Characterization of marine venoms

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I came out the other side.

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Abstract

Many venomous marine creatures cause painful and lethal envenomations worldwide, and the number of stings is predicted to increase as warming ocean temperatures drive the expansion of geographic distribution. Jellyfish and stonefish encompass two medically important groups of venomous marine animals, causing a high number of stings annually in the Indo-Pacific region. The severity of jellyfish and stonefish stings is generally dose-dependent, where severe envenomation is linked to cardiovascular pathologies, such as tachycardia, bradycardia, hypotension or hypertension, depending on the species, and can be fatal. Venoms are typically complex mixtures of many components, including proteins, peptides and organic molecules that have bioactivity towards neuronal, muscular and cardiac systems, leading to pathologies and symptoms that cause morbidity and/or mortality in victims. The high diversity in venom components leads to a wide array of pharmacological activities which can target various biological functions in human systems. The current understanding of the venom compositions in jellyfish and stonefish and the link to the sting pathologies is very limited. This warrants closer investigation of jellyfish and stonefish venoms to further understand their molecular composition and related mechanisms of action. Furthermore, toxins with high potency and selectivity for biological targets offer a rich resource of natural products with potential as physiological tools or therapeutic leads.

This dissertation was conducted in two stages. The first stage investigated stings caused by jellyfish, focusing on two different species of cubozoans, the sea wasp, *Chironex fleckeri*, and the Irukandji Syndrome-causing jellyfish, *Carukia barnesi*. Envenomation by the former is painful, causes dermonecrotic lesions and appears to directly affect cardiomyocytes, whereas stings by the latter seem innocuous at first, with more severe pathologies occurring after a latent period, which indicates an indirect cardiotoxicity by the venom. Currently, the pathophysiology of these venoms is mostly unknown, resulting in controversy surrounding appropriate treatment protocols for victims. This dissertation attempted to understand the mechanisms of action of these venoms through a non-invasive murine model, which used pulsed-wave Doppler technology to monitor anaesthetized mice in real time from venom injection to death. The findings indicated that *C. fleckeri* venom causes significant effects on both the systolic and diastolic functions of mice hearts, which is consistent with a toxin that acts directly on the heart. *C. barnesi* venom, however, appears to cause a stress induced cardiomyopathy in the animals. This study indicated the venoms have differing modes of action and validated the Doppler technology as a method that can be used to investigate toxicity mechanisms and venom dose response in live animals.

The second stage of this dissertation studied stonefish venoms and their potential as a source of novel therapeutics. Stonefish stings also cause local and systemic edema in victims, which indicates the presence of inflammatory components within the venom capable of modulating immunological responses. Therefore, immunological effects and properties of stonefish venoms were investigated in two distinct species, the reef stonefish *Synanceia verrucosa* and the estuarine stonefish *Synanceia horrida*. An ecological assessment comparing the effect of time between successive milkings and the effect of starvation on venom yield was investigated. The results showed that although venom yield was significantly affected by starvation, individuals could restore the main venom components in intervals as short as four weeks post venom extraction.

Immunological assays performed *in vitro* revealed for the first time that stonefish venoms have immunosuppressive properties. This surprising result, given that stonefish venom produce inflammatory effects in both experimental models and clinical reports, was investigated at both the protein and gene levels. Crude venoms downregulated a number of pro-inflammatory cytokines, which are proteins secreted by immunological cells, and are responsible for a range of inflammatory processes which aim to protect the host. Furthermore, analytical analyses were combined with immunological assays to investigate the compounds found within stonefish venoms and their relative role in immunosuppression. These analyses revealed the presence of small molecules and small proteins that are reported for the first time in these venoms and are likely related to the immunosuppression observed.

Collectively, the results within this dissertation offer new avenues of treatment modalities and diagnostic tools for the treatment of jellyfish and stonefish stings in live models of envenomation with the pulsed-wave Doppler technology. Additionally, this dissertation also revealed the potential of stonefish venoms to be used as physiological tools and as a source of novel drug leads. I encourage future researchers to dive into the world of marine venom research with focus on mitigating venom effects in sting victims and/or discovering novel therapeutics within venoms.

Challenges and Limitations

Apart from the inherent challenges that venom researchers usually face, such as the ones previously described, the COVID-19 pandemic posed yet another hurdle to be surpassed. The pandemic has slowed research progress by casting a shadow on research projects underway and shifting the focus of research to the development of vaccines and viral therapeutics (Wickremsinhe et al., 2021). Apart from that, the pandemic was responsible for paralyzing many industries and disrupting supply chain worldwide due to shortages in research supplies, production of goods coming to a complete halt and lack of reactivity by the suppliers themselves, amongst other issues (El Baz and Ruel, 2021). These led to the global shortage of laboratorial consumables (Moreyra et al., 2022) and, in the case of our laboratory, we experienced significant shortages in filtered pipette tips of various sizes that took months to be replenished, severe delays in reagents shipments, and greatly affected delivery of mice. At the time of writing (February 2022), we are facing yet another mice shortage due to unavailable space in flights and currently there is a global supply issue of 225 cm^2 of tissue culture flasks.

Apart from shortage of consumables and reagents brought by the pandemic, serious financial issues have now become evident. Direction of research undertaken at a given locality is explained by incentives, team composition and available infrastructure and tools (Zhang et al., 2021), which means that research requires funding. One of the main funding for Australian universities is the recruitment of international students, which accounted for over AUD$ 40 billion dollars in 2019, being Australia's fourth largest export (Thatcher et al., 2020). Unfortunately, this has shifted during the COVID-19 pandemic. The education industry in Australia, particularly the university sector, has shown a decline in growth due to lockdowns and international border closures (Thatcher et al., 2020). Additionally, the main source of government funding for Australian Universities is through the Commonwealth Grant Scheme (CGS), but its funding is capped at 2017 levels (Thatcher et al., 2020). With the decline of the level of international students and available funding for academic research, it was estimated that more than 21,000 jobs would be at risk at the beginning of 2020, and it was also predicted that academic research output will be severely impacted as a result of COVID-19 (Thatcher et al., 2020).

Naturally, these quantifiable changes put added pressure on researchers worldwide at all career levels (Moreyra et al., 2022), but there is also the intangible factors to be included in the equation. Many people suffered psychologically and silently due to the relative lack of progress in their areas, especially post-graduate students (Sands, 2021). Supervisors had to adapt to working from home or quarantining after conferences and other engagements that required their presence, while taking care of their post-graduate students' and postdoctoral fellows' mental wellbeing and psychological needs while maintaining lab morale (Sands, 2021). Nonetheless, the members of our laboratory managed to

make progress towards finalizing research and completing PhD students, thanks to the resilience of our group and the brilliant management and constant mentorship applied by our supervisors.

Bringing these issues from a macro perspective and focusing on this thesis and its related research, the impacts that COVID-19 have had are evident. The pandemic restrictions and the lack of laboratory consumables have changed how this research was planned and conducted. Many experiments had to be rethought or cancelled altogether due to shortages in reagent supply or lack of available staff members to oversee the experiment, advise or teach a particular technique. Staff and resources had to be redirected to doing the most urgent, informative and necessary experiments, especially regarding those that were already underway, which had priority over experiments that had not yet started. Additionally, since this research is based on venom and depends on the supply of venom, the experiments have also been impacted due to the lack of this precious material. Nonetheless, adaption, resilience, will power and a plasticity in mindset shown by this student allowed this research to move forward, focusing on different areas that needed further investigation and were financially available to be investigated. My ability to adjust and learn new skills rapidly and effectively allowed research progression, where data were collected where funding and technical support were available, which meant that I was only able to collect certain data sets as I moved through different research areas.

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Chapter 1

1 Introduction

1.1 Marine venoms

The marine environment is often viewed as beautiful, mysterious and as a source of dangerous creatures. Apart from the obvious and infamous larger animals, such as sharks, whales and crocodiles, danger lurks in the form of much smaller, venomous, inconspicuous, and potentially lethal animals, such as corals, octopus, cone snails, stingrays, jellyfish, and fish. These venomous marine animals are the culprits of a large number of accidental stings annually, which cause morbidity and mortality in human beings worldwide, but especially in Australia (Balhara and Stolbach, 2014; Berling and Isbister, 2015). Australian venoms and toxins, as a group, are the deadliest in the world (Sutherland and Tibballs, 2001), placing Australia in a unique toxinological position and is arguably the perfect place to study marine venoms.

Venoms are primarily used to subdue prey items, defend against predators or aid in prey digestion (Sutherland and Tibballs, 2001). Unfortunately, humans are often inadvertently envenomed by animals, which can cause deleterious effects in victims. Venoms are a mixture of complex chemicals that affect various bodily functions due to the wide variety of components found within a single drop (Fry et al., 2009; King, 2011). This diversity arises from the numerous combinations of proteins, peptides, organic molecules, and salts which comprise the venom (Fry et al., 2009; King, 2011). Upon envenomation these compounds can interfere with neuronal transmissions, disrupt cell metabolism and signaling, and produce inotropic and chronotropic effects, which ultimately lead to pathologies and symptoms such as severe pain, paralysis of the affected limb or complete immobility, dermatitis, respiratory failure, cardiovascular collapse, amongst others (Balhara and Stolbach, 2014; Berling and Isbister, 2015).

Synergism between individual venom components plays an important role in the overall venom activity, as they interact with an array of targets that ultimately enhance venom potency and subsequent pathologies. For instance, stingray venoms seem to have synergism between translationally controlled tumour proteins and hyaluronidases, which might aid in the severe necrosis seen in victims (Kirchhoff et al., 2021). In cone snails, a group of peptides seem to act synergistically to inhibit neuromuscular transmission (Olivera, 1997). Due to this high diversity in venom compounds, their synergism and potential polypharmacology (action on multiple targets), studying, discovering, characterizing, and understanding each venom compound are important steps. To further our knowledge and improve treatment modalities to reduce morbidity and/or mortality in envenomed victims, understanding the mechanisms of action of each venom component and their synergistic

actions is paramount. Although numerous studies have been performed over past decades, much is still to be learned about marine venom compounds, including their molecular composition, activity, synergy and mechanistic actions in human beings. It is with this in mind that this doctorate thesis started, to shed light on the unknown and bridge gaps to improve our current understanding of marine venoms.

1.2 Jellyfish and stonefish venoms

Of all the venomous marine organisms that inhabit Australian waters, two major venomous taxa are medically important not only locally, but in the Indo-Pacific region: bony fish and jellyfish. Several venomous fish species produce painful and potentially lethal human envenomations. Fish species comprise over 50% of all venomous vertebrates, terrestrial or aquatic, and generally the venom pharmacology is similar between taxa. The Synanceiidae family, which incorporates the estuarine and reef stonefish *Synanceia horrida* and *Synanceia verrucosa*, respectively, is of particular note. These are regarded as the world's most venomous fish species, found throughout the warm waters of the Indo-Pacific region, the Persian Gulf, and the Red Sea (Saggiomo et al., 2021). Independent reports place stonefish stings as a) one of the major causes of hospitalizations in Australia in 2017-2018 (Pointer and Harrison, 2021), b) the second-most implicated fish in envenomations in Hong Kong from 2005 to 2008 (Chan et al., 2010), and c) responsible for a high number of stings in Singapore (Khoo, 2002; Ngo et al., 2009), and there is growing concern that sting numbers will increase in Japan due to warmer ocean temperatures (Hifumi et al., 2020). As with many venomous fish, the venom apparatus lacks musculature, so envenomation happens involuntarily while the animal is defending itself. Upon envenomation in humans, severe pain can radiate from the affected limb to the regional lymphatics, where edema and erythema may happen. Apart from these localized symptoms, systemic complications may also develop, such as weakness, tachycardia, pulmonary edema, convulsions, respiratory and/or cardiac failure (Saggiomo et al., 2021). Severe stings can lead to death, with an 11-year-old boy in Bora Bora in November 2018 being the latest recorded death (Maillaud et al., 2020).

Many of these symptoms have been linked to cytolysins identified and purified in stonefish venoms. Fish cytolysins are multifunctional proteins and stonustoxin, purified from *S. horrida* venom, is an example, being responsible for the lethal, hemolytic, cardiovascular, and neuromuscular activities seen both *in vitro* and *in vivo* (reviewed by Campos et al., 2021). In the case of *S. verrucosa* venom, verrucotoxin is responsible for the lethal, hemolytic, and cardiovascular activities (reviewed by Campos et al., 2021). Although research has evolved and knowledge has improved in the past few decades, the mechanisms through which stonefish venoms give rise to the observed pathologies and symptoms remain poorly understood. Chapter 3 is a review of the literature and highlights the current

state of knowledge of stonefish venoms, including experimental and clinical effects, which the reader is encouraged to read for further information.

In jellyfish species, prey acquisition relies on envenomation via nematocysts, an explosive organelle found inside cells that are mainly located in the animal's tentacles (Williamson et al., 1996). Nematocysts are highly specialized penetrative structures, usually a few micrometers in length (Östman, 2000) that deliver venom through one of the fastest biological mechanisms known (Nüchter et al., 2006). When the nematocysts are triggered by a physicochemical stimulus (e.g. encounter with prey or bather's skin), their coiled penetrative threads are inversed and discharged, penetrating and depositing venom across the epidermis, dermis and/or lymphatics of the target (Burnett, 2001) (Figure 1.1A and B). Of all jellyfish species, cubozoans (named after the cube-like shape of their bell) cause the majority of hospitalisations and deaths in Australian waters (Carrette, 2014). Within the cubozoans, two groups are of medical concern: the small box jellyfish that cause Irukandji Syndrome (Figure 1.1C), with symptoms ranging from nausea and headache, through pulmonary edema and severe cardiac insufficiency to death (Carrette et al., 2012); and the large box jellyfish known as the "sea-wasp" (*Chironex fleckeri*) (Figure 1.1D), whose stings have been responsible for over 70 deaths in Australia (Currie and Jacups, 2005). Following 15 years of no recorded deaths in Australian waters by *Chironex fleckeri*, a teenage boy died in March 2021 as a result of severe envenomation by this species (Tiemensma et al., 2021). Although there is much evidence pertaining to the cardiovascular effects of venoms from *Carukia barnesi* (one of the Irukandji Syndrome-causing jellyfish) and *C. fleckeri*, the precise mechanisms of action of these venoms are still largely unknown.

Stings caused by *C. fleckeri* are painful, cause dermonecrotic lesions, and death can occur within a few minutes (Currie, 1994; Endean et al., 1969). Animal and laboratory models, together with clinical evidence, suggest a direct, rapidly progressive cardiotoxic effect of *C. fleckeri* venom (Beadnell et al., 1992; Currie, 1994; Pereira and Seymour, 2013; Saggiomo and Seymour, 2012; Williamson et al., 1980). Poor cardiac output through extremely low or unrecordable blood pressure has been previously reported to be associated with this effect (Currie, 1994). This creature is well-known for the speed and effectiveness through which the venom acts, and unattended victims can potentially succumb to lethal consequences within minutes of the sting (Beadnell et al., 1992). The most critical and effective first aid for envenomed victims stung by this jellyfish is cardiopulmonary resuscitation, which has proven to be lifesaving in the past (Balhara and Stolbach, 2014; Berling and Isbister, 2015; Williamson et al., 1980).

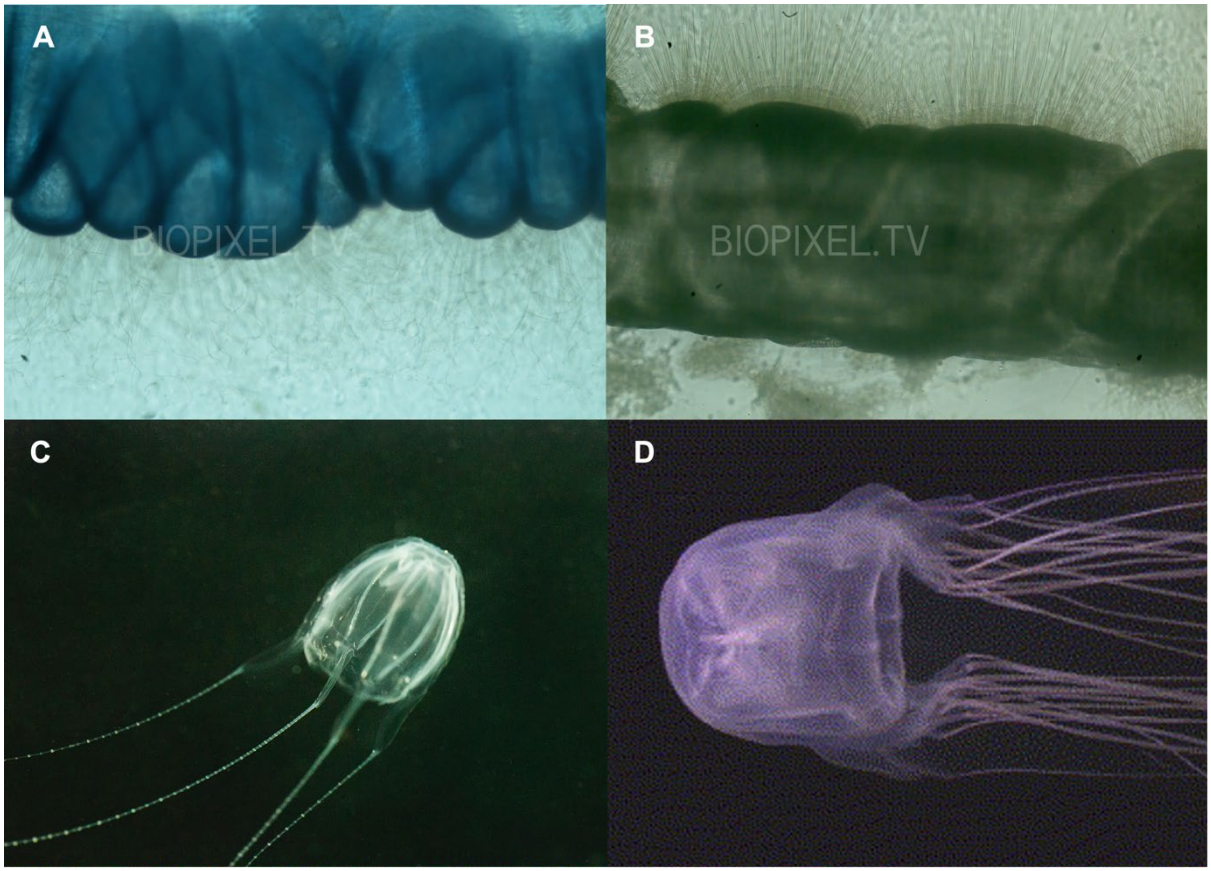

Figure 1.1: Box jellyfish species and tentacles with discharged nematocysts. *(A) Nematocysts discharged from tentacle of a Irukandi Syndrome-causing jellyfish (Biopixel TV, 2017), (B) Nematocysts discharged from* C. fleckeri *tentacle (Biopixel TV, 2021), (C)* C. barnesi *jellyfish (Photo: Dr. David Wilson), (D)* C. fleckeri *jellyfish (Tibballs, 2006).*

In contrast to the painful stings caused by *C. fleckeri*, *C. barnesi* stings can initially appear relatively innocuous and sometimes victims do not realise they have been stung until they start experiencing symptoms (Carrette and Seymour, 2013). Upon envenomation, victims can experience back pain, agitation, feeling of impending doom, and symptoms of cardiotoxicity, such as tachycardia, hypertension and cardiac failure (Little et al., 2020). In contrast to venom from *C. fleckeri*, the cardiotoxicity pathologies seen in sting victims of *C. barnesi* are regarded as an indirect effect of the venom toxins (Huynh et al., 2003; Little et al., 2003; Pereira and Seymour, 2013; Winkel et al., 2005). This indirect effect might be due to massive catecholamine release, with some clinical evidence of sudden onset of ventricular dysfunction (Tiong, 2009), but the precise mechanism through which this happens is still unknown and there is no consensus as to how best treat a victim (Carrette and Seymour, 2013).

In addition to the deleterious health effects experienced by victims, Irukandji Syndrome represents a major financial problem to Australian communities regarding public health and tourism. Approximately 50% of victims require hospital admission, where a significant number of resources and

time are allocated to retrieving victims (many are stung offshore and need helicopter retrieval). It is estimated that the financial burden of Irukandji Syndrome stings costs between AUD$ 1-3 million per year in Australia (Carrette and Seymour, 2013). However, species responsible for this syndrome are found around the globe, including Hawaii, Fiji, Puerto Rico, the Gulf Sea, Key West Florida, and the Caribbean, so this problem is not isolated to Australia (Carrette and Seymour, 2013) and studying the venoms from these animals might aid victims worldwide.

Similarities exist in the pathologies and symptoms experienced by victims of both cubozoan jellyfish and stonefish envenomation, where the severity is generally related to the amount of venom injected, being dose-dependent (Khoo, 2002; Williamson et al., 1996). Deaths linked to both cubozoan and fish envenomations are usually attributed to cardiovascular and respiratory effects, or from a result of cardio pathology (Carrette et al., 2012; Fenner, 2005; Maillaud et al., 2020; Ziegman and Alewood, 2015). Currently, the pathophysiology of these marine venoms and the mechanisms of action through which death occurs remain largely unknown. Consequently, appropriate treatment modalities and protocols of these marine envenomations remain controversial or based on anecdotal evidence, which often leads to confusion and to the incorrect treatment application for an envenoming (Bailey et al., 2003).

