (TGG). For this assay the negative control, which represents a fully generated spontaneous clot, was conducted using 7 μL of ddH_2O and glycerol at a 50:50 ratio in replacement of venom. All assays were conducted in triplicate.

3.6. Inhibitory coagulation assays

Additional coagulation assays were carried out on a Stago STA-R Max coagulation analysing robot (Stago, France), to investigate the mechanism of action for the anticoagulant activity of fresh *S. verrucosa* venom. Pooled frozen human plasma was thawed and warmed to 37 ºC for 5 min in a water bath before being placed in the Stago STA-R Max machine. To identify the target in the clotting cascade, either plasma or individual factors of the clotting cascade were incubated with the venom following methodology adapted from previously validated protocols (Bittenbinder et al., 2018; Youngman et al., 2019a, b; Zdenek et al., 2020). The incubation step is included to allow time for the venom to bind and inhibit its target. All assays were conducted in triplicate. Supplementary Table 1 details the methodology used for each of the inhibitory assays conducted.

3.7. Statistical analysis

All statistical analyses were conducted using Prism8.0 software (GraphPad Software Inc., La Jolla, CA, USA). The statistical analysis for the nAChR binding data (Fig. 1) was achieved using a one-way ANOVA with a Dunnett's multiple comparisons post-hoc test comparing to the control. For the $Ca_V1.2$ binding (Fig. 2) a one-way ANOVA with a Tukey's multiple comparisons post-hoc test was conducted. The analysis for the anticoagulant venom activities (Fig. 3) was also a one-way ANOVA with a Tukey's multiple comparisons post-hoc test.

Author contributions

Conceptualisation, R.J.H.; data acquisition, R.J.H., N.J.Y.; funding acquisition, B.G.F.; investigation, R.J.H., N.J.Y.; methodology, R.J.H., N.J.Y., F.B.; resources, R.J.H., N.J.Y., W.C., K.L.C., B.G.F.; writing—original manuscript, R.J.H.; writing—review and editing, R.J.H., N.J.Y., W.C., F.B., K.L.C., B.G.F.; supervision, B.G.F. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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