1.3 Immunotoxicology of marine venoms

It is becoming increasingly clear venoms can have a significant impact on the immune system (Ryan et al., 2021). The immune system has evolved for a thousand million years to protect the host from both external and internal elements, acting as a ubiquitous physiological tool used by many living organisms (Minutti-Zanella et al., 2021). Immune systems are composed of an array of soluble molecules, specialized cells, and organs with the specific functions of preventing, recognising, and eliminating pathogens, infections, and neoplasia (Minutti-Zanella et al., 2021). The immune system is mainly divided in two classes: innate, the system that is inherent to our bodies; and adaptive, the system that is acquired over time. Innate immunity is the first line of defence and the quickest to help the body fight infections or pathogens (Dinarello, 2010; Voskoboinik et al., 2010). The innate system protects through physical and chemical barriers, utilising specialized cells called phagocytes, dendritic cells, and natural killer lymphocytes, as well as via blood proteins, and other inflammatory mediators (Minutti-Zanella et al., 2021). By contrast, adaptive immunity takes several days to reach its peak maturity but is specific and responsible for generating immune memory (Dinarello, 2010; Voskoboinik et al., 2010). Adaptive immunity can be further divided into humoral immunity, which is mediated by B lymphocytes whose function is to secrete antibodies; and cellular immunity, mediated by T lymphocytes whose activation depend on antigen-presenting cells (Minutti-Zanella et al., 2021). Despite their biological

differences, both types of immunity initiate an acute response to protect the body from threats (Voskoboinik et al., 2010).

Like in the presence of pathogens, envenomation can also start an acute immune defensive response, where a variety of innate signalling receptors are activated to eliminate or neutralise the threat (Mogensen, 2009). Pro-inflammatory events are initiated by a variety of actions, including the release of enzymes that degrade proteins, the release of antimicrobial peptides that kill pathogens directly, or the production of cytokines or chemokines that recruit immune cells to the targeted area (Mogensen, 2009). Cytokines are small proteins that mediate immune and inflammatory responses by binding to receptors in a hormone-like manner (Dinarello, 2010). The length and intensity of the immunological response is controlled by cytokines through antibody regulation, the proliferation, activation and differentiation of a variety of cells, or by the secretion of other cytokines (Cohen and Cohen, 1996). Cytokines are known to display pleiotropic (individual cytokines can exert various effects) and redundant behaviours (different cytokines may exert the same effects), depending on the immunopathological process at hand (Cohen and Cohen, 1996). Chemokines, in turn, are a large family of chemotactic cytokines. Their major role is to stimulate cell migration into antigen affected areas, particularly leukocytes, playing an important role in pathological processes, including inflammatory responses (Hughes and Nibbs, 2018). Therefore, the detection of toxins or venom compounds by the immune system initiates several inflammatory chain reactions which promote detoxification, symptom resolution and overall host protection (León et al., 2011; Palm and Medzhitov, 2013).

1.3.1 Drug discovery from venom components

Through evolution and natural selection, toxins have evolved with a wide range of pharmacological activities, targeting an array of biological functions, and resulting in human pathologies (Bordon et al., 2020). Due to this broad activity, toxins have the potential to be used as physiological tools for understanding pathophysiological and biochemical processes and have also become increasingly more important in recent years as sources of potential novel therapeutics and anti-cytokine treatments (King, 2011; Ryan et al., 2021). One classic example is the commercial drug called Captopril, which was developed after the realization that peptides found in the venom of the Brazilian viper *Bothrops jararaca* had potent hypotensive effects (Koch-Weser et al., 1982). Research into the components and activities of venoms has subsequently increased, with focus on treating diseases such as type 2 diabetes, gastrointestinal conditions, chronic pain, and stroke (Pennington et al., 2018). Additionally, the properties of venom components have been exploited in a variety of circumstances, such as diagnostic tools, cosmeceuticals, and to validate targets of postulated therapeutics leading to improved drug libraries (Bordon et al., 2020).

Studying the active compounds present in venoms is the first step to making use of this rich pharmacological and physiological pool, and marine venoms present a prime opportunity for such studies. Previous research found active compounds that could potentially help against endocrine, nervous, cardiovascular and immune diseases, and other investigations have looked at antibiotic, antitumor and anti-inflammatory properties of several different marine venoms (Minutti-Zanella et al., 2021). For example, a conotoxin isolated from the venom of cone snails (*Conus* spp.) has been used for the treatment of chronic pain since 2004 (Bordon et al., 2020). Ziconotide, a synthetic analogue of the peptide toxin MVIIA, blocks Ca^{2+} channels, inhibiting the nerve impulse conduction and its subsequent neurotransmitter release, which leads to nociception. Moreover, ziconotide (Prialt®) is more potent than morphine and does not cause dependence (Bordon et al., 2020). In addition to this well-known marine venom peptide, other peptides from marine sources have been discovered and tested. Sea anemones, which are a cousin of the jellyfish, inspired the design of a different synthetic peptide (dalazatide) that inhibits K^{2+} channels and showed a reduction in the clinical score of multiple sclerosis in a rat model (Bordon et al., 2020). Dalazatide completed phase I trials in patients with plaque psoriasis in 2015, but no phase II studies were started (Bordon et al., 2020). Tetrodotoxin (TTX), a guanidium neurotoxin found in Tetraodontidae pufferfish, octopuses, sea stars and many other marine organisms, blocks Na^{+} channels, ultimately leading to neuromuscular paralysis (Bordon et al., 2020). TTX went to phase III clinical trials under the name Tectin® in 2018 to treat pain in patients undergoing chemotherapy and it has also been used to develop anesthetic pharmaceuticals (Bordon et al., 2020).

1.3.2 Immunomodulation by fish venoms

Although many studies have investigated venoms and their components, and technological advances have increased the throughput of toxin discovery, research on the immunomodulatory activity of fish venoms still remains largely unexplored and underrepresented. Immunomodulation therapy can be achieved through natural products that can regulate, stimulate or suppress a range of immunoactivities (Minutti-Zanella et al., 2021). This therapy has been successfully used in the treatment of many diseases, from pathogen-driven infections to cancer (Minutti-Zanella et al., 2021). The majority of studies on fish venoms have mainly focused on the pharmacological and chemical properties of venoms from a few fish species, including scorpionfish, weeverfish, toadfish and stonefish (Sivan, 2009). Only a handful of examples exist in the literature in relation to immunomodulation by fish venoms. One example is a toxin from the scorpionfish *Scorpaena plumieri* that induced local inflammation in mice, with increased levels of pro-inflammatory cytokines and chemokines (TNF, IL-6, MPC-1) (Menezes et al., 2012). Another example was the discovery of novel immunomodulators in the venom of the Brazilian toadfish *Thalassophryne nattereri*. A C-type lectin,

nattectin, found in the venom modified biological responses, controlling both the recruitment and activation of macrophage cells, which allowed them to differentiate into cells that have similar functions to dendritic cells and is useful for antigen-specific immunity and for long-term immunologic memory (Saraiva et al., 2011). Additionally, nattectin can also recruit neutrophils via activation and release of chemotactic factors (IL-1ß, LTB4, KC, MMP-9, and MMP-2) (Lopes-Ferreira et al., 2011). Another family of peptides derived from the venom of *T. nattereri*, called *Tn*P, has been used for drug discovery and development (Komegae et al., 2017). Mouse models have shown the potential for *Tn*P to control inflammation and demyelination and could potentially be used to treat multiple sclerosis (Komegae et al., 2017).

Research into marine venoms is important. To date, hundreds of different marine venom compounds have been discovered and described, ranging from proteins to small molecules. Our understanding of these venom compounds and their physiological pathways continues to expand, and this understanding of the venom composition, properties and biological activities might lead to improved treatment of bites and stings for victims, as well as lead to the discovery of new therapeutics. Technological improvements in the methodologies for studying venom allow enhanced definition of venom composition and toxin characterization, and this thesis takes advantage of both quantitative and analytical tools to examine marine venoms.

1.4 Challenges related to researching marine venoms

To date, the primary focus of venom studies has been on terrestrial animals such as snakes, scorpions, and spiders (Fry et al., 2015). This bias exists for two primary reasons. Firstly, these creatures pose the greatest threat to humans through potential exposure, and so have higher clinical and medical priorities. Secondly, terrestrial venomous creatures are generally easier to procure, compared to marine animals, and perhaps the largest hurdle to studying marine venoms is locating the targeted specimen in the vast ocean (Church and Hodgson, 2002). Stonefish are a prime example as masters of crypsis, completely camouflaging themselves amongst coral or sand substrates (Smith and Heemstra, 1986). These fish are extremely hard to detect, having the perfect coloration of bare rocks, or being brightly colored to mimic coral covered by algae, depending on the habitat in which they are found (Endean, 1961). They often have hydroids or other sessile organisms growing on the surfaces of their body due to their sedentary lifestyle, remaining half-buried and motionless, even when approached, looking much like an innocuous rock (Endean, 1961). Cubozoan jellyfish are another example of inconspicuous venomous animals. These creatures are composed of over 95% water, so they are translucent and extremely difficult to locate in the water (Hamilton, 2016). Jellyfish that give rise to the Irukandji Syndrome are small, their bell measuring approximately 2.5 cm in diameter, presenting

four thin tentacles of up to 1.3 m in length (Courtney et al., 2015). These animals are incredibly difficult to find and collect, since they are found in open-water and their presence is sporadic, unpredictable, and often happens for only a short period of time in a small area (Fenner, 2006; Fenner et al., 1988, 1986).

In addition to the difficulties locating and collecting marine venomous creatures, maintaining marine animals in aquaria is a difficult, costly, and labor-intensive endeavor compared to keeping invertebrates and reptiles (Jackson and Thomas, 1980; King, 2011; Steiner, 1966). Marine creatures require the right range of salinity, pH, and temperature, which need to be regularly checked and adjusted with chemicals or other supplies to keep the water quality at their specific optimal range (Alderton, 2012). Reptiles need terraria with only a few items, such as a log, some soil, and a source of heat (Jackson and Thomas, 1980), and invertebrates can be easily kept in small plastic containers (Duran et al., 2020; Evans et al., 2020). There is the possibility of extracting venom and subsequently culling the animal; however, this can be ethically challenging and is not sustainable. Additionally, many venomous marine creatures are seasonal (e.g. box jellyfish), so keeping the animals in aquaria is the only option to access a reliable, year-round supply of fresh venom for studies of the venom from individual specimens over time (e.g. cone snails (Dutertre et al., 2014)).

After successfully locating and collecting marine venomous creatures, other challenges become evident. Many creatures are relatively small and provide miniscule amounts of venom (King, 2011), such as Irukandji Syndrome-causing jellyfish. Due to the minuscule size of nematocysts, the amount of venom discharged from each of these organelles is incredibly small. As a result, collection of adequate amounts of venom to perform research and data analysis requires several millions of nematocysts (Carrette and Seymour, 2004).

Once the marine venom has been collected, developing the right technique for venom extraction while maintaining activity is a difficult task. While many studies were undertaken in the 1980s and 1990s and much was discovered about marine venoms, the technology available then is considered rather primitive, and so the pharmacological, chemical, physiological and biological characterization of venoms have not been fully appreciated until more recently with the advent of improved technology (Vetter et al., 2011). This is true in the case of both jellyfish and stonefish venom research. For cubozoan jellyfish, many venom extraction techniques have been devised, but their implementation is somewhat difficult, which produces venom of dubious quality (Carrette and Seymour, 2004; Ramasamy et al., 2005). Consequently, conclusions and findings could not be accurately interpreted (Ramasamy et al., 2004) and have yielded discrepant results between research groups (Tibballs, 2006). In 2004, a repeatable and reliable venom extraction technique was devised

(Carrette and Seymour, 2004), and has been widely adopted in the jellyfish research community. This extraction technique was used for the experiments performed in this thesis. Stonefish do not present the same venom yield problems as Irukandji Syndrome-causing jellyfish; however, both jellyfish and stonefish venoms appear to have biochemical properties that hinders research. The most prominent factor is the instability of these venoms. They are thermolabile, pH-sensitive, unstable when mixed with certain chemicals, and they also tend to bind to non-toxic apparatus, aggregate and dissociate (Burnett et al., 1990; Othman and Burnett, 1990; Ramasamy et al., 2003). Therefore, the differences in venom extraction methods, the storage procedures (i.e. fresh, frozen or lyophilized), and the inherent issues in the performance of different bioassays have all made it difficult to study marine venoms (Russell, 1965).

Nonetheless, this thesis attempted to bridge gaps in the literature of marine venoms. The venomous species used in this project were the jellyfish *C. fleckeri* and *C. barnesi* (Irukandji Syndrome jellyfish), the estuarine stonefish *S. horrida* and the reef stonefish *S. verrucosa*. These species were chosen because they represent two of the most medically important taxa in the marine environment in Australia, and there is limited information on the molecular composition of their toxins and the specific bioactivities of those toxins. These four species of venomous animals are found locally in Cairns, Australia, and it was possible to either keep them in the University's aquarium (in the case of stonefish) or to collect their venom, process it and store it in lyophilized form in -80°C (in the case of jellyfish), making this research project possible. Importantly, these species are known to cause severe envenomation in the Indo-Pacific region, so the findings from this research project are applicable across a wide range of locations.

1.5 Thesis aims and structure

The overall aim of this thesis was to characterize the functions and compositions of jellyfish and stonefish venoms. The first aim was to further the understanding of some of the cardio-physiological pathologies of jellyfish envenomation using non-invasive pulsed wave Doppler technology. This was achieved through a live mouse model, where Doppler sonograms of anaesthetized mice were obtained. This model showed that it is possible to use this technique and technology to assess cardiac function in a sedated mouse model and could have implications in the application of this technique to investigate current and future treatment modalities for jellyfish envenomation, which is necessary and currently lacking.

The second aim was to investigate the immunological activities of stonefish venoms. Systematic screening of the venoms through both immunological assays and analytical tools uncovered new immunomodulatory components that could lead to a better understanding of the illnesses seen in

victims and to the discovery of new drug leads. These studies were achieved using enzyme linked immunosorbent assay, cytometric bead array assays, reverse-transcriptase quantitative polymerase chain reaction, liquid chromatography-mass spectrometry and nuclear magnetic resonance analyses of crude venom and venom fractions. Recent advances and improvements in technologies, especially regarding sensitivity of assays and various spectrometry machinery allowed unprecedented access to the molecular components of these venoms.

Chapter one explains the background to this research and justifies the need to do this project. The current knowledge of marine venoms is summarised with focus on envenomings caused by Australian marine venomous species, such as the two cubozoans *C. fleckeri* and *C. barnesi*, and the two species of stonefish *S. verrucosa* and *S. horrida*.

Chapter two elucidates and describes the differences in the cardio-physiology of envenomed live mice through pulsed wave Doppler sonograms. This was performed using a live animal model, where mice received intra-arterial venom injections and were continuously and non-invasively monitored throughout the experiments using the Doppler Flow Velocity System and the MouseMonitor™ System by Indus Instruments. Mice were envenomed with venoms from two species of box jellyfish, *C. fleckeri* and *C. barnesi*. This work was published in *Toxicon* in 2020.

Chapter three reviews the current state of knowledge of stonefish from the genus *Synanceia.* The review presented the first global map of the current distribution of all *Synanceia* spp., collated, also for the first time, clinical case reports that placed stonefish as the culprit for human envenomations around the globe, provided updated information about stonefish antivenom and reviewed the literature in terms of the biomolecular composition of stonefish venoms. In addition, the review highlighted that further research on these venoms may lead to the discovery and elucidation of novel molecules that could be useful for drug discovery or as physiological tools. This work was published in *Marine Drugs* in 2021.

Chapter four focuses on the effects of diet consistency on venom yield in the estuarine stonefish *S. horrida*. Although venom production seemed to be affected by starvation, the venom components seemed to be replenished quickly. This was an important step for the research implemented for the remaining chapters of this thesis, as findings were used as a road map to extract venom from the same individual consistently and frequently without loss of activity for the research performed in chapters five and six. These findings were published in *Toxicon* in 2017.

Chapter five assesses the potential for immunomodulation in the venoms from both *S. horrida* and *S. verrucosa* stonefish on human peripheral mononuclear cells (hPBMCs). As venoms are known to possess immunomodulatory activity, these venoms were tested for the first time in whole blood T cell lymphocytes in an attempt to increase scientific interest in this area. Whole venoms were screened using well-known cell stimulants such as lipopolysaccharides and PMA-ionomycin, which stimulate cells to express numerous cytokines and chemokines.

Chapter six further assesses the immunomodulation and analyses the different components found within the venoms of both *S. horrida* and *S. verrucosa*. After seeing the immunosuppressive activity in these venoms, a systematic screening of venom fractions and small molecules found in these venoms could potentially uncover new immunosuppressive compounds that could later be developed as drug leads. This investigation used high-throughput technologies combined with immunological assays.

Chapter seven is a general discussion of the outcomes and conclusions of this research. It summarises what was added to the literature, outlies the limitations of the study, and uncovers possible topics for future research.

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Chapter 2

2 Non-invasive assessment of the cardiac effects of *Chironex fleckeri* and *Carukia barnesi* venoms in mice, using pulsed-wave Doppler

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2.1 Abstract

Both *Chironex fleckeri* venom (CFV) and *Carukia barnesi* venoms (CBV) are known to cause significant cardiac morbidity and mortality. Many animal studies have demonstrated cardiac dysfunction with these venoms. This study specifically examines the systolic and diastolic cardiac functions using non-invasive pulse wave Doppler. Mitral and aortic Doppler sonograms of anaesthetised mice were obtained utilising a 10 MHz Doppler probe. These continuous sonograms were analysed to ascertain changes in cardiac function before and after the parenteral administration of the test venoms. We found that CFV administration causes rapid cardiac dysfunction without a change in heart rate. Analysis of the resulting sonograms shows both systolic and diastolic dysfunction which together is suggestive of a progressively poorly compliant, contracted left ventricle. Additionally, the rapidity of cardiac dysfunction suggests a direct effect of CFV on myocardial cells. In contrast CBV showed a moderate immediate inotropic and chronotropic effect that was sustained until precipitous cardiac decompensation. This is consistent with the hypotheses of a toxin induced stress cardiomyopathy from sustained catecholaminergic activity.

2.2 Introduction

In Australia, over 70 deaths have been reported due to *Chironex fleckeri* (Currie and Jacups, 2005), and there are reports of two deaths attributed to *Carukia barnesi* (Fenner and Hadok, 2002; Anavekar and Oh, 2009; Pereira et al., 2010). Rapidly progressive cardiorespiratory collapse followed by cardiac arrest is well documented for *C. fleckeri* envenomation, as is life threatening cardiac failure for *C. barnesi* envenomation (Little et al., 2003).

The clinical literature shows that severe *C. fleckeri* envenomation is associated with tachycardia, dyspnoea, hypertension and hypotension (Williamson et al., 1980; Beadnell et al., 1992). Additionally, a prospective study of jellyfish stings in the Northern Territory (Currie and Jacups, 2005) showed that although 90% of *C. fleckeri* stings were minor, the last 10 fatalities from *C. fleckeri* were children. Paediatric victims only constituted 37% of their *C. fleckeri* cases. This over representation in fatalities suggests a dose-dependent relationship where a smaller body mass leads to worse clinical outcomes. Furthermore, animal and laboratory models suggest a direct cardiotoxic effect of *C. fleckeri* venom (CFV) (Currie, 1994; Saggiomo and Seymour, 2012; Pereira and Seymour, 2013).

In contrast, many of those stung by *C. barnesi* develop hypertension and tachycardia, with 2% progressing to congestive cardiac failure over the ensuing 12–24 h (Huynh et al., 2003). As distinct from *C. fleckeri* envenomation, there is an implication of an indirect toxin induced stress cardiomyopathy through elevated catecholamine levels (Winkel et al., 2005; Pereira and Seymour, 2013). Injection of crude *Carukia barnesi* venom (CBV) into piglets was associated with a 4000-fold rise in noradrenaline levels and a 1000-fold increase in adrenaline levels (Winkel et al., 2005). Furthermore, clinical echocardiographic findings on victims experiencing congestive cardiac failure demonstrate marked reduction in cardiac ejection fraction and myocardial wall motion abnormalities consistent with Takotsubo cardiomyopathy (Little et al., 2003; Tiong, 2009).

There is substantial *in vivo* and *in vitro* evaluation of heart rate (HR) and blood pressure (BP) changes associated with CFV and CBV (Ramasamy et al., 2004; Ramasamy et al., 2005; Hughes et al., 2012), while Winter et al. showed that CFV caused a sustained contraction of rat isolated aorta (Winter et al., 2007). There, however, is little literature regarding the direct cardiac dysfunction impacted by these venoms. Severely envenomed patients require intensive supportive care and, as such, a better understanding of the effect of the venom may lead to new medical strategies to manage these critically ill patients.

Pulse wave Doppler studies have been validated and used in assessing cardiac function in mammalian models for over 40 years (Lewis, 1975; DeMaria et al., 1991; Anavekar and Oh, 2009). Clinical echocardiography routinely uses pulse wave Doppler analysis as an integral part of myocardial assessment. The aim of this study was to utilise pulse wave (PW) Doppler to directly measure cardiac function after parenteral administration of CVF and CBV into anaesthetised mice.

2.3 Methods

2.3.1 Venom preparation

Live *C. fleckeri* were sampled from Weipa, while live *C. barnesi* were sampled from waters around Double Island and Palm Cove in Tropical North Queensland. Established techniques were used for nematocyst isolation and venom extraction from tentacles of live medusae, followed by lyophilisation for storage and subsequent reconstitution for use (Bloom et al., 1998; Carrette and Seymour, 2004). For this research CFV was obtained from a single animal, while CBV was pooled from a number of animals to obtain appreciable amounts.

Lyophilised venom reconstitution occurred with phosphate buffered saline (PBS, standard solution pH of 7.4) to achieve a stock solution which was stored in an ice bath and used within 4 h.

2.3.2 Venom analysis

Venom protein compositions were not determined until after the data was collected and were calculated using Bradford's protein determination as CFV 0.442 g/mL and CBV 0.39 g/mL.

2.3.2.1 Size exclusion chromatography (FPLC)

Between 200 µL and 500 µL of reconstituted venom was passed through a 0.22 µm filter and individually run over an AKTA™ fast-performance protein liquid chromatography (Superdex 200 TM 10/ 300 GL; Tricorn; 13 µm, 10 mm - 200 mm) at a flow rate of 0.3 mL/min and wavelength set at 280 nm. Degassed Dulbecco's phosphate buffered saline was used as a running buffer to fractionate the venoms for venom profile generation.

2.3.2.2 NuPAGE

Proteins and their molecular masses found in venom from tentacular nematocysts of both species were determined using SDS-PAGE. Samples were run on a NuPAGE 12% Bis-Tris Mini Precast Protein gel. Bio-Rad Kaleidoscope Protein Standards were used for comparison of molecular weights. Gels were then stained with 16% Fast Blue-20% acetic acid solution for 1 h. Banding patterns were visually assessed for similarities and differences between and within sample types.

2.3.3 Mouse preparation

Ten week old male laboratory mice (strain BALB/c), bred by Animal Resources Centre (www.arc.wa.gov.au) and maintained at JCU Mouse House, were anaesthetised by intraperitoneal injection of ketamine 4 mg (mean 163 mg/kg, range 129–203 mg/kg) and pentobarbital 0.2 mg (mean 7.8 mg/kg, range 6.2–9.7 mg/kg). When sufficiently anaesthetised, as determined by absence of response to firm tail pressure, the mice were weighed (mean 24.86 g, range 19.7–30.9 g) and then placed supine on a warmed (37°C) monitoring mat (Indus Instruments Rodent Surgical Monitor), where heart rate (HR) and electrocardiogram (ECG) readings were continuously monitored.

A 10 MHz Doppler probe, (Indus Instruments Doppler Flow Velocity System, manufactured by Indus Instruments 721 Tristar Drive Webster, Texas, 77598, USA), was focused on either the aortic valve (AV) or the mitral valve (MV). Only one valve dataset was obtained on each mouse.

Pulse wave Doppler sonograms of blood flow through the selected valve were recorded for analysis (Figures 2.1, 2.2, 2.3 and 2.4). Each sonogram describes the sequentially collected velocities of individual blood cells passing through the selected valve. The waveforms produced can be used to accurately measure blood flow and blood volume through the left ventricle. Furthermore, left ventricular systolic function is assessable by examining morphological changes to the aortic Doppler sonogram, while left ventricular diastolic function is assessable by examining the mitral Doppler sonogram (DeMaria et al., 1991; Anavekar and Oh, 2009). A brief explanation of the relevant cardiac indices is provided in Appendix A.

2.3.4 Controls

Eight mice were anaesthetised with intraperitoneal injections of ketamine and pentobarbital after which they were weighed (mean 23.04 g, range 20.6–24.8 g). All animals remained fully anaesthetised for 60 min without additional anaesthetic, following which all were euthanized with a fatal dose of pentobarbital 3.2 mg via intracardiac injection. The controls established a baseline for anaesthetised mice. ECG and PW Doppler sonograms were recorded for the duration of 60 min. Study mice were controlled against themselves as percentage change (Δ %) from their baseline.

All cardiac parameters derived from the aortic and mitral PW Doppler sonograms showed no statistical variation through 60 min of observation. Interestingly, the control mice had a baseline E/A Ratio of 1.5 when compared to the study mice which had baselines of around 2.4 for both CFV and CBV groups. Although we cannot account for this difference, we stress that the study mice were controlled against

their baselines. Similarly, we highlight that for 60 min after induction of anaesthesia there was no deterioration in cardiac function identified in controls.

2.3.1 Study mice

After anaesthesia was achieved, a baseline set of observations and PW Doppler readings were taken. Following this, the study animal's tail ventral artery was identified and injected (27 G Terumo insulin syringe) with either 0.02 mL CFV (0.009 mg, mean 0.35 mg/kg, range 0.29-0.45 mg/kg) or 0.02 mL CBV (0.008 mg, mean 0.31 mg/kg, range 0.27-0.33 mg/kg). The HR, ECG and PW Doppler sonograms were continuously recorded until death.

2.3.1 PW Doppler waveform analysis

For the purpose of analysis, T_0 was time at or immediately before injection. During data collection it was observed that the development of asystole was preceded by progressive bradycardia, sometimes briefly with rapid progression to asystole, while in others the bradycardia persisted despite minimal effective sonographic flow with each contraction. Using asystole as an objective endpoint, therefore, would introduce significant periods in some mice where cardiovascular collapse had already occurred confounding the study's aim to examine the progressive cardiovascular effects of these venoms. As a consequence, it was decided that an endpoint based on the percentual reduction of cardiac output rather than the time to asystole would provide an alternative objective endpoint, and arbitrarily, a 75% reduction in cardiac output was selected. Consequently, the time at which cardiac output was identified to be < 25% was used as T_{100}.

On identifying T_{100}, the best four contiguous waveforms at 10% intervals from T_0 were selected (T_{10} – T_{90}) and data points on each waveform were manually identified (Figures 2.1, 2.2, 2.3 and 2.4) and mapped into the Indus Instruments Signal Analysis software (Doppler Signal Processing Workstation V1.625, Indus Instruments). This provided the cardiac indices outlined and graphed in the results (Figure 2.5).

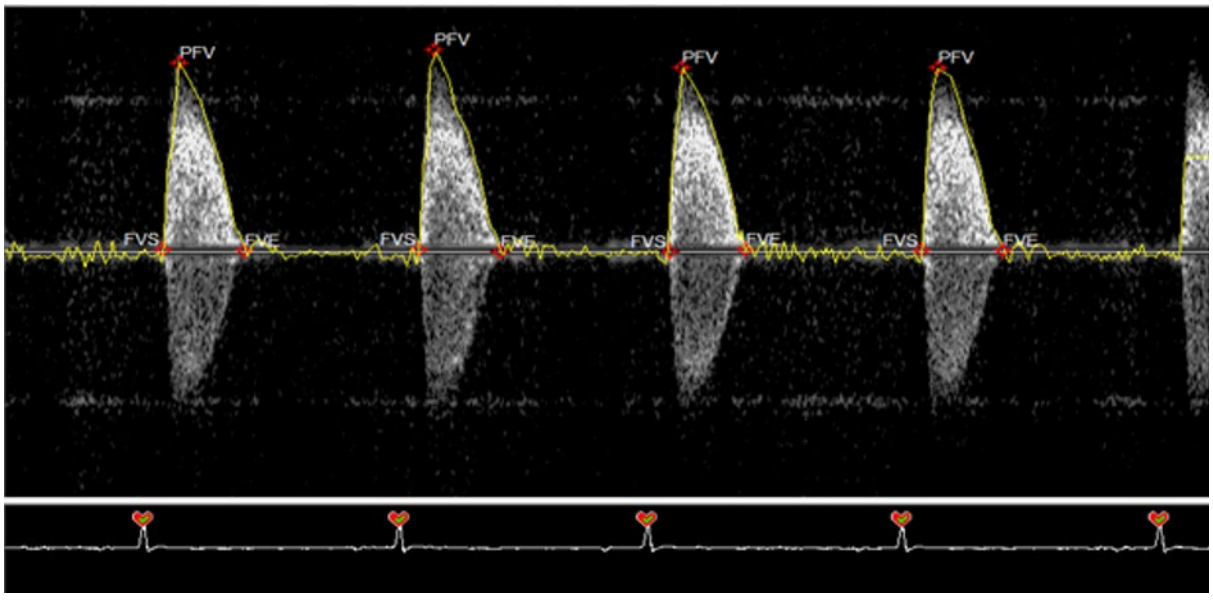


Figure 2.1: Pulse wave Doppler aortic flow sonogram at T_0. *PFV: Peak Flow Velocity; FVS: Flow Velocity Start; FVE: Flow Velocity End.*

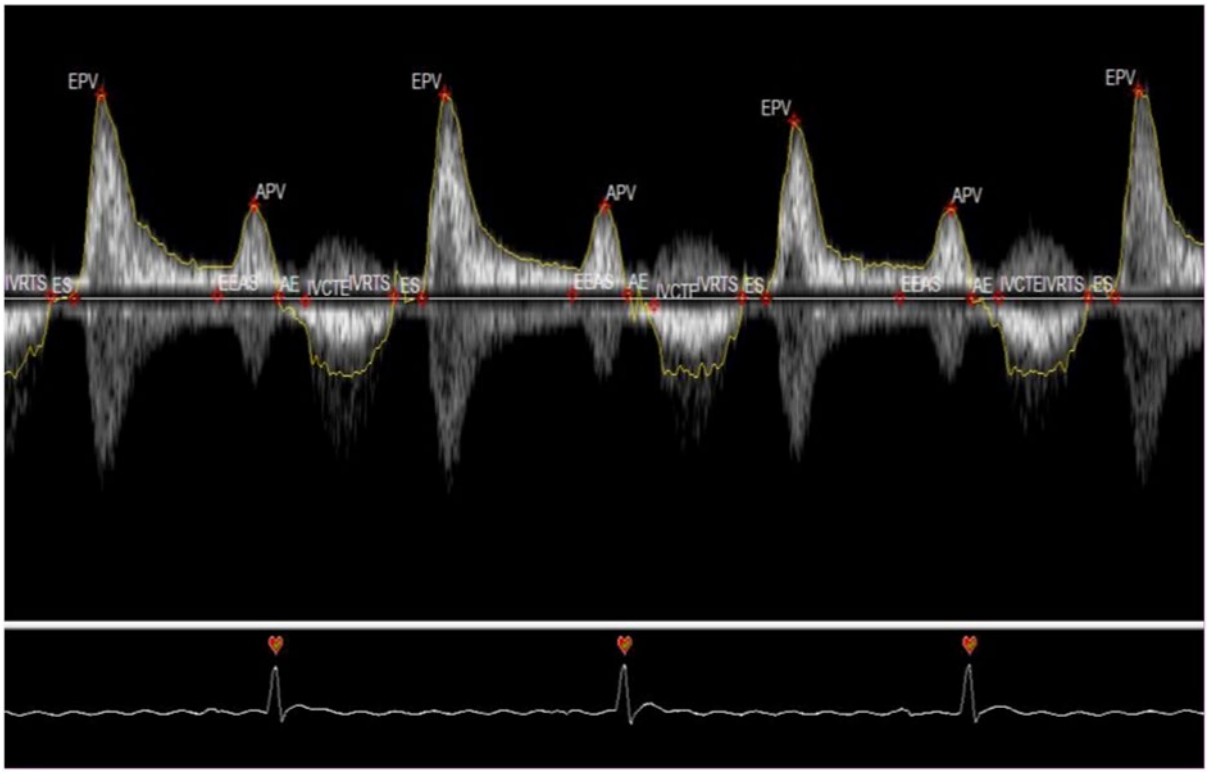
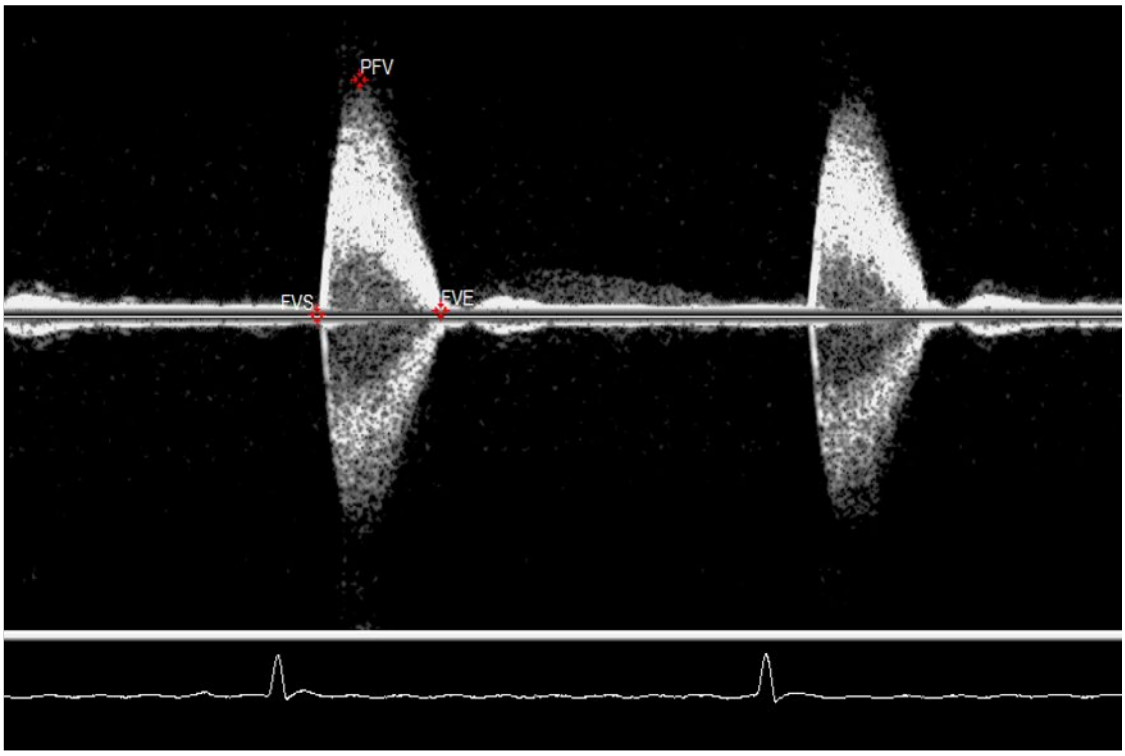


Figure 2.2: Pulse wave Doppler mitral flow sonogram at T_0. *EPV: Early flow Peak Velocity; APV: Atrial flow Peak Velocity; EEAS: Early flow velocity End & Atrial flow velocity Start; IVCTE: IsoVolumetric Contraction Time End; IVRTS: IsoVolumetric Relaxation Time Start; ES (and IVRTE): Early flow velocity Start (and IVRT End); AE (and IVCTS): Atrial flow velocity End (and IVCT Start).*

A



B

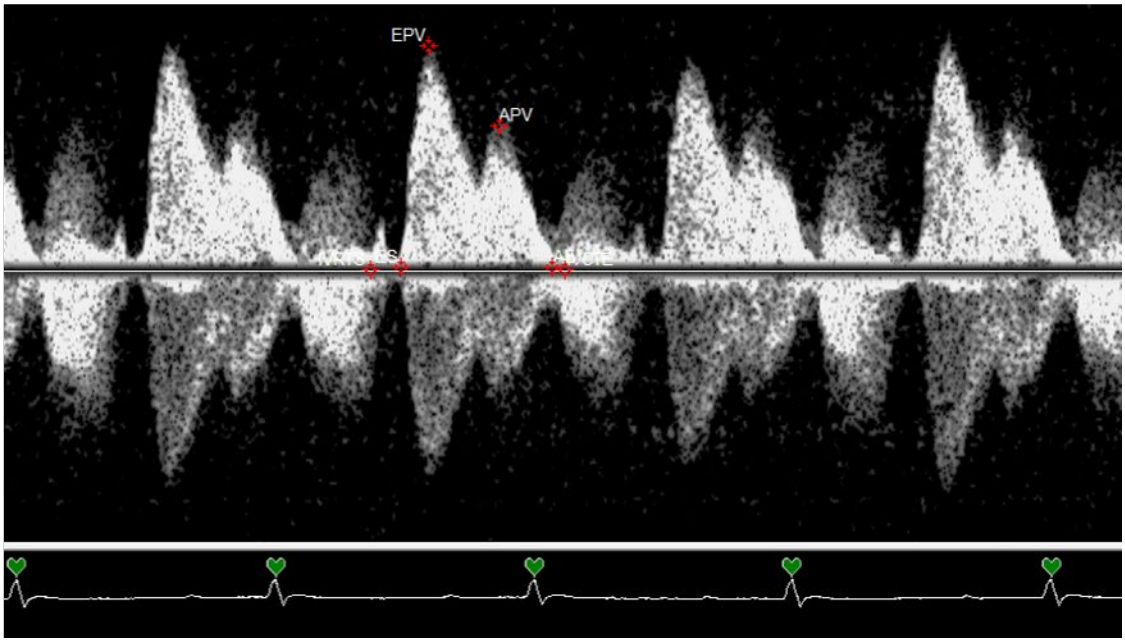
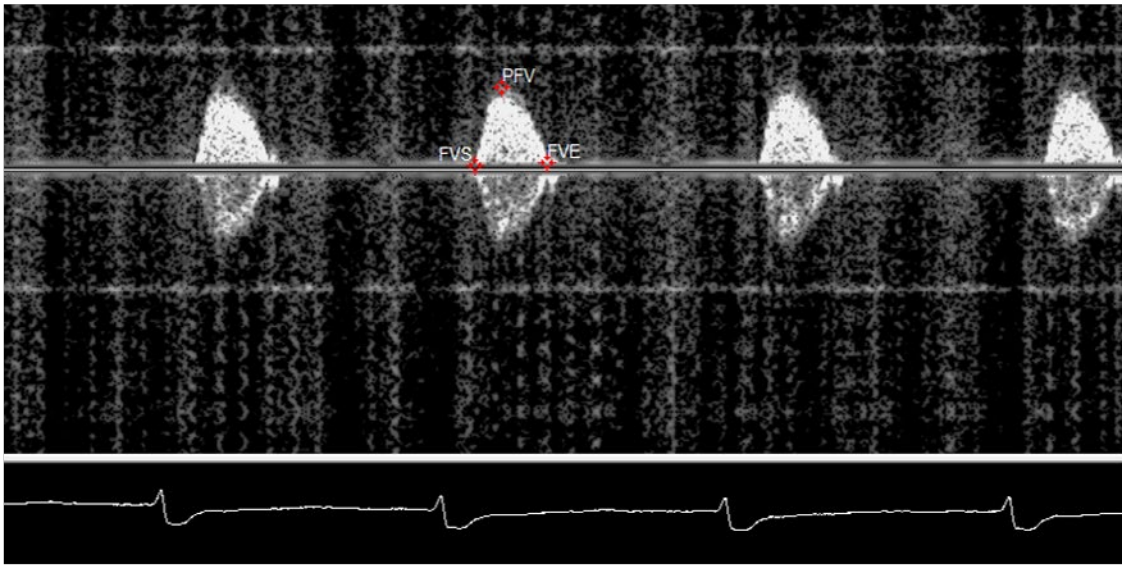


Figure 2.3: Pulse wave Doppler sonogram of CFV at T_{80}. *(A) Pulse wave Doppler aortic flow sonogram at T_{80}. (B) Pulse wave Doppler mitral flow sonogram at T_{80}.*

A



B

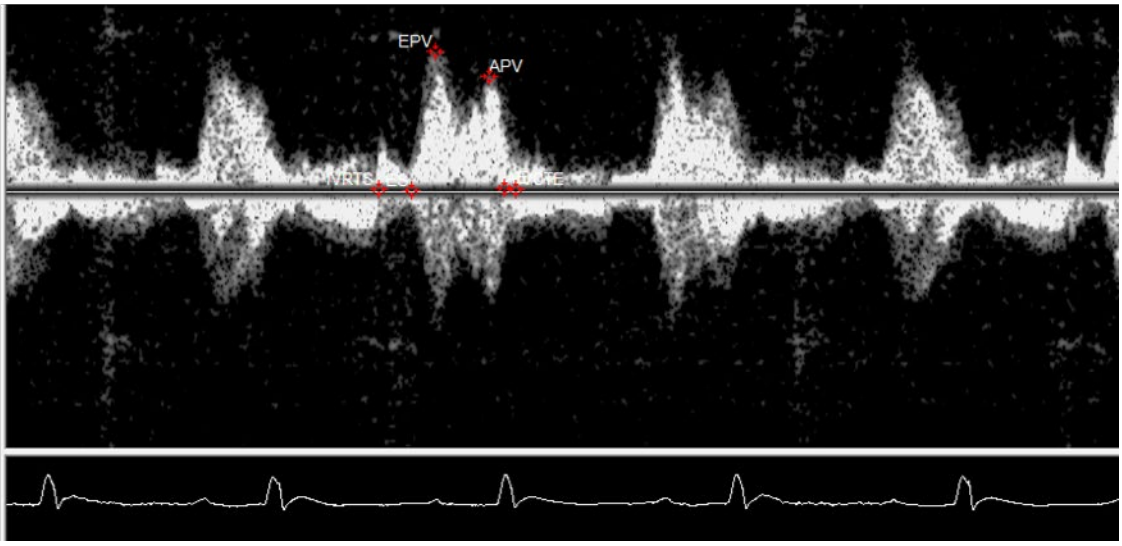


Figure 2.4: Pulse wave Doppler sonogram of CBV at T_{80}. *(A) Pulse wave Doppler aortic flow sonogram at T_{80}. (B) Pulse wave Doppler mitral flow sonogram at T_{80}.*

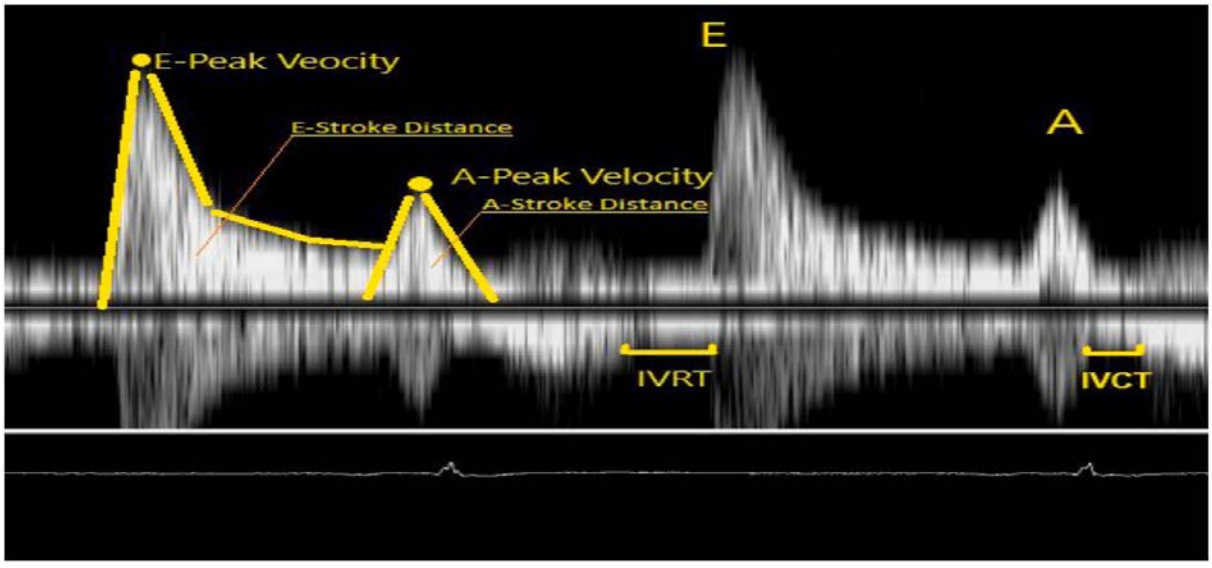


Figure 2.5: PW Doppler mitral flow sonogram. *E-Stroke Distance = Area under E-Wave; A-Stroke Distance = Area under A-Wave; IVRT = IsoVolumetric Relaxation Time; IVCT = IsoVolumetric Contraction Time.*

2.3.2 Statistical analysis

The effect of time since venom application on various dependent cardiac parameters (such as heart rate, stroke distance, etc.) were determined using a repeated measures one-way analysis of variance. In cases where sphericity was violated, Greenhouse-Geisser correction was applied. All analyses were performed using IBM SPSS Statistics Ver 25. Results of all analyses of variances are displayed as F statistic (F = ***), degrees of freedom for the independent and error term (e.g. D.f. = 2*12) and the probability of the effect '*p*' (in summary, F = ****, D.f. = ****, *p* = ****).

2.4 Results

2.4.1 Venom analysis

2.4.1.1 Size exclusion chromatography (FPLC)

The FPLC graphs show larger peaks for CBV in the 20 to 30 min range, however CBV does not have a peak at 15 min (Figure 2.6).

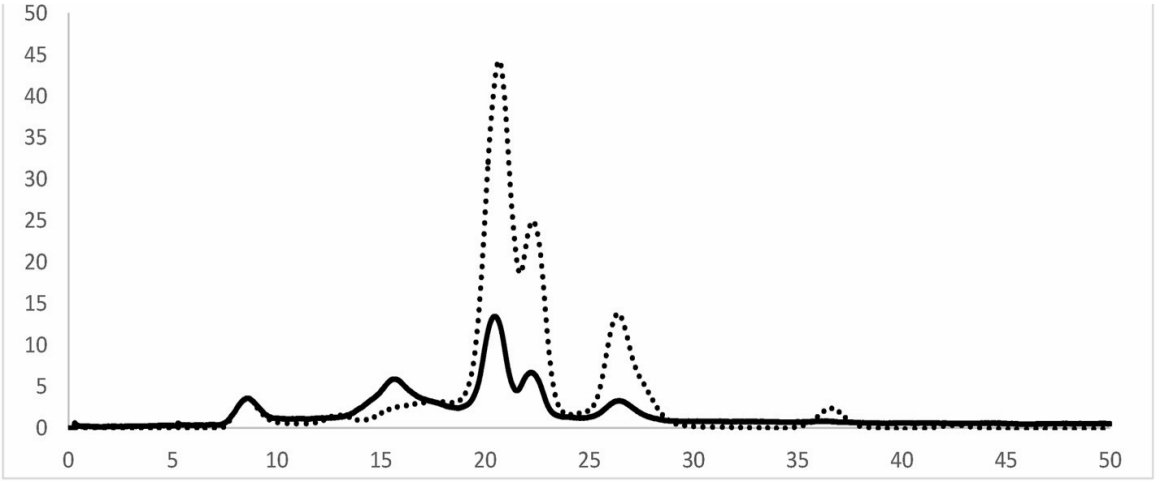


Figure 2.6: FPLC spectra for CFV (–) and CBV (- - -). *Time of elution of the column is displayed on the x axis (min) and UV absorbance on the y axis (%). Lethal vertebrate cardiac component in CFV is shown in peak 2, at approximately 15 min elution time.*

2.4.1.2 NuPAGE

Unlike FPLC, the NuPAGE results show significantly different bandings between CBV and CFV. This reflects differences in the protein structure between venoms after they have been reduced (Figure 2.7).

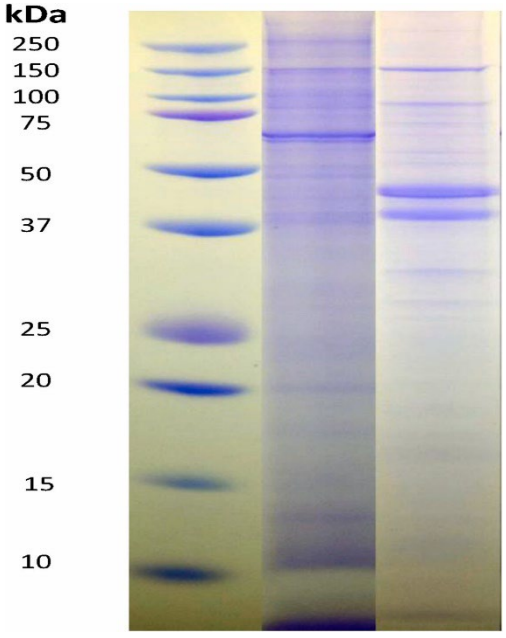


Figure 2.7: NuPAGE gel of control, CBV and CFV, respectively. *12% Bis-Tris mini precast protein gel. Y-*axis is in kilo Daltons (kDa).

2.4.2 Control mice

All animals in the control group maintained unchanged heart rate and Doppler parameters for the 60 min of induced anesthesia, whereupon they were euthanized. Cardiac parameters showed no change

throughout their period of anesthesia (Tables 2.1, 2.2 and 2.3). As there were no deaths the data are provided as mean data at 20 min intervals from T_0 to T_{60}.

Table 2.1: Mean values for aortic cardiac parameters at 0, 20, 40 and 60 min for control mice. Time (T), heart rate (HR), stroke distance (SD), extrapolated cardiac output (CO – Θ = SD x HR x Valve area (Θ)), and aortic rise time (ART). Raw values and associated 95% C.I. are included in brackets for each parameter.

T (min)	HR (bpm)	SD (mm)	CO – Θ (mm/min)	ART (msec)
0	100% (404 ± 12)	100% (5.4 ± 0.6)	100% (2200 ± 218)	100% (29.5 ± 1.3)
20	100% (403 ± 12)	96 (5.2 ± 0.8)	95% (2096 ± 289)	101% (29.8 ± 2.2)
40	101% (408 ± 17)	94 (5.1 ± 0.8)	95% (2093 ± 279)	92% (27.4 ± 1.8)
60	101% (408 ± 25)	94 (5.1 ± 1.0)	91% (2008 ± 343)	99% (29.2 ± 1.3)

Table 2.2: Mean values for mitral cardiac parameters at 0, 20, 40 and 60 min for control mice. Time (T), E-stroke distance (E-SD), E-peak velocity (E-PV), A-stroke distance (A-SD), A-peak velocity (A-PV), E/A ratio, isovolumetric contraction time (IVCT), isovolumetric relaxation time (IVRT). Raw values and associated 95% C.I. are included in brackets for each parameter.

T (min)	E-SD (mm)	E-PV (cm/s)	A-SD (mm)	A-PV (cm/s)	E/A Ratio	IVCT (msec)	IVRT (msec)
0	100% (3.8 ± 0.5)	100% (144 ± 13)	100% (1.6 ± 0.1)	100% (96 ± 11)	100% (1.5 ± 0.1)	100% (8.6 ± 1.9)	100% (14.9 ± 0.9)
20	97% (3.7 ± 0.5)	100% (144 ± 17)	94% (1.5 ± 0.2)	99% (95 ± 14)	101% (1.5 ± 0.1)	136% (11.7 ± 1.9)	95% (14.2 ± 1.0)
40	97% (3.7 ± 0.6)	99% (142 ± 17)	94% (1.5 ± 0.2)	93% (89 ± 14)	107% (1.6 ± 0.1)	107% (9.2 ± 1.2)	102% (15.2 ± 1.0)
60	95% (3.6 ± 0.8)	99% (142 ± 18)	88% (1.4 ± 0.2)	89% (86 ± 18)	114% (1.7 ± 0.2)	86% (7.4 ± 2.0)	101% (15.1 ± 0.5)

Table 2.3: Number of subject mice in each group.

	Controls	CFV	CBV
Aortic	4	5	6
Mitral	4	5	4
Total	8	10	10

2.4.3 Study mice

All study animals died after CFV or CBV venom within 30 min of injection. CFV mice reached their endpoint of 25% CO significantly faster than CBV mice ($F = 13.57$, D.f. = 1*18, $p < 0.05$) (Figure 2.8).

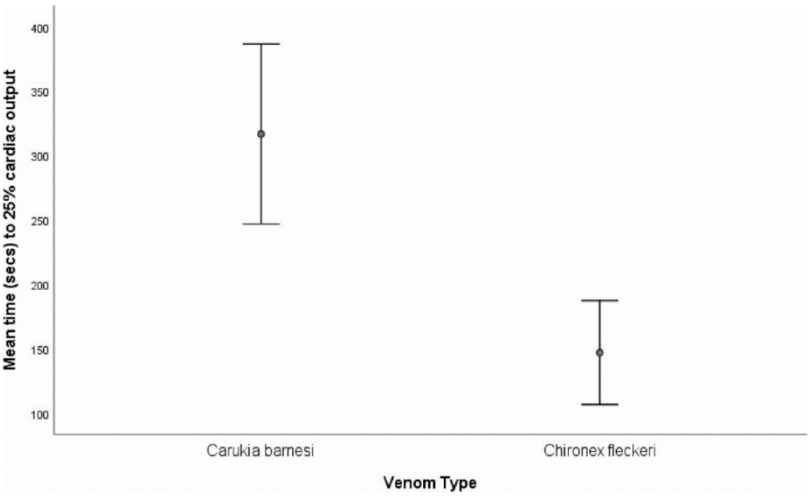


Figure 2.8: Mean time (sec) to endpoint of 25% cardiac output. Time vs CBV or CFV. Error bars are 95% confidence limits.

2.4.4 Assessment of cardiac output

After CFV administration (Figure 2.9A) there was a progressive linear deterioration in stroke distance (% SD) from T_{30} ($F = 37.6$, D.f. = 9*17, $p < 0.001$). Cardiac output (% CO) was calculated as a product of HR and SD. There was no significant change to % HR until T_{90} ($F = 39.2$, D.f. = 9*32, $p < 0.001$), while % SD deteriorated substantially from T_{40}, with concomitant reduction in % CO ($F = 69.7$, D.f. = 9*32, $p < 0.001$).

In Figure 2.9B, CBV administration induced a sustained increase in % HR ($F = 21.3$, D.f. = 9*59, $p < 0.001$), while % SD was maintained to T_{70} ($F = 6.2$, D.f. = 8*21, $p < 0.001$). The calculated increase in % CO of 25% appeared to be directly related to HR and this was maintained until T_{90} ($F = 15.5$, D.f. = 9*59, $p < 0.001$), after which there was a precipitous fall in both.

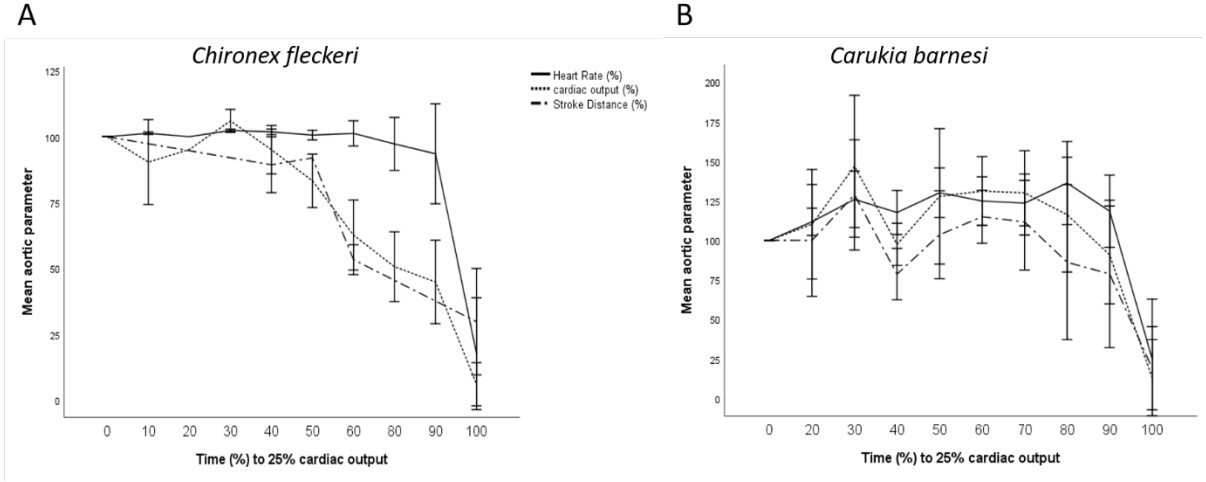


Figure 2.9: Parameters of venom effects on aortic valve. *Time vs % HR (–), % CO (…) and % SD (_.) for (A) CFV and (B) CBV. % CO = 100((HRxSDxΘ)/HRxSDxΘ)), where Θ is the cross-sectional area of the aortic valve. Error bars are 95% confident limits.*

2.4.4.1 Assessment of systolic function

Figure 2.10A shows that CFV was associated with an increase in the isovolumetric contraction time (% IVCT) from T_{20} until T_{90}, peaking at 150% at T_{30} (F = 11.5, D.f. = 9*15, $p < 0.001$), after which it was not measurable. Aortic rise time (% ART) did not demonstrate any statistically significant change until T_{90} (F = 0.36, D.f. = 4*10, $p = 0.83$, not significant), while peak aortic velocity (% PAV) showed deterioration from T_{50} (F = 11.7, D.f. = 4*10, $p = 0.001$).

Figure 2.10B shows a modest reduction in % ART (though this did not reach statistical significance, F = 0.52, D.f. = 9*29, $p = 0.845$) with an accompanying increase in % PAV (F = 6.2, D.f. = 9*29, $p < 0.001$) after administration of CBV. The expected reduction in % IVCT, consistent with inotropy was not demonstrated (F = 1.1, D.f. = 8*20, $p = 0.38$) presumably because of the large spread of data points.

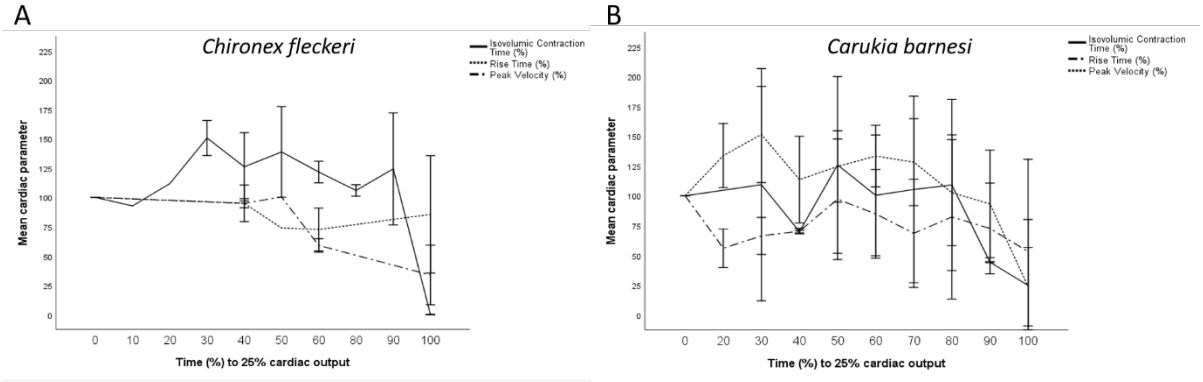


Figure 2.10: Parameters of venom effects on aortic valve. *(A) CFV: Time vs % IVCT (–), % ART (…) and % PAV (_.); (B) CBV: Time vs % IVCT (–), % ART (_.) and % PAV (…). Error bars are 95% confidence limits.*

2.4.4.2 Assessment of diastolic function

Figure 2.11A shows that following CFV administration there was an increase in isovolumetric relaxation time (% IVRT) from T_{50} (F = 27.9, D.f. = 9*15, $p < 0.001$), associated with a concomitant progressive reduction in E-stroke distance (% E-SD) (F = 10.9, D.f. = 9*17, $p < 0.001$). A-stroke distance (% A-SD) was maintained until T_{80} after which it too deteriorated (F = 37.6, D.f. = 9*17, $p < 0.001$).

Figure 2.11B shows that CBV was associated with a significant reduction in % IVRT after T_{40} (F = 3.8, D.f. = 8*21, $p = 0.007$), however both % E-SD and % A-SD remained unchanged until after T_{90} (F = 5.1, D.f. = 8*21, $p = 0.001$, and F = 6.2, D.f. = 8*21, $p < 0.001$, respectively).

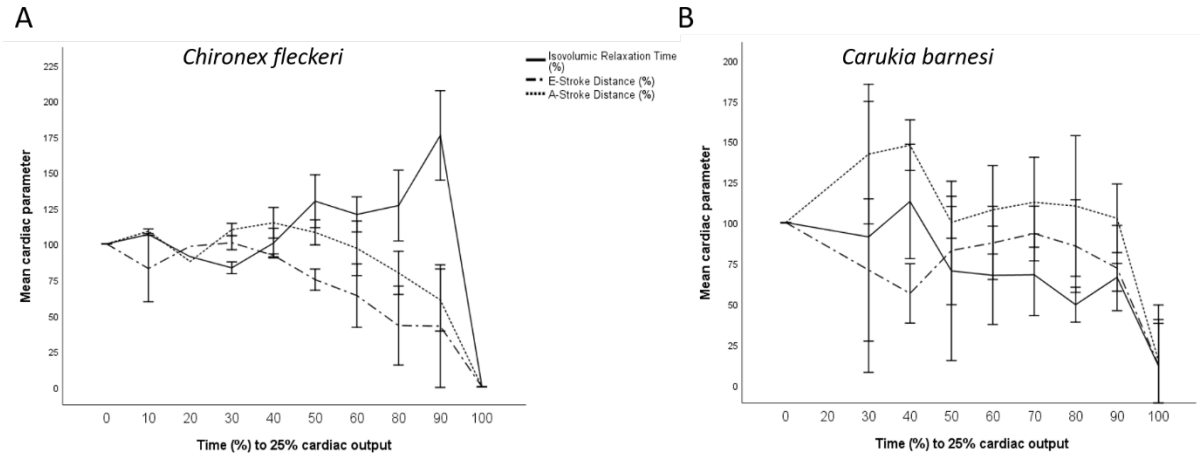


Figure 2.11: Parameters of venom effects on mitral valve. *Time vs % IVRT (–), % E-SD (–), % A-SD (_.) for (A) CFV and (B) CBV. Error bars are 95% confidence limits.*

CFV administration was associated with a significant early reduction in % E-PV from T_{30} ($F = 17.1$, D.f. = 9*17, $p < 0.001$), while % A-PV was maintained until T_{80} ($F = 12.3$, D.f. = 9*17, $p < 0.001$) (Figure 2.12A). CBV administration, however, showed an immediate increase in % A-PV ($F = 8.4$, D.f. = 8*21, $p < 0.001$) with little concomitant change in E-PV ($F = 16.3$, D.f. = 8*21, $p < 0.001$) (Figure 2.12B). This was maintained until T_{90}, where both started deteriorating.

With CFV, E/A Ratio showed an immediate modest decrease from 2.4 to 1.8 ($F = 2.9$, D.f. = 9*29, $p = 0.013$) which was maintained until T_{90} (Figure 2.12C). In contrast, CBV was associated with a significant immediate reduction in E/A Ratio to 1 by T_{30} ($F = 20.1$, D.f. = 9*32, $p < 0.001$) maintained until T_{90} (Figure 2.12C).

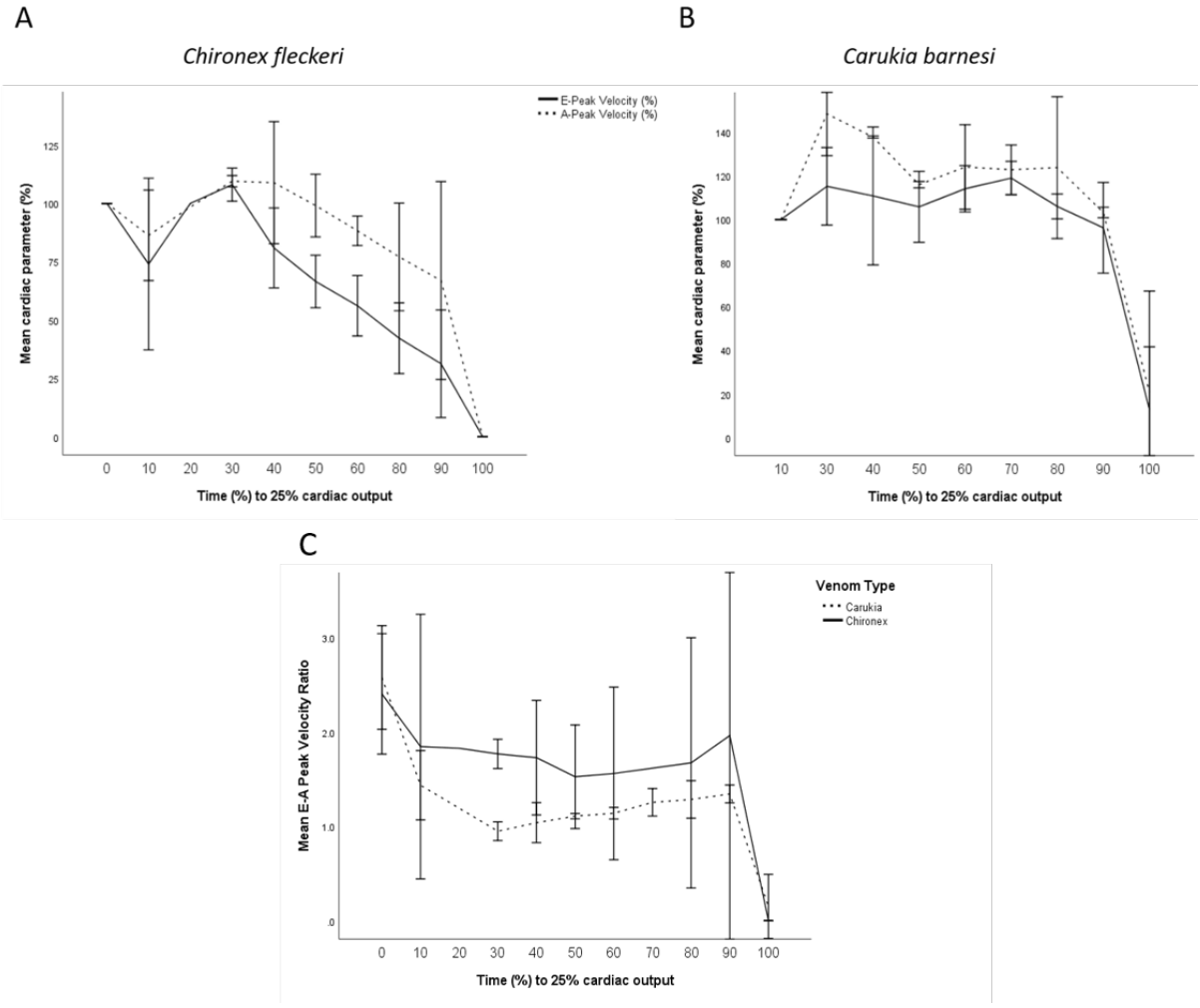


Figure 2.12: Parameters of venom effects on mitral valve. *Time vs % E-PV (–) and % A-PV (…) for (A) CFV, (B) CBV and (C) Time vs E/A Ratio for CFV (–) and CBV (…). Error bars are 95% confidence limits.*

2.5 Discussion

2.5.1 Overview

Our study suggests that CFV is associated with a rapid reduction in cardiac output, and due to the rapidity of onset, suggests a direct myocardial action. Morphological analysis demonstrates both systolic and diastolic dysfunction, which we believe is consistent with a progressive loss of compliance, possibly from a contracted myocardium. In the absence of direct visualisation (echocardiographic or otherwise), this remains a reasonable speculation. CBV produced significant chronotropic and inotropic effects that were sustained until a precipitous deterioration to death. This sudden deterioration after a sustained period of increased work is consistent with previous suggestions of a stress induced cardiomyopathy, although confirmation will require dynamic echocardiographic evidence of progressive wall motion abnormalities.

2.5.2 CFV

Although respiratory rate was not documented, there was no gross change to respiratory rate or effort with CFV administration, until after pre-terminal bradycardia had ensued whereupon agonal breathing was observed until complete cardiorespiratory arrest.

There were significant changes in cardiac output following injection of CFV. Cardiac output was maintained until T_{50} after which there was progressive deterioration to 40% at T_{90} (Figure 2.9A). The HR was maintained until T_{80} following which bradycardia ensued and CO dropped below 25%. Given that there was no change to heart rate until T_{80}, the reduced cardiac output appears to be solely from a reduced stroke volume (directly proportional to the measured SD). The cause of this reduced stroke volume becomes apparent when the force of ventricular contraction and filling of the left ventricle is examined through analysis of the aortic and mitral waveforms.

2.5.2.1 Aortic waveform (Figure 2.10A)

Ventricular contraction (systolic function) is reflected in morphological measures of the left ventricular (aortic) outflow waveform, particularly the % IVCT, % PAV and % ART. Percent IVCT increased after CFV injection, reaching 150% of control at T_{30}. An increase in % IVCT was maintained until cardiovascular collapse at T_{100} where it could no longer be measured. The increased % IVCT describes dysfunctional left ventricle contraction, reflecting the time taken to attain adequate intra-ventricular pressure to open the aortic valve and eject its volume into the aorta.

Consistent with this is the opposite pattern with % PAV. A reduction to <50% at T_{60} was seen, indicating reduced left ventricular contractility, where ventricular contraction effectively took longer to achieve peak velocity. This parameter is independent of HR and is a reliable measure of systolic dysfunction

(Doppler Flow Velocity System, User Manual). An accompanying reduction in % ART did not reach statistical significance.

Our findings demonstrate that CFV produces significant systolic ventricular dysfunction that appears rapidly after injection. This strongly supports the presence of a direct acting myotoxin.

2.5.2.2 Mitral waveform (Figures 2.11A, 2.12A and 2.12C)

This shows that CFV was associated with a moderately reduced E/A Ratio from 2.4 to 1.8. In the absence of an increase in HR this indicates diastolic ventricular dysfunction (dysfunctional ventricular relaxation). Figure 2.11A shows a progressive increase in % IVRT from T_{50} reaching 175% at T_{90} and unmeasurable at T_{100}. The increase in % IVRT indicates diastolic ventricular dysfunction and consequent reduction in % E-SD. Interestingly, there was no compensatory increase in % A-SD. Similarly, Figure 11A shows that the % E-PV deteriorated from T_{40} while % A-PV was maintained until T_{90}. Together these suggest dysfunctional ventricular relaxation extending throughout diastole.

In summary, CFV administration was associated with significant reduction in cardiac output. There was measurable left ventricular systolic dysfunction evidenced by an increase in % IVCT and a reduction in % PAV. Furthermore, there is evidence of diastolic dysfunction with a progressive increase in % IVRT and reduction in passive and active ventricular filling. These findings of systolic dysfunction together with sustained dysfunctional diastolic relaxation strongly supports hypotheses that CFV induces its cardiac effect through a progressively poorly compliant myocardium, possibly contracted. The rapid development of these measurable findings also supports the notion that CFV exerts its effect through a direct myocardial toxin.

The mechanism by which this occurs is not examined in this study, though there are many possibilities including channelopathies or local metabolic dysfunctions, for instance as a result of ischemia from vasospasm. A number of hypotheses involving Ca^{2+} have previously been proposed. Given the complex and focal dependence of effective myocardial contraction on Ca^{2+} ion movements between the sarcoplasmic reticulum and the tropomyosin contractile complex, Currie proposed the possibility of the governing homeostatic mechanisms of Ca^{2+} movement being disrupted by CFV (Currie, 1994). This may be through an increase in intracellular Ca^{2+} influx (directly or indirectly), or through a reduction in Ca^{2+} efflux by disruption of the complex reuptake of intracellular Ca^{2+}.

Persistently contracted ventricles (ischemic contracted ventricles, stone heart) are sometimes identified at autopsy following failed surgery involving cardiopulmonary bypass (Hutchins and Silverman, 1979). In 2002, Klouche et al., described a progressive loss of left ventricular (LV) compliance, thickening of the LV walls and reduced diastolic volumes evolving under direct trans-oesophageal echocardiographic observation during induced cardiac arrests in a porcine model

(Klouche et al., 2002). Autopsy findings confirmed the presence of ischaemic contracture of the myocardium, or stone heart. The relevance to our study is that we consistently observe this phenomena with CFV administration in a cane toad model where the anterior thorax has been removed for direct cardiac observation; and provide a link to one of the Authors' YouTube video demonstrating this: https://www.youtube.com/watch?v=sf ZuKXNPqFs.

2.5.3 CBV

Although respiratory rate was not measured, it was subjectively appreciated that respiratory rate and effort were increased after CBV administration. Additionally, ocular proptosis was frequently observed, while oral/nasal secretions forming foamy bubbles were noted in all CBV subject mice. Although these secretions appeared similar to those encountered in clinical situations where patients experience fulminant cardiac failure, these were too small to sample in appreciable amounts for analysis.

Unlike CFV, the injection of CBV produced an increase in HR that is sustained until T_{80}, followed by a precipitous deterioration to death (Figure 2.9B). This is an immediate effect and suggests either a direct chronotropic effect or a direct stimulus for catecholamine release.

2.5.3.1 Aortic waveform (Figures 2.9B and 2.10B)

An increase in calculated % CO was observed with CBV (Figure 2.10B) and this was maintained until T_{80}. As % SD remained unchanged until then, the increased % CO appears to be due to the increased chronotropy. Percent SD is directly proportional to stroke volume and consequently it is reasonable to conclude that CBV does not increase the ejected volume of each ventricular contraction. Instead, the force of contraction is increased, as indicated by an increase in % PAV (Figure 2.10B). A measured reduced % ART would be further evidence, however, this did not reach statistical significance. Similarly, an accompanying reduction in % IVCT was not statistically observed.

CBV produced an increase in systolic function primarily as a result of increased chronotropy. The measured change to % PAV is evidence of increased contractility that necessarily accompanies the increased chronotropic effects with CBV administration. These findings are in keeping with toxicity models that associate CBV toxicity with measured increases in serum catecholamines (Winkel et al., 2005).

2.5.3.2 Mitral waveform (Figures 2.11B and 2.12C)

Measures of diastolic function are clouded by the expected, normal fusion of E and A waves at HR above 500 beats/min (Doppler Flow Velocity System, User Manual). Figure 2.11B shows progressive reduction in % IVRT, which is consistent with increased chronotropy. Similarly, there was an

accompanying reduction in % E-SD and an increase in % A-SD, resulting in a substantial reduction in the E/A Ratio (Figure 2.12C). It is not surprising that with increased chronotropy the left ventricle's reliance on passive filling is reduced and instead is increasingly reliant on active atrial contraction.

We have direct evidence of significant chronotropic and inotropic effects which are sustained until T_{90} after which there was cardiac collapse and death. Positive inotropy and chronotropy are consistent with hypotheses that promote a catecholaminergic aetiology, however, our research was not structured to investigate this as a potential mechanism. Winkel's research with animal models demonstrate large increases in catecholamine levels following administration of crude CBV (Winkel et al., 2005). As we did not measure catecholamine levels, we can only speculate that our findings were due to similarly elevated catecholamine levels.

Huynh, in his study of 116 Irukandji victims, identified that patients with persistent symptoms and signs of catecholamine excess were at increased risk for cardiac injury identified by raised serum markers, some with ECG changes or echocardiographic abnormalities (Huynh et al., 2003). Persistently elevated catecholamine disease states demonstrate similar signs and symptoms and are associated with the development of a Takotsubo type myocardial injury (Ghadri et al., 2018). In support of this is Tiong's finding that a patient with severe Irukandji syndrome with diagnostic echocardiographic patterns of Takotsubo stress induced cardiomyopathy (Tiong, 2009).

In our study, the precipitous nature of the collapse after sustained increased chronotropy and inotropy suggests the possibility of a stress related myocardial failure, however in the absence of echocardiographic findings this remains purely speculative. Stress induced cardiomyopathy is generally considered a reversible endpoint of extreme stress. Notably, our study identified only moderate changes in heart rate, and consequently moderate changes in cardiac output and work. Tiong, however, showed that mid ventricular wall abnormalities consistent with Takotsubo cardiomyopathy was present in his case report of a 24M with Irukandji Syndrome, in the presence of mild persistent elevations in HR (Tiong, 2009). It suggests that HR is a poor measure of cardiovascular stress. Our study used an anaesthetic protocol that utilised ketamine and pentobarbital. While ketamine is a known cardiac stimulant, it is possible that the latter agent could have blunted a larger increase in HR.

It is recognised that Mg^{2+} is effective at reducing adrenal catecholamine release as well as peripheral adrenal nerve terminals (Douglas and Rubin, 1963). Although there is reported variation in the success of Mg^{2+} in the treatment of Irukandji Syndrome (Corkeron et al., 2004; McCullagh et al., 2012), there is some theoretical cardioprotective rationale for its administration as an anti-catecholaminergic agent.

2.5.4 Other observations

All tested mice developed bradycardia followed by agonal breathing and cardiorespiratory arrest, and notably there were no observed respiratory arrests preceding bradycardia. Fatal *C. fleckeri* envenomation occurs rapidly at the time of envenomation, and respiratory arrest was understandably described as a presenting feature; note that this is invariably in the absence of cardiac monitoring. Our research indicates that respiratory arrest is secondary to the cardiac effects.

2.6 Conclusions

Our study demonstrates that CFV was associated with a rapid reduction in cardiac output that strongly suggests a direct myocardial action. Previous suggestions of associated respiratory arrest was not demonstrated in our mouse envenomation model. Respiratory arrest only occurred after cardiovascular collapse had ensued. Analysis of the PW Doppler sonograms indicates both a systolic and diastolic dysfunction that we believe is best explained by a progressively poorly compliant, contracted left ventricle.

CBV did not produce the same immediate deterioration in cardiac function. Instead, there was immediate increased chronotropy and accompanying inotropy that was sustained until a precipitous cardiac collapse leading to death. This temporal relation, together with subjective observations of increased respiratory efforts and frothy oral secretions appear to be consistent with clinical models of stress induced cardiomyopathy. Our research does not shed any light on whether this is a direct or indirect effect of CBV.

2.7 Further directions

This study demonstrates that PW Doppler can be used for non-invasive assessments of cardiac function in ketamine/pentobarbital sedated mouse envenomation models. We believe there is scope to apply this technique in assessing the effectiveness of current and future treatment modalities. Perhaps by utilising larger animals invasive measures can be added to this technique and there is scope for an investigation into toxic mechanisms and also dose response.

2.8 Limitations

This work was performed on a small number of anaesthetised mice, using ketamine/pentobarbital as the anaesthetic. Ketamine does have an inotropic effect and as such may have contributed to some chronotropic and inotropic bias to the results. However, control mice (ketamine only) did not demonstrate any deterioration in cardiac function over the study period and all required euthanasia

at the end of the 60 min study period. The data should be interpreted with caution when interpreting this for human envenoming.

In addition, there was significant variation in time to death within venom groups. This was seen within both CFV and CBV groups and presumably is a combination of a variation in venom dose/kg (variation in animal size), individual variation in susceptibility, and variation in intravascular dosage from venom administration through vascular puncture (possible extravasation), rather than through cannulation where extravasation is less likely. Variation in time of death was negated by using time to 25% CO as the analysis endpoint, which allowed the study to compare between test mice.

This study did not aim to examine dosage and instead was targeted to cause death so that myocardial dysfunction leading to this endpoint could be studied. Nevertheless, all animals received sufficient venom to demonstrate cardiac dysfunction and death within 30 min.

Ethics: Ethics application was approved by the JCU Ethics committee in Oct 2015 (Ethics: A2233).

Funding: Emergency Medicine Foundation (EMPG-205R22-2014-LITTLE) funded this research.

Ethics statement: All 28 mice used in this experiment were housed by the JCU mouse house, a facility that looks after all mice for the JCU to a national standard. All mice were bred by the Animal Resources Centre (www.arc. wa.gov.au) with national and international quality standards. All mice were anaesthetised with a single dose of ketamine and pentobarbital sufficient to maintain anaesthesia, analgesia and free from any distress for sufficient duration.

Declaration of competing interest: The authors declare that there are no conflicts of interest.

CRediT authorship contribution statement: Jamie Seymour: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Silvia Saggiomo:** Formal analysis, Investigation, Data curation, Writing - review & editing. **Willis Lam:** Formal analysis, Writing - review & editing. **Peter Pereira:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Mark Little:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Chapter 3

Review

3 The geographic distribution, venom components, pathology and treatments of stonefish (*Synanceia* spp.) venom

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3.1 Abstract

Stonefish are regarded as one of the most venomous fish in the world. Research on stonefish venom has chiefly focused on the *in vitro* and *in vivo* neurological, cardiovascular, cytotoxic and nociceptive effects of the venom. The last literature review on stonefish venom was published over a decade ago, and much has changed in the field since. In this review, we have generated a global map of the current distribution of all stonefish (*Synanceia*) species, presented a table of clinical case reports and provided up-to-date information about the development of polyspecific stonefish antivenom. We have also presented an overview of recent advancements in the biomolecular composition of stonefish venom, including the analysis of transcriptomic and proteomic data from *Synanceia horrida* venom gland. Moreover, this review highlights the need for further research on the composition and properties of stonefish venom, which may reveal novel molecules for drug discovery, development or other novel physiological uses.

Keywords: stonefish; *Synanceia*; venom; toxins; pharmacology; distribution

3.2 Introduction

There are five species of stonefish within the genus *Synanceia*: *Synanceia horrida* (previously referred to as *S. trachynis*), *S. verrucosa*, *S. alula, S. nana* and *S. platyrhyncha*. *Synanceia* spp. can grow 35-50 cm in length and have evolved grey and mottled skin to camouflage themselves amongst encrusted rocks and coral for predation and defense (Smith and Heemstra, 1986). *Synanceia* species have up to 15 dorsal fin spines that are erected when the fish is disturbed (Eschmeyer and Rao, 1973). Stings from this medically important group of fish are known to cause painful and lethal human envenomations.

Although clinical reports of stings and injuries from *Synanceia* species have been reported since the end of the 19th century (Halstead et al., 1956), studies into their venom biology, chemistry and pharmacology have been scarce, and the mechanisms of action of the venom remain mostly unknown. Fortunately, much has changed in this field in recent years due to technological advances, which have spurred new avenues of research. This review attempts to comprehensively summarize and update the current state of knowledge of *Synanceia* spp., with a focus on geographic distribution, human impact, disease burden, venom composition and the mechanisms of action of venom components.

3.3 Disease burden of stonefish envenomation

Statistics from independent retrospective clinical studies of stonefish stings from hospitals in the Indo-Pacific region showed that most envenomations occurred in young adult males with occupational or recreational exposure to stonefish habitat (Bradley, 2008; Chan et al., 2010; Hifumi et al., 2020; Poon et al., 2020). Stonefish were the second most common fish involved in human envenomations in Hong Kong (Chan et al., 2010), and were responsible for one-third of all fish envenomations in Australia in 2008 (Bradley, 2008). A recent report by the Australian Institute of Health and Welfare stated that, of all hospitalizations due to injury from contact with venomous marine plants and animals from 2017-2018, 81% were caused by stinging fish, where 30 out of 320 cases (9.3%) were due to stonefish envenomation (Pointer and Harrison, 2021). Additionally, stonefish have been responsible for a high number of stings in Singapore (Khoo, 2002; Ngo et al., 2009), and there are concerns that the number of stings will grow in Japan due to warmer ocean temperatures and increasing tourism (Hifumi et al., 2020). These studies also report that victims usually sought medical assistance within 2 h of being stung due to intense pain (Hifumi et al., 2020; Poon et al., 2020). The majority of the cases was regarded as mild envenomation, with average hospital stays ranging from hours (Poon et al., 2020) to days (Lee et al., 2004). In more severe cases, hospitalizations can last for over a week (Hifumi et al., 2020) and surgical interventions may be necessary in some instances (Lee et al., 2004; Ngo et al., 2009; Poon et al., 2020).

While these reports highlight the burden of fish stings in the Indo-Pacific region, stonefish envenomation is grossly underreported in the literature (Poon et al., 2020). This makes it difficult to precisely quantify the number of victims and the global impact of stonefish envenomation on an annual basis. Another confounder is that the diagnosis of a stonefish sting usually relies on the presence of puncture wounds and the clinical and systemic features that follow envenomations. Only rarely is the animal brought to the medical center, hindering the actual identification of the offending animal (Lee et al., 2004). Stonefish envenomation case reports from literature to date have been reviewed and summarized in Appendix B. Caution in interpreting these numbers is further advised as generally, while case reports may state that the victim was stung by a *Synanceia* species, there is frequently no confirmation or validation of which marine creature caused the sting (Poon et al., 2020). To date, there has been no research comparing or contrasting the effects of venom from different species of stonefish following envenomation. Thus, species differentiation by clinical presentation is currently not viable and it is not known if venom effects differ across the genus.

3.4 Geographic distribution of *Synanceia* spp.

Species of the *Synanceia* genus have a broad overlapping distribution throughout the shallow waters of tropical and temperate areas of the Indo-Pacific (Figure 3.1 and Appendix C). *S. verrucosa* (reef stonefish) has the broadest distribution, with confirmed sightings off the coasts of Turkey, Mozambique, Sri Lanka, Singapore, Japan, Australia, and throughout the islands of the Pacific Ocean to French Polynesia. *S. horrida* (estuarine stonefish) has the second largest distribution, with confirmed sightings from mid to Northern Australia, throughout Malaysia, Indonesia, Singapore, Thailand and the Philippines. *S. nana* appears to be confined to the waters of the Persian Gulf and the Red and Arabian Seas, whereas *S. alula* and *S. platyrhyncha* have been observed only occasionally (Figure 3.1; Appendix C). Interestingly, only one holotype of *S. platyrhyncha* was located, and upon further examination, Eschmeyer and Rao (1973) stated that *S. platyrhyncha* may be *S. horrida*, but more specimens are required for validation. Although there have been some suggestions of *Synanceia* species present in the Florida Keys and the Caribbean Sea (Diaz, 2015), no evidence was found in the literature to support this.

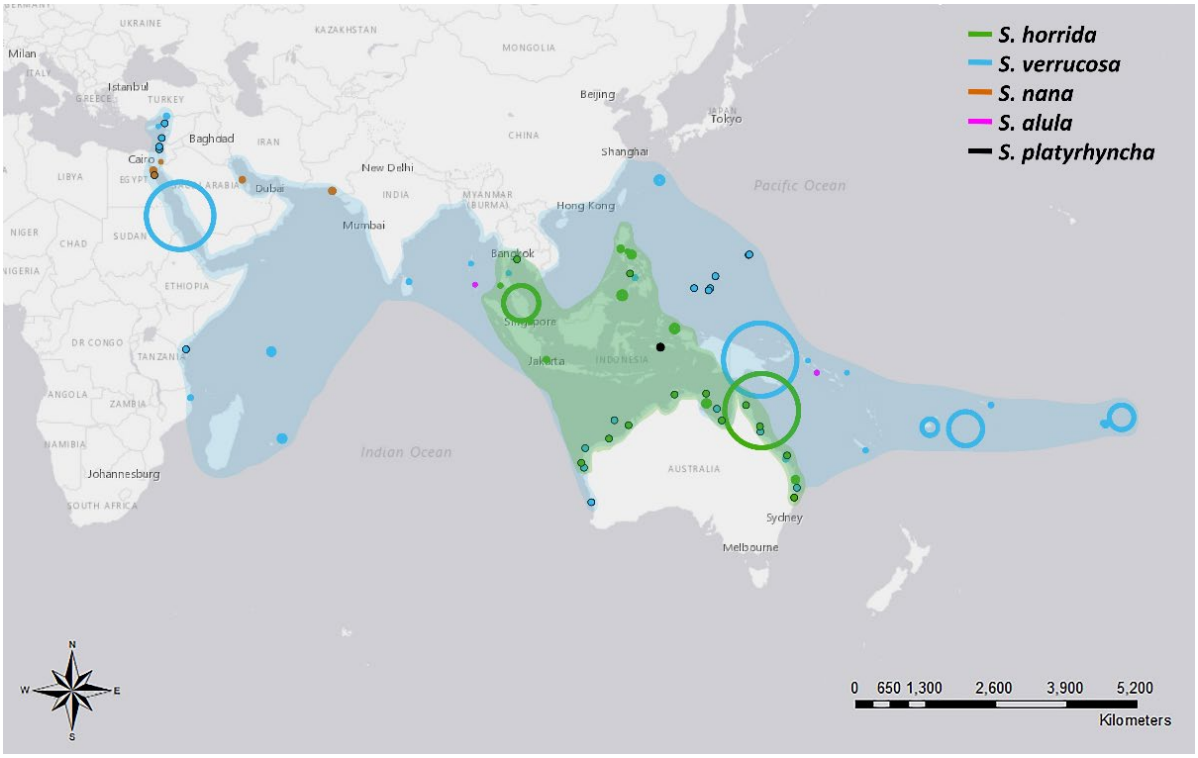

***Figure 3.1: Distribution of five different species of stonefish of the genus* Synanceia**. *Points with black outlines represent sightings where coordinates were provided. Circle size depicts the accuracy of the reported location; for example, large circles depict reports where only the country name was given, and small circles depict reports where the precise location was stated. Shades indicate the hypothesized distribution of both* S. horrida and S. verrucosa *based on collated sighting data. Animals would be found in the shallow regions within the shaded areas. References listed in Appendix C. Figure generated with ArcMap 10.7 and Photoshop 2021.*

Although there are five species of *Synanceia*, only two have been extensively studied: *S. horrida* and *S. verrucosa*. These species are considered the world's most venomous fish species (Halstead et al., 1956). *S. horrida* has 13 to 14 dorsal venom spines, and is commonly found in estuaries, sheltered bays, shoal reef areas and tide pools (Endean, 1961; Eschmeyer and Rao, 1973) (Figure 3.2A). The head is depressed, large, and the most distinguishable features are the deep pits below the eyes and the eyes in elevated stalks with high crests joining them (Eschmeyer and Rao, 1973). *S. verrucosa* can have 12 to 14 dorsal venom spines and is commonly found in coral reef areas (Endean, 1961; Halstead et al., 1956) (Figure 3.2B). The head is broad, large and depressed, and the eyes are marginally elevated (Eschmeyer and Rao, 1973). Both species are sluggish, ambush predators that often sit motionless and partially buried in the substrate (Endean, 1961; Grobecker, 1983).

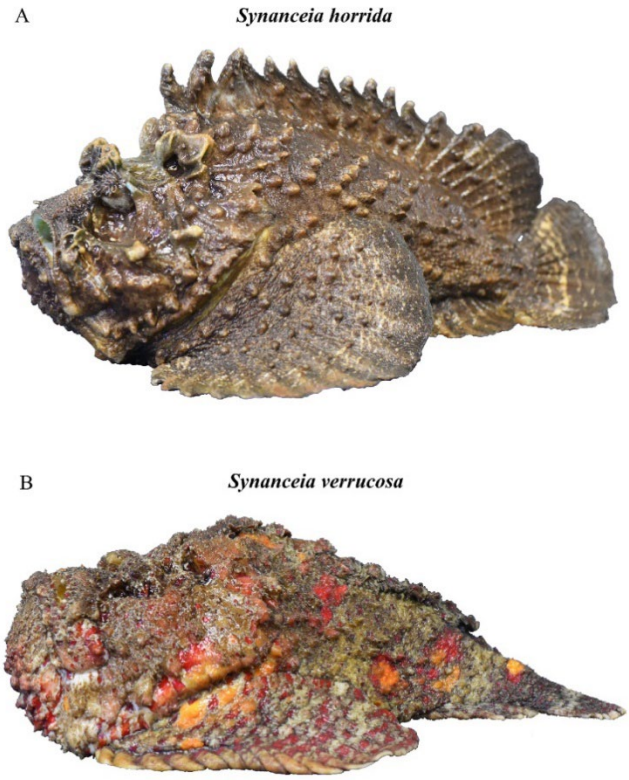

Figure 3.2: Side profiles of (A) **Synanceia horrida** ***and (B)*** **Synanceia verrucosa**. *Photo: Prof. Jamie Seymour.*

3.5 Envenomation

3.5.1 Venom apparatus

One of the remarkable physical attributes of stonefish is their grooved hypodermic-like dorsal spines (Figure 3.3). These spines are used purely as a defensive mechanism, as they are erected by the animal when threatened (Southcott, 1977). Each dorsal spine is associated with a pair of venom glands, which are covered by a loose and thick integumentary sheath (Williamson et al., 1996). When force is applied vertically on the dorsal spines, the integument is ruptured, and the surrounding tissue of the predator or the victim compresses the dual venom glands, involuntarily releasing venom through the spinal venom duct (Williamson et al., 1996). Each pair of dorsal venom sacs contains approximately 5-10 mg of dried venom (Wiener, 1959a), thus the severity of symptoms is typically associated with the number of spines involved in envenomation and the depth of spine penetration (Khoo, 2002).

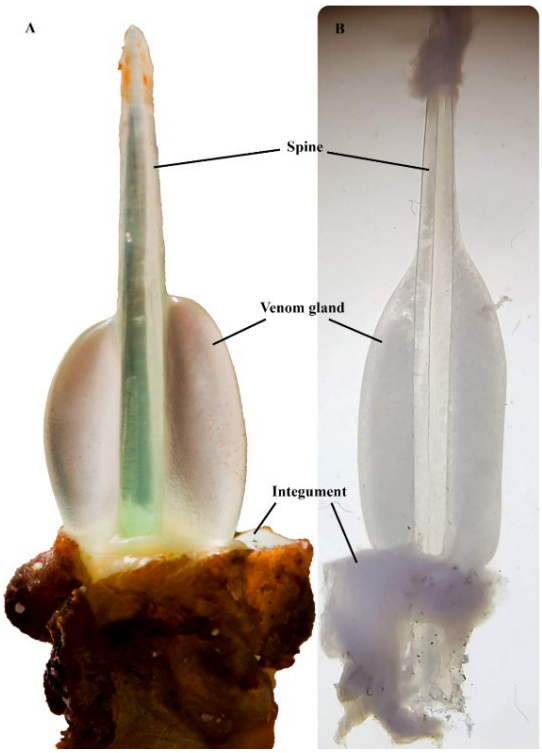

Figure 3.3: **Synanceia *venom glands.*** *(**A**) The thick integument sheath was removed from the second spine (longest) of* S. horrida*, revealing the venom gland* in situ (*Photo: Prof. Jamie Seymour) and (**B**) Venom gland dissected out from the last spine (shortest) of* S. verrucosa, *under the microscope (Photo: Silvia Luiza Saggiomo). Figures are not to scale.*

3.5.2 First aid, clinical and systemic features of stonefish stings

The pain associated with stonefish stings is intense, excruciating, disproportionate to the size of the injury, and may spread to include the whole limb and associated lymph glands (Halstead et al., 1956). The envenomed limb displays puncture wounds with a blue coloration, gross edema and local morbidity (Lee et al., 2004). Successful first aid for fish stings usually focuses on pain alleviation, treatment of the injury site and the effects of envenomation (Tintinalli et al., 2011). Although there seems to be no consensus on venomous fish sting first aid, hot water therapy is recommended as an effective pain management tool (Atkinson et al., 2006; Hornbeak and Auerbach, 2017). For stonefish envenomation in particular, it has been suggested to keep the affected limb immersed in water at 42^{o}C for at least 20 min to inactivate the venom (Barnett et al., 2017). Victims should also seek medical aid, where treatments might include radiography, ultrasound, debridement of the wound, local anesthetics, tetanus prophylaxis, and administration of stonefish antivenom (Gwee et al., 1994; Lee et al., 2004; Williamson et al., 1996). Systemic symptoms include fever, delirium, muscle weakness and paralysis, pulmonary edema, respiratory difficulties, hypotension, bradycardia, arrhythmia, convulsions, heart failure and death (Auerbach, 1991; Gwee et al., 1994; Phoon and Alfred, 1965; Saunders, 1959; Williamson et al., 1996).

In recent years, several clinical reports of stonefish envenomation indicate that victims may have lingering complications after being stung (Appendix C). Some symptoms, such as asthenia and trophic disorders, may persist for months in the affected limb, and authors recommended ongoing care of the puncture site and broad-spectrum antibiotics for a few days post envenomation (Lee et al., 2004;

Lehmann and Hardy, 1993; Maillaud et al., 2020). Additionally, acute compartment syndrome due to edema has been reported in stonefish sting victims, thus surgical procedures may be required to avoid permanent neurological damage (Ghanem et al., 2019; Ling et al., 2009; Tay et al., 2016) and amputations due to necrosis have been necessary in some cases (Dall et al., 2006; Nistor et al., 2010; Téot et al., 2015). Deaths caused by stonefish envenomation have been the subject of great debate, as specific details of the recorded deaths have been poor or missing altogether (Smith, 1957; Williamson et al., 1996). Unfortunately, a recent case report discussing three cases of stonefish envenomation described the death of an 11-year-old boy after being stung in the foot in 2018 (Maillaud et al., 2020). It appears, therefore, that stonefish envenomation causes serious symptoms and pathologies, where long-lasting effects may affect victims worldwide, and lethal envenomations may in fact occur.

3.5.3 Antivenom

Synanceia antivenom works by neutralizing the hemolytic, lethal and vascular permeability-increasing properties of the venom (Shiomi et al., 1989; Southcott, 1977). The efficacy of stonefish antivenom has been demonstrated both *in vitro* and *in vivo* (Shiomi et al., 1989; Southcott, 1977), and its clinical efficacy at combating venom-induced tissue damage and providing analgesia is well-established (Church and Hodgson, 2003; Lehmann and Hardy, 1993; Shiomi et al., 1989; Sutherland, 1992). Antivenom can be injected intramuscularly or administered through intravenous infusion in severe cases (Gwee et al., 1994). An ampoule containing 2,000 units of antivenom neutralizes approximately 20 mg of venom, and dosages are managed according to the number of puncture wounds present in the affected limb (Currie, 2003).

Interestingly, *Synanceia* antivenom has shown cross-reactivity with different species of venomous fish in both *in vitro* and *in vivo* models (Table 3.1). In particular, this antivenom appears to exhibit cross-reactivity with venoms from the South Australian cobbler (*Gymnapistes marmoratus*) and the red lionfish (*Piterois volitans*) in immunoblotting analysis (Church and Hodgson, 2003). This cross-reactivity may be explained by the close phylogenetic relationship between these species and *Synanceia* species (Theakston et al., 2003), or perhaps these venoms and their constituents possess similar structures and/or modes of action (Ledsgaard et al., 2018). Interestingly, the antivenom used for the aforementioned studies was raised against venom from *S. horrida* only (i.e. monospecific). Currently, however, stonefish antivenom is produced using both *S. horrida* and *S. verrucosa* venoms at Seqirus Pty. Ltd., Melbourne, Australia (Commonwealth Serum Laboratories) (Williamson et al., 1996). Therefore, it may be possible to create a polyvalent (i.e. polyspecific) antivenom that would be effective at treating stings from the most medically important species of venomous fish, increasing the antivenom efficacy, as seen with snake antivenoms (Ciscotto et al., 2011).

Table 3.1:* In vivo *and* in vitro *cross-reactivity studies between venomous fish species and* Synanceia *antivenom.

Fish Species	Neutralizing Action	Reference
Synanceia verrucosa	Lethal and hemolytic	(Shiomi et al., 1989)
Inimicus japonicus	Lethal and hemolytic	(Shiomi et al., 1989)
Pterois lunulata	Lethal and hemolytic	(Shiomi et al., 1989)
Pterois antennata	Lethal and hemolytic	(Shiomi et al., 1989)
Dendrochirus zebra	Lethal and hemolytic	(Shiomi et al., 1989)
Pterois volitans	Lethal, hemolytic and pharmacological	(Church and Hodgson, 2002; Shiomi et al., 1989)
Gymnapistes marmoratus	Pharmacological	(Church and Hodgson, 2002)
Scorpaena plumieri	Inflammatory and cardiovascular	(Gomes et al., 2011)
Notesthes robusta	No effect	(Hahn and Connor, 2000)

3.6 Composition of *Synanceia* spp. venoms

Lability is a key characteristic of stonefish venom. Stonefish venom has shown susceptibility to changes in pH, temperature, storage conditions, lyophilization and repeated freezing and thawing (Church and Hodgson, 2000; Harris et al., 2021; Saunders and Tokes, 1961; Wiener, 1959b). Additionally, there are reports of fish-to-fish variation in toxicity (Garnier et al., 1995), and a recent study into *S. horrida* venom profile associated with different feeding regimes found that venom production is also affected by starvation (Saggiomo et al., 2017). Individual variation in venom composition has been shown in several different species, including snakes (Chippaux et al., 1991), jellyfish (Winter et al., 2010), spiders (Duran et al., 2020) and scorpions (Kalapothakis and Chávez-Olórtegui, 1997), which can affect the efficacy of the venom. Therefore, it is possible that other potential variables may influence venom composition. Factors such as geographical location, season, gender and age of fish may play a significant role; however, none of these have been investigated or reported for *Synanceia* venom yet. Not surprisingly, therefore, early attempts at purifying stonefish venom for experimental purposes were largely unsuccessful.

Progress in fish venom research has rapidly advanced with improvements and advances in biochemical separation processes and molecular analyses (Russell, 1965; Wiener, 1959a). In particular, advances in fractionation, purification and isolation of the toxic elements in *Synanceia* venoms have revealed that all of the lethal activity appears to be caused by only a few toxins or proteins (Garnier et al., 1995; Poh et al., 1991). Stonustoxin (SNTX), isolated from *S. horrida* crude venom, was shown to be a dimeric protein consisting of a α- and β-subunit each, with calculated molecular weights (MW) of 79 kDa (Ghadessy et al., 1996) (Table 3.2). SNTX has a p*I* of 6.9, can comprise as much as 9% of the total protein content of crude venom and has been shown to be 22-fold more toxic than crude venom (Poh

et al., 1991) (Table 3.3). In addition, a cytolysin with a p*I* of 5.7 and a MW of 158 kDa has also been purified from *S. horrida* venom, and was found to possess lethal activity and cell membrane-damaging properties in mice (Table 3.2) (Kreger, 1991).

Table 3.2: Toxins found in stonefish venom with their corresponding molecular weights (MW).

***Synanceia* Species**	**Toxins**	**MW (kDa)**	**Subunit MW (kDa)**	**Reference**
S. horrida	SNTX	148	α-subunit—79 β-subunit—79	(Ghadessy et al., 1996)
	Cytolysin	158	-	(Kreger, 1991)
	SFHYA1	62	-	(Poh et al., 1992)
	Peroxiredoxin-6	24	-	(Ziegman et al., 2019)
S. verrucosa	VTX	322	2x α-subunit—83 2x β-subunit—78	(Garnier et al., 1995)
	NeoVTX	166	α-subunit—79 β-subunit—79	(Ueda et al., 2006)
	Cardioleputin	46	-	(Abe et al., 1996)
	Con A-I-PS-I	42.1 100 110	-	(Kato et al., 2016)
	45 kDa lectin	45	-	(Kato et al., 2016)
	Hyaluronidase	59	-	(Madokoro et al., 2011)

Verrucotoxin (VTX), isolated from *S. verrucosa*, is a tetrameric toxin (two α- and two ß-subunits) with a MW of 322 kDa (Garnier et al., 1995) (Table 3.2). Each α-subunit has a MW of 83 kDa and each ß-subunit 78 kDa. Less than 135 ng/g body weight of VTX is capable of causing immediate death in mice (Garnier et al., 1995). A proteic complex has also been isolated from VTX, termed p-VTX, which is more stable than VTX but exhibits no lethal or hemolytic activities (Garnier et al., 1997b). Furthermore, a glycoprotein (neoVTX) which has both hemolytic and lethal properties has also been isolated from *S. verrucosa* venom (Ueda et al., 2006). Like SNTX, neoVTX is a dimeric protein with a MW of 166 kDa and is composed of two subunits, α- and ß-, each with a MW of 79 kDa by deduced amino acid sequence (Ueda et al., 2006) (Table 3.2). Table 3.3 lists the different LD_{50} values for these toxic components or venom preparations in relation to the stonefish species of origin and route of administration in anaesthetized mice. The isolated toxins appear to be more potent than crude or reconstituted venoms and are believed to be responsible for much of the venom's lethal and hemolytic activities.

Remarkably, no significant amino acid sequence similarity has been identified between *Synanceia* toxins and other animal toxin proteins, suggesting that *Synanceia* toxins are novel molecules (Garnier et al., 1997a). Not surprisingly, however, significant sequence similarity has been found within the active proteins that comprise different stonefish venoms. For example, the VTX ß-subunit shares 96%

amino acid sequence similarity with the SNTX ß-subunit across the first 72% of the protein sequence (Garnier et al., 1997a), and neoVTX α- and ß-subunits share 87% and 95% amino acid similarity with the SNTX α- and ß-subunits, respectively (Ueda et al., 2006). Interestingly, neoVTX appears to differ from SNTX only in the number of cysteine residues and free thiol groups, whereas it appears to differ considerably from the composition of VTX (Ueda et al., 2006).

Table 3.3: LD_{50} values in mice from two different species of* Synanceia *venoms.

***Synanceia* Species**	**Toxic Component**	**Route**	**LD_{50} (µg/kg)**	**Reference**
S. horrida	Crude venom	IV	0.4–0.6	(Deakins and Saunders, 1967)
	Reconstituted venom	IV	220	(Saunders et al., 1962)
			300–666	(Khoo et al., 1992; Poh et al., 1991; Wiener, 1959b)
		SC	2666–4000	(Wiener, 1959b)
		IC	266	(Wiener, 1959b)
		IP	1333–2000	(Kreger, 1991; Wiener, 1959b)
	Fraction 1	IV	35	(Poh et al., 1991)
	SNTX	IV	17	(Poh et al., 1991)
S. verrucosa	Crude venom	IV	360	(Shiomi et al., 1993)
			180	(Shiomi et al., 1989)
			125 (estimated)	(Garnier et al., 1995)
	Crude venom	IM	107	(Wahsha et al., 2017)
			38	(Khalil et al., 2018)
	NeoVTX	IV	47	(Shiomi et al., 1993)

IC–intracerebral; IM—intramuscular; IP—intraperitoneal; IV—intravenous; SC—subcutaneous.

A comprehensive characterization of the venom gland transcriptome and the proteome of crude venom from *S. horrida* has recently been published (Ziegman et al., 2019). This analysis revealed that the venom proteome is primarily composed of proteins identified as C-type lectins and SNTX, with additional putative hyaluronidase and peroxiredoxin present (Ziegman et al., 2019) (Table 3.2). C-type lectins appear to be the most abundant component in the proteome and are likely responsible for the hemagglutinating activity of the venom that contributes to the inflammation observed in envenomations (Ziegman et al., 2019). Peroxiredoxins are antioxidant proteins believed to be involved with the functional and structural diversification of toxins through disulfide bond formation (Calvete et al., 2009). Hyaluronidases are found in the extracellular matrix of many different organisms, binding water molecules, metal ions and salts, and functioning as an intercellular cement (Kemparaju and Girish, 2006). Hyaluronidase is found in human body fluids and organs, as well as in the venoms of scorpions, lizards, spiders, snakes, amongst other organisms (Kemparaju and Girish, 2006). Although

hyaluronidases are not toxins *per se*, they are believed to act as spreading factors which mediate the diffusion of toxins throughout the body (Tu and Hendon, 1983). Purified hyaluronidase (SFHYA1) from *S. horrida* venom is a heat labile glycoprotein with a p*I* of 9.2 and a MW of 62 kDa (Table 2), and has not been associated with the lethal or hemorrhagic activities of venom (Poh et al., 1992). SFHYA1 appears to be specific to hyaluronic acid (HA) (Sugahara et al., 1992) and resembles the PH-20 hyaluronidase family, which is a group of multifunctional proteins with unique enzymatic properties and expression patterns in different tissues (Ng et al., 2005). Interestingly, the purified hyaluronidase showed a 261-fold increase in activity than the crude venom when measured by turbidimetric assays (Poh et al., 1992). When a comparison was made between snake venom hyaluronidase and SFHYA1, SFHYA1 showed activity that was many-fold higher when hydrolysing 50% of HA. Further measurements showed that SFHYA1 was present at concentrations approximately 10^5-fold higher in the estuarine stonefish venom than the hyaluronidases in snake venoms (Poh et al., 1992).

S. horrida venom also contains other enzymatic proteins including acetylcholinesterase, alkaline phosphomonoesterase, phosphodiesterase, arginine amidase, 5' nucleotidase, arginine ester hydrolase, proteases, amongst others (Khoo et al., 1992; Ziegman et al., 2019). Analysis of the venom gland transcriptome found that the majority of the assembled contigs (59.3%) showed no homology to any existing protein in the Swiss-Prot database, while 40.3% were similar to non-toxic housekeeping genes responsible for cellular maintenance and function, and 0.4% were homologous to known venom components (Ziegman et al., 2019). The percentage of contigs from the complete venom gland transcriptome of *S. horrida* found to be homologous to putative venom components were much lower compared to those found in the venom gland transcriptomes of other venomous species such as snakes (24-27%) and scorpions (53%) (Ziegman et al., 2019). Additionally, a relatively low proportion of putative animal toxin families were identified in the venom, which might be explained by the defensive role the venom plays in the ecology of the animal, where a low diversity of distinct toxins would be sufficient to deter predators (Ziegman et al., 2019). The low number of distinct toxins found in the venom could also be explained by mechanical damage to the venom gland during milking in this particular study, causing an up-regulation of the proteins needed to rebuild and repair the gland itself relative to proteins responsible for the toxic activity of the venom (Ziegman et al., 2019). Overall, the venom composition of *S. horrida* appears to be unique when compared to other venomous species, containing several proteins that have not been previously recognized in proteomic studies of other venoms (Ziegman et al., 2019).

Crude *S. horrida* venom also contains small molecules such as norepinephrine, dopamine and tryptophan, which are known regulators of cardiac physiology and may play a role in the symptoms and pathologies experienced by sting victims (Garnier et al., 1996). In addition, negligible amounts of

histamine, a potent inflammatory mediator associated with pain and edema, are present (Hopkins et al., 1994). Investigation into whether or not stonefish venom elicits the release of endogenous stores of histamine found that this is unlikely, as it appears that the venom does not act on histamine receptors in guinea pig smooth muscles (Hopkins et al., 1994). Interestingly, serotonin 5-HT, which was previously thought to be linked to the pain caused by stonefish envenomation and contribute to bronchoconstriction and vasodilation, has not been detected in any *Synanceia* venom thus far, even though these symptoms are consistent with stonefish envenomation (Garnier et al., 1996; Hopkins et al., 1994).

Fewer studies have been performed on the venom of *S. verrucosa*; however, a 59 kDa hyaluronidase (Table 3.2) has been partially purified and, like SFHYA1, shows activity only against HA. It was also the first fish hyaluronidase reported to act as a spreading factor (Madokoro et al., 2011). *S. verrucosa* hyaluronidase appears to be structurally and enzymatically similar to SFHYA1, with 92% amino acid sequence identity, but it exhibits less than 50% identity with the honeybee or snake hyaluronidases, for example (Madokoro et al., 2011). Other enzymes, including lipases and aminopeptidases have also been identified in this venom (Garnier et al., 1995). Similar to *S. horrida*, *S. verrucosa* crude venom also contains norepinephrine, dopamine and tryptophan (Garnier et al., 1996). Apart from those, cardioleputin, a cardiotoxic 46 kDa protein (Table 3.2) has been isolated from *S. verrucosa* venom. It is primarily composed of glycine, serine and glucosamine, with few basic amino acids and no cystine (Abe et al., 1996). Similar to the crude venom, cardioleputin toxicity can be lost at high temperatures, as well as during dilution and freeze-thawing (Abe et al., 1996). Its cardiotoxic activity is described in the next section. *S. verrucosa* venom has also been shown to contain novel lectins, which are proteins that bind specific carbohydrates to mediate several different biological processes (Vijavan and Chandra, 1999). Experimental fractions from crude *S. verrucosa* venom named Con A-I and Con A-II were shown to have hemagglutinating and mitogenic activities, where Con A-I showed stronger activity compared to Con A-II and the unfractionated crude venom. (Kato et al., 2016). Further analysis of Con A-I revealed the presence of two sub-fractions, Con A-I-PS-I and Con A-I-PS-II (Kato et al., 2016). The first sub-fraction contained three proteins (42.1, 100 and 110 kDa in MW), while the second showed only one protein of 45 kDa, named 45 kDa lectin (Table 3.2). Both Con A-I-PS-I and the 45 kDa lectin did not show toxicity to human leukemia cells (K562) (Kato et al., 2016).

3.7 Cardiovascular and respiratory effects of *Synanceia* envenomation

While crude *S. verrucosa* venom has been shown to produce positive inotropic and chronotropic responses in isolated frog atrial fibers (Sauviat et al., 1995), the isolated compounds from *S. verrucosa* venom produce a range of reactions *in vivo* and *in vitro*. For example, purified VTX causes a marked

dose-dependent hypotensive effect in anaesthetized rats, and cardiac and respiratory failure in mice *in vivo* (Garnier et al., 1995). In contrast, *in vitro* investigations have revealed that both VTX and p-VTX produce negative chronotropic and inotropic effects in frog atria, where p-VTX is postulated to open K^+ channels during the cardiac cycle and reduce the amount of Ca^{2+} that enters the cells through competitive inhibition of Ca^{2+} binding sites (Garnier et al., 1997b). VTX seemingly inhibits K_{ATP} currents in a voltage-independent and dose-dependent manner in guinea-pig ventricular myocytes (Wang et al., 2007). The reason for this discrepancy between the effects of VTX on frog and guinea pig hearts is unclear, but it is possible that these two species have different regulatory K_{ATP} pathways (Wang et al., 2007), as there seems to be a difference between ß$_1$- or ß$_2$- mammalian adrenoceptors and atrial amphibian ß-adrenoceptors (O'Donnell and Wanstall, 1982). Furthermore, when the effects of cardioleputin on guinea pig atria were investigated, irreversible positive inotropic and chronotropic effects were identified, which suggests lack of phospholipase A_2 activity, as well as a possible action on atrial membrane Ca^{2+} channels (Abe et al., 1996), supporting the Sauviat et al. (1995) results.

Similarly, reconstituted *S. horrida* venom also appears to cause a range of effects depending on the envenomation model and the methods used. This venom seems to have potent myotoxic effects on cardiac, skeletal and involuntary muscles (Austin et al., 1961). These effects can be observed following intravenous injection of reconstituted venom in anaesthetized rabbits, where muscular paralysis, respiratory distress and hypotension are observed (Austin et al., 1961). In contrast, reconstituted venom appears to cause a biphasic response in anaesthetized rats, where the initial pressor response is partly mediated by α_1-adrenoceptors and leukotriene receptors, and the depressor response is mediated by β_2-adrenoceptors, creating an overall hypertensive effect (Hopkins et al., 1996). The reasons for these discrepancies are not yet understood.

In vitro studies using purified SNTX on rat aortic rings have demonstrated potent vasorelaxant activity (Low et al., 1993). This vasorelaxation effect appears to be mediated by an endothelium-dependent mechanism, most likely facilitated by the L-arginine-nitric oxide synthase pathway (Low et al., 1993). Endogenous hydrogen sulfide (H_2S) acts in synergy with nitric oxide (NO) (Liew et al., 2007) to bind to endothelial Substance P neuropeptide receptors, producing NO and activating K^+ channels, ultimately leading to the SNTX-induced muscle relaxation observed (Sung et al., 2002). Furthermore, SNTX was found to contain a B30.2 domain, which is present in multiple intracellular, transmembrane and secreted proteins (Henry et al., 1997; Seto et al., 1999). This domain was shown to indirectly increase NO production through competitive binding of the NO synthase inhibitor, xanthine oxidase, in bovine cerebellum, which might explain the role of NO and the SNTX-induced muscle relaxation (Henry et al., 1997).

The respiratory failure observed in experimental *Synanceia* envenomation may also contribute to the lethal activity of stonefish venom (Low et al., 1993). Respiratory failure caused by *S. horrida* envenomation could ensue from direct skeletal muscle paralysis, as neuromuscular conduction through the phrenic nerve-diaphragm junction is not inhibited even when respiration has stopped in anaesthetized rabbits (Austin et al., 1961). In the case of *S. verrucosa* venom, irregular or weakened respiration is observed, often followed by cessation of respiration when lethal doses of crude venom or VTX are administered in anaesthetized rats (Garnier et al., 1995). Furthermore, pathological activity can be observed in mouse lung after intramuscular injections of *S. verrucosa* crude venom (Wahsha et al., 2017). Apart from these experimental envenomation studies, a case report described three instances where patients were admitted to hospital after being envenomed by *S. verrucosa*. One of the victims required CPR and continued to show dyspnea and bilateral rattles, where acute pulmonary edema was hypothesized, although not confirmed (Maillaud et al., 2020). In two other cases, pulmonary hypertension and acute pulmonary edema were diagnosed, evidenced by interstitial edema and bilateral pleural effusions. In one of those instances, the pathologies were severe, where the patient also suffered from cardiorespiratory arrest, left ventricle dysfunction, pericardial and pleural effusions, and did not survive (Maillaud et al., 2020).

3.7.1 G-protein-coupled receptors

G-protein-coupled receptors (GPCR) are one of the largest families of membrane proteins (Rosenbaum et al., 2009). This family mediates the majority of physiological responses, including neurotransmission and hormonal responses, amongst other functions (Rosenbaum et al., 2009). *S. verrucosa* venom appears to act on several GPCRs. For example, when examining venom effects in guinea pig ventricular myocytes, VTX appeared to modulate ion channels by stimulating the ß-adrenoceptor-cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway (Yazawa et al., 2007). When stimulated by agonist binding, this ß-adrenoceptor leads to increased cAMP production via G-protein dissociation and adenylyl cyclase activation. The cAMP signaling then activates PKA causing phosphorylation of several proteins downstream of this cascade including L-type Ca^{2+} channels, which promotes an inotropic effect in cardiomyocytes (Madamanchi, 2007).

Reconstituted crude *S. horrida* venom also appears to act on bradykinin receptors. A B_2 receptor antagonist, acting as a noncompetitive inhibitor of bradykinin, was shown to inhibit the relaxation response induced in pig coronary arteries by reconstituted crude *S. horrida* venom or bradykinin (Church and Hodgson, 2000; Rizzi et al., 1997). Bradykinins are peptides involved in several pathophysiological processes (Calixto et al., 2004), such as inflammation, pain, increase in capillary permeability and vasodilation, and decrease in vascular resistance (Lewis, 1964). Therefore, some

cardiovascular symptoms might be explained by the venom-induced effects on bradykinin receptors and may also contribute to the excruciating pain experienced by victims (Church and Hodgson, 2000). In addition to bradykinin stimulation, *S. horrida* venom also seems to act on muscarinic receptors by stimulating endogenous acetylcholine production or mimicking its effect on the cardiac membrane (Church and Hodgson, 2000; Khoo et al., 1992; Sauviat et al., 2000). When porcine coronary arteries were exposed to venom, a cholinergic-like endothelium-independent contraction was observed. Atropine, an antimuscarinic drug, inhibited this effect, which further supports the suggestion that *Synanceia* venom has some action at muscarinic receptors (Church and Hodgson, 2000). Research supporting this finding showed that purified SNTX induces the release of acetylcholine from atrial nerve terminals in frog atrial fibers, indirectly activating muscarinic receptors (Sauviat et al., 2000). Given what is known about muscarinic receptors and the physiological effects of their activation, the responses observed in both experimental models and clinical reports suggest that muscarinic activity is a significant contributor to the cardiovascular pathophysiology of *Synanceia* envenomation. VTX can also stimulate muscarinic receptors and activate Ca^{2+} currents, leading to arrhythmia and hypoxia, preventing the activation of cardio protective K_{ATP} currents, and promoting cardiovascular collapse (Wang et al., 2007; Yazawa et al., 2007). VTX does not seem to inhibit K_{ATP} current through activation of adenosine receptors or α_1-adrenoceptors. Instead, it was strongly suggested that VTX activates the M_3 receptor-Protein Kinase C (PKC) pathway (Wang et al., 2007).

There is evidence that venom from *S. horrida* stimulates the release of endogenous tachykinins. *S. horrida* venom-induced bronchoconstriction responses were reduced by an NK_1 receptor antagonist in anaesthetized guinea pigs *in vitro* (Hopkins et al., 1996), indicating that the venom acts on NK_1 receptors possibly through the release of Substance P (Hopkins et al., 1994). Substance P is an important neurotransmitter released from nerve-endings and transmits nociceptive signals by immune cells and various non-neuronal cells (Zieglgänsberger, 2019). The venom may also further stimulate the endogenous release of cyclooxygenase products, which play an important role in increasing the activities of pain-producing inflammatory mediators and acetylcholine (Hopkins et al., 1994).

3.8 Vascular permeability and cytolytic effects of *Synanceia* envenomation

Crude *S. horrida* and *S. verrucosa* venoms produce potent inflammatory responses in *in vivo* studies and in patients, where stonefish victims show signs of tendon inflammation, pulmonary edema and other cardiac complications (Lehmann and Hardy, 1993; Lopez et al., 2019; Maillaud et al., 2020). Research shows that *S. horrida* venom causes edema when administered intradermally into rat hind paws and mouse footpads (Austin et al., 1965; Khoo et al., 1992; Kreger, 1991). The increase in

vascular permeability associated with *Synanceia* envenomation does not appear to be triggered by histamine release because diphenhydramine, an antihistamine, does not seem to have an effect on the edema-inducing properties of SNTX (Poh et al., 1991). Instead, it has been suggested that stonefish hyaluronidase might be responsible, at least in part, for this activity as it enhances the capillary-increasing activity of neoVTX when co-injected intradermally in mice (Madokoro et al., 2011).

Crude venoms from *S. verrucosa* and *S. horrida*, as well as VTX and SNTX, exhibit strong lytic actions against diluted blood and washed erythrocytes of a variety of mammal species (Garnier et al., 1995; Khoo et al., 1992; Kreger, 1991; Poh et al., 1991). Crude venom from *S. horrida* does not cause hemorrhage or have any measurable dermonecrotic effects in mice but seems to possess some anticoagulant activity in rabbit blood (Khoo et al., 1992). Additionally, reconstituted crude *S. horrida* venom appears to prevent clotting of human fibrinogen when Ca^{2+} is present, and this anticoagulant activity seems to be concentration-dependent (Khoo et al., 1992). When examining the effects of SNTX on whole blood, some hemolysis and platelet aggregation were observed *in vitro* in blood collected from rats and rabbits (Khoo et al., 1995). Notably, no hemolytic or coagulation modulating properties were observed in mouse or human studies, although *S. horrida* venom appears to cause cell lysis in cultured murine cortical neurons (Church and Hodgson, 2002). This lack of lytic activity in mouse erythrocytes may indicate that the direct cause of death in experimental animals may not be due to hemolysis (Poh et al., 1991).

Apart from these, thromboelastography assays were performed using fresh and lyophilized *S. verrucosa* venoms on recalcified human plasma (Harris et al., 2021). Although fresh venom did not have an effect on clot strength, it exhibited anticoagulant properties, demonstrated by a delay in time until clot formation. Lyophilized venom, in turn, did not show significant anticoagulant activity (Harris et al., 2021). Fibrinogen levels can be assessed by clot strength and, since clot strength was not affected, this indicates that the anticoagulation activity seen likely happened upstream in the clotting cascade instead of being directly related to fibrinogen cleavage (Harris et al., 2021). Further testing could not unravel the mechanism of action, but Harris et al. suggested that the venom possibly degrades phospholipids, which would ultimately lead to the anticoagulation seen (Harris et al., 2021).

The observed hemolytic activity produced by *Synanceia* venom in some animals has been linked to the formation of pores in cell membranes (Chen et al., 1997; Ouanounou et al., 2000). Experiments on the function and structure of mice kidneys using intramuscular injections of crude *S. verrucosa* venom resulted in elevated levels of the oxidative stress marker malonaldehyde caused by lipid peroxidation, which indicates that venom-induced damage to the cell membrane may be caused by the generation and action of free radicals (Wahsha et al., 2019). Moreover, the SNTX complex of *S. horrida* venom, in

particular, is one of the largest naturally occurring toxins isolated to date that possess pore-forming activity (Chen et al., 1997). SNTX subunits belong to the perforin superfamily of pore-forming immune effectors (Ellisdon et al., 2015). Each SNTX protein has four domains, where the *N*-terminal domain is homologous to the Membrane Attack Complex-Perforin/Cholesterol-Dependent Cytolysin (MACPF/CDC) pore-forming domains (Ellisdon et al., 2015). Proteins from the MACPF/CDC superfamily are typically promiscuous, suggesting that SNTX may form pores in a variety of tissues, likely having a central role in SNTX-induced pathologies and physiological effects (Ellisdon et al., 2015). Interestingly, it was suggested that SNTX irreversibly binds to the membrane, where a certain amount of toxin is presumably required to change membrane permeability (Ouanounou et al., 2002). Additionally, a smaller protein has been isolated from the crude venom of *S. horrida* that is homologous to perforin-1-like proteins found in several teleost fish species (Ziegman et al., 2019). This protein has a C2 domain that targets cell membranes and binds to phospholipids (Ziegman et al., 2019), which constitutes the majority of the lipid components in mammalian membranes (Fagone and Jackowski, 2009), and may further enhance the venom's pore forming cytolytic activities (Ziegman et al., 2019).

To understand the mechanism behind the toxicity of *Synanceia* venoms and the functional structure of their isolated fractions, inactivation studies have been performed with SNTX and neoVTX. The hemolytic activity from both neoVTX and SNTX are inhibited by modified anionic lipids (Chen et al., 1997; Ueda et al., 2006). Furthermore, SNTX's hemolytic activity is also inhibited the presence of some anionic lipids and chemical modification of tryptophan, thiol groups, lysine and arginine residues within the protein (Chen et al., 1997; Khoo et al., 1998a, 1998b; Yew and Khoo, 2000). Additionally, SNTX's lethal properties are inhibited by chemical modification of lysine residues, thiol groups and some cationic amino acids in the protein (Chen et al., 1997; Khoo et al., 1998b, 1998a). Interestingly, the hemolytic and lethal activities of SNTX might not originate from the same domain or region of the protein (Khoo et al., 1998b), as the lethal activity is lost after lyophilization or temperature changes, while the hemolytic activity is retained (Khoo et al., 1998b; Yuen et al., 1995).

3.9 Neuromuscular effects of *Synanceia* envenomation

Experiments to determine the neuromuscular effects of crude *S. horrida* venom have demonstrated that the venom may also block neurotransmitter synthesis, ultimately leading to a depletion in neurotransmitter stores (Khoo et al., 1992). When applied to extracted mouse hemi-diaphragms, *S. horrida* venom can cause irreversible neuromuscular blockages, which may lead to fatal respiratory paralysis (Low et al., 1990). Interestingly, SNTX irreversibly blocks both nerve- and muscle-evoked twitches of mouse isolated nerve-hemidiaphragm and chick biventer cervicis muscle preparations (Low et al., 1994). This inhibitory action of SNTX on neuromuscular function was likely the result of

direct myotoxic effects of SNTX in the muscle as the contractile responses to acetylcholine, carbachol and potassium chloride were completely blocked (Low et al., 1994). SNTX has also shown to act directly on the muscle by producing contractures in the mouse hemidiaphragm in the presence of tubocurarine. Furthermore, blockade of the SNTX-induced contractures by dantrolene sodium, which causes blockade prior to activation of the contractile proteins in the muscle, indicates SNTX does not act directly on the contractile muscle proteins (Low et al., 1994). This is further evidence that *Synanceia* venom is myotoxic and likely to induce the permanent damage observed by electron microscopy at the neuromuscular apparatus in skeletal muscle (Kreger et al., 1993; Low et al., 1994).

Recent research on crude fresh and lyophilized venoms from *S. verrucosa* investigated the activity of the venoms on mimotopes of nicotinic acetylcholine receptors (nAChRs) in four different taxa (Harris et al., 2021). The fresh venom was able to bind to all mimotopes, whereas the lyophilized venom was significantly reduced in activity (Harris et al., 2021). This binding action indicates that *S. verrucosa* venom might act on postsynaptic nAChRs, agreeing with previous research on chick biventer cervicis nerve preparations using *S. horrida* venom (Church and Hodgson, 2000). This difference between the activities of lyophilized and crude fresh venoms could be due to the size of the toxins responsible for the nAChR binding, likely being labile and large (Harris et al., 2021).

Further testing on mammalian L-type $Ca_v1.2$ channel mimotopes of domains I-IV (DI-DIV) using biolayer interferometry (BLI) showed that both lyophilized and fresh venoms bound only to DIV, indicating that the toxins responsible for this particular activity might be small peptides or amine-type molecules, which are more stable than proteinaceous or enzymatic molecules (Harris et al., 2021). These results also support previous research that suggested that *Synanceia* venom activates Ca^{2+} channels (Abe et al., 1996; Garnier et al., 1997b). It was not possible to verify by BLI if the venom had antagonist or agonist actions towards Ca^{2+} channels (Harris et al., 2021), so there is still much to be studied.

In addition, SNTX promotes secretion of catecholamine from neuroendocrine cells through Ca^{2+}-dependent exocytosis of large dense core vesicles in the presence of extracellular Ca^{2+} in bovine chromaffin cells (Meunier et al., 2000). The Soluble *N*-ethylmaleimide-sensitive fusion protein Attachment Protein REceptor (SNARE)-dependent exocytosis was shown to be independent of Ca^{2+} influx through voltage-activated Ca^{2+} channels, as blockade of the L, N and P/Q type channels resulted in a minimal change in catecholamine secretion (Meunier et al., 2000). Intracellular fluorescence experiments also showed that SNTX-induced catecholamine secretion required sufficient internal stores of Ca^{2+}, as depletion of intracellular Ca^{2+} with caffeine, thapsigargin or ryanodine resulted in a reduced SNTX-induced catecholamine response (Meunier et al., 2000).

Many of the effects of *Synanceia* venom have a time- and dose-dependent relationship. *S. horrida* venom, for example, causes permanent depolarization of muscle fibers at high concentrations, whereas at low concentrations it causes massive release and depletion of neurotransmitters in frog nerve-muscle preparations (Kreger et al., 1993). Additionally, high concentrations of venom cause both muscle and nerve damage, with nerve terminal swelling and no synaptic vesicles evident. These effects, combined with the observation of a total absence of miniature endplate potentials following prolonged periods of high-frequency potentials, indicates *Synanceia* venom also blocks the recycling of synaptic vesicles (Kreger et al., 1993). When comparing *Synanceia* venom to other animal toxins with similar neuromuscular blocking activity via the release and depletion of neurotransmitter from the nerve terminal, such as α-latrotoxin from the spider genus *Latrodectus* (Rosenthal and Meldolesi, 1989), *Synanceia* venom is differentiated by also causing muscle depolarization and microscopic muscle damage. An exception to this is the pardaxins, toxins found in the dorsal and anal fin gland secretions of the Pacific sole (*Pardachirus pavoninus*) and Red Sea Moses sole (*P. marmoratus*). *Synanceia* venom mimics the action of the pardaxins, acting as a presynaptic neurotoxin at low concentrations and severely damaging skeletal muscle fibers at high concentrations (Renner et al., 1987; Spira et al., 1976). Interestingly, although both *Synanceia* venom and the pardaxins lyse dog erythrocytes, only the pardaxins lyse human erythrocytes (Kreger et al., 1993; Shai, 1994).

Stonefish venom-elicited release of neurotransmitters has been reported not to involve voltage-gated sodium channels (Na_V) (Kreger et al., 1993). This was determined from experiments where sodium channel currents blocked by tetrodotoxin (TTX) did not affect venom-elicited neurotransmitter release. It is, however, possible that neurotransmitter release may involve the TTX-resistant $Na_V1.8$ (Akopian et al., 1996) or $Na_V1.9$ (Tate et al., 1998) sodium channel sub-types, both discovered subsequent to the Kreger et al., 1993 study. Activation of these channel sub-types is associated with pain (Jami et al., 2018) and is consistent with the symptoms of stonefish envenomation.

3.10 Conclusions and future directions

Stings caused by venomous fish species occur worldwide. While the causative agent of many of these stings are attributed to “stonefish”, it should be noted that the identity of the offending animal cannot be confirmed in most cases. As a result, reports of stonefish stings should be viewed with caution when the animal has not been captured. Notably, individuals belonging to the genus *Synanceia* are widely distributed, and while stonefish cause serious envenomations worldwide, the extent of involvement of other venomous fish species in serious envenomations around the globe is not fully understood.

Studies investigating the pathophysiological mechanisms and effects of *Synanceia* venom at the cellular, tissue and organ levels have significantly advanced since the 1990s. Of note, all published work on *Synanceia* venom has been performed only on two species thus far: *S. horrida* and *S. verrucosa*. Characterizing the venom of additional species for future research is likely to increase understanding of the pathophysiological mechanisms and effects of stonefish venoms.

Of note, while the cytolytic activity of stonefish venom appears to be the result of pore-forming action on cell membranes, the precise mode of action of the venom and its individual components still remains largely unknown. It has become clearer that the biological symptoms and pathologies observed in sting victims cannot solely be attributed to the presence of a single venom protein, such as SNTX or VTX, as initially thought. Instead, these effects are likely a result of the combined activity of the many enzymes and proteins that have now been discovered in *Synanceia* venom, many of which appear to be novel. Indeed, only one of these enzymes, hyaluronidase, has been characterized on a molecular level, and yet its role in the envenomation effects remains poorly understood. Although the rich bioprospecting potential of *Synanceia* venoms has yet to be realized, the results of recent transcriptomic- and proteomic-based approaches to venom characterization highlight the need for more research into these medically important fish species. The study of *Synanceia* venom variability, composition and immunological cross-reactivity may also have a direct impact on human health by contributing to the development of improved antivenoms, thereby reducing the burden of venomous fish species on human health worldwide.

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Chapter 4

4 Relationship between food and venom production in the estuarine stonefish *Synanceia horrida*

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4.1 Abstract

Background: The potential costs of venom production may be significant to many marine venomous taxa. In general, the parameters that influence the rate of venom production are poorly understood but seem to be related to feeding frequency.

Methods: This study examines the effects of starvation on venom profile and venom yield on the estuarine stonefish (*Synanceia horrida*). In total, the venom of eight stonefish individuals was tested under two feeding regimes. One of the two groups underwent an episode of suspended feeding over a four-week period. The effect of time on venom replacement was determined by a paired T-test. ANOVA was performed to analyse differences in venom weight between fed and unfed treatments.

Results: Nutritional suspension was found to have a significant effect on the quantity of venom produced. SDS-PAGE gel and FPLC revealed that the components of the venom collected from both groups were similar, indicating that four weeks is an adequate time to regenerate key venom components but not replenish initial venom quantities.

Conclusions: Venom production was found to be affected by starvation.

4.2 Introduction

Venom is a widespread trait amongst marine taxa. Its evolution sometimes is related to prey acquisition (i.e. jellyfish) and/or defence (i.e. marine venomous fish, stingrays). This trait, although advantageous, comes as a trade-off between energetic production costs and the ultimate benefit it brings to the animal. The potential production costs, in turn, may be quite significant and venom is used in a conservative way in many different taxa, possibly as an attempt to minimise energetic expenditure on stock renewal (Hayes, 1993, 1995; Inceoglu et. al, 2003). Likewise, the rate and quantity of venom being produced may be influenced by its metabolic production costs and also may depend on the necessity and the ecology of the animal producing it (Mirtschin et. al, 2002; Kintner et. al, 2005; Mebs, 2001). In general, however, the ecological parameters influencing the rate of venom production in venomous animals are poorly understood. Although concentrated focus is often maintained on variation in venom composition, venom yield remains a relevant feature to examine, as it may reveal a greater understanding of the general venom ecology of an organism as well as the consequences associated with envenomations.

An example of venomous marine taxa is the Synanceiidae family, which encompasses the highest number of venomous fish species known to date (Haddad, 2016). Some of its members are the stonefish (genus *Synanceia*), regarded as the most venomous fish in the world (Russell, 1965; Tang et. al, 2006). All species possess dark coloured skin covered in wart-like projections (tubercles) and frequently embed themselves under rocky soils using their large pectoral fins (Endean, 1961). Naturally, they can camouflage themselves very effectively (Isbister and Caldicott, 2004), due to their coloration and shape, blending in with rocks, corals and seaweed (Auerbach, 1991). Stonefish are bottom dwelling and remain motionless in the shallow tropical and warmer temperate waters, waiting for prey to swim by, moving only when disturbed in a clumsy and reluctant manner (Isbister and Caldicott, 2004; Cooper, 1991).

Stonefish have 13 dorsal spines, closely associated with twin venom glands, used for defence and can deliver large quantities of venom from each dorsal spine when stepped upon or mishandled, as close to 100% of known envenomations occur in the hand and foot (Ngo et al., 2009). Venom is involuntarily expelled from both venom glands when the gland is depressed, releasing up to 10 mg of venom per spine (Ngo et al., 2009). With an estimated LD_{50} value of 0.36 μg/g in mice, it is predicted that only 6 depressed spines, containing a total of 18 mg of venom, has the potential to cause fatality in a 60 kg human (Garnier et. al, 1995). Apart from this primary mode of defence of the stonefish, the epidermal tubercles that cover the animal's body release a milky substance (icthyocrinotoxin) when compressed (Cameron et. al, 1981). Icthyocrinotoxin and the venom found in the dorsal spines are known to be

different in both structure and function (Cameron et al., 1981), while icthyocrinotoxins possess distasteful and debilitating properties to predators (Cameron and Endean, 1973). Although investigations into the icthyocrinotoxins and the venom found in the dorsal spines of stonefish have been conducted, an inadequate amount of information is known about these organisms. As such, inquiries into their general venom ecology and the factors influencing venom yield warrant further investigation.

This study aims to examine the effects of starvation on venom yield and profile in the estuarine stonefish *Synanceia horrida*. In general, nutrition provides the critical elements for an organism to maintain and produce required protein based body tissue, such as secretory glands and venom. Since the consumption of food is necessary for sustained maintenance, it is suspected that withholding nutritional resources would result in a reduction of venom production, thus influencing total venom yield. In addition to diminished venom quantities, a difference in the venom profile may also occur, as nutritional supplementation is thought to supply the raw materials needed to synthetize venom components.

4.3 Methods

4.3.1 Study species

Although *S. horrida* are known to be sexually dimorphic, external gender determination is impossible and as such, sex was not determined for any of the animals used in this study. Two groups of four individuals were housed in similar conditions and maintained normal feeding regimes for six months prior to milking. Individuals were offered prawns once daily until they rejected additional food. Initial milking of all eight stonefish occurred after six months and then fed for a subsequent four weeks and milked once more. All animals were then split into two groups, one group (n = 4, mean weight = 951 g, ranging from 453 g to 1619 g) fed daily and one group (n = 4, mean weight = 1096 g, ranging from 416 g to 1841 g) starved for four weeks. At the end of the fourth week both groups were milked for a third time.

4.3.1.1 Collection of venom from dorsal spines

Venom was extracted from all 13 dorsal spines of all eight stonefish by inserting a small hypodermic needle (Terumo™ 26 G x ½" with 1 mL syringe) into each side of the venom sac on each spine. Extracted venom was immediately frozen, lyophilized, weighed and stored at a -80°C.

4.3.2 Fast performance liquid chromatography, size exclusion chromatography, and SDS-PAGE

Lyophilized venoms were rehydrated with a DPBS running buffer and passed through a fast performance liquid chromatography column (Superdex™ 10/300 L Column (13 µm, 10 x 300 mm)) at a flow rate of 0.3 mL/min.

Similarly, samples of rehydrated venom were mixed with 2% 2-Mercaptoethanol and added into a 96-well PCR plate and heated to 90°C for five min. Equal volumes of samples and 2 µL of Dual Colour Precision Plus Protein Standards (Bio-Rad Laboratories) were added to wells in a 4-20% LongLife gels (NuSep) and underwent electrophoresis in Tris-HEPES running buffer at 120 V for approximately 60 min. Gels were then washed three times for seven min in a 45% methanol 10% acetic acid solution, and stained using Rapid Coomassie Electrophoresis Stain (NuSep) for 15 min. Gels were destained using 6% acetic acid solution.

4.3.3 Statistical Analysis

To determine whether there was an effect of time on venom replacement, the weight of venom (transformed to mg/g of stonefish) for the two time periods (six months and four weeks) from each stonefish was analysed using a paired T-test. An ANOVA was then performed to compare the weight of venom (mg/g of stonefish) between fed and starved treatments over a four-week period. Analysis was completed using SPSS Ver 2.0.

4.4 Results

4.4.1 The effect of time and feeding frequency on venom production

A significant difference ($T = 3.201$, $df = 7$, $p < 0.01$) was observed between the quantities of venom (mg venom/g stonefish) collected in response to time since last milking (six months) (Figure 4.1).

Stonefish from which food was withheld produced significantly less venom compared to stonefish receiving daily nourishment ($T = -5.542$, $df = 3$, $p = 0.012$) (Figure 4.2).

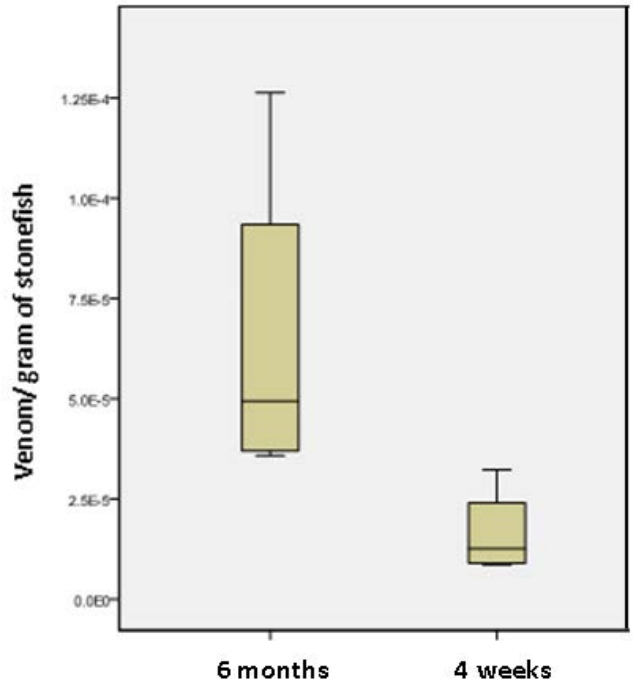

Figure 4.1: **Comparison of time effect between successive milkings on venom yield (mg of venom/g of stonefish) of *S. horrida* individuals (95% C.I.).**

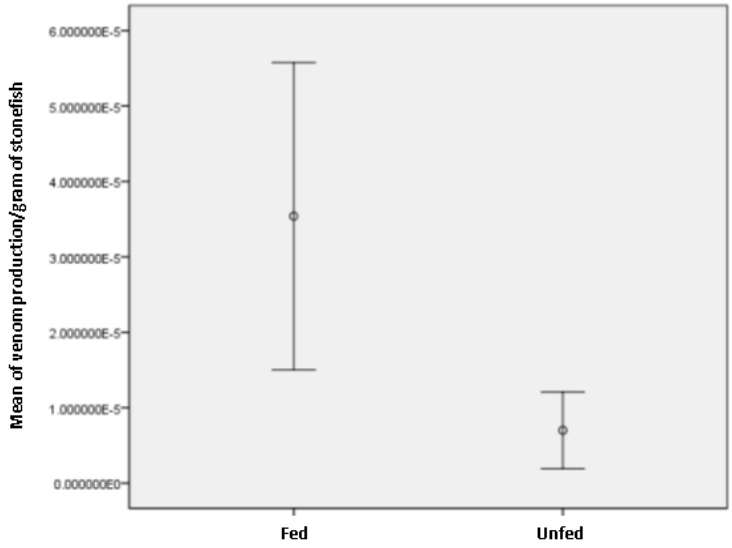

Figure 4.2: **Comparison of mean venom production (mg of venom/g of stonefish) on fed and unfed *S. horrida* individuals (95% C.I.).**

4.4.2 Profiles between starved and fed stonefish

There was little difference in the FPLC traces of venom extracted from fed or unfed individuals (Figure 4.3). Similarly, the SDS-PAGE gel revealed similar protein bands in both fed and unfed individuals, lanes b and c, respectively (Figure 4.4). The protein band around 75 kDa appears to be present in both fed and unfed individuals and, although not individually distinct, the band is consistent with the size of the lethal components SNTX-α and SNTX-β.

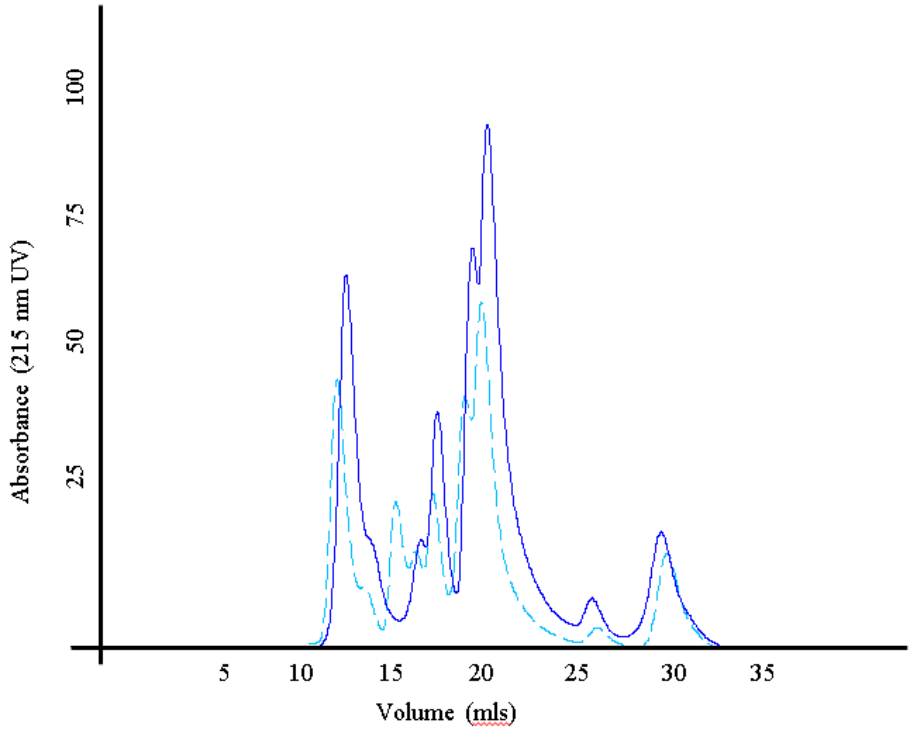

Figure 4.3: **FPLC trace of fed (solid line) and unfed (dashed line) venom profiles from *S. horrida.***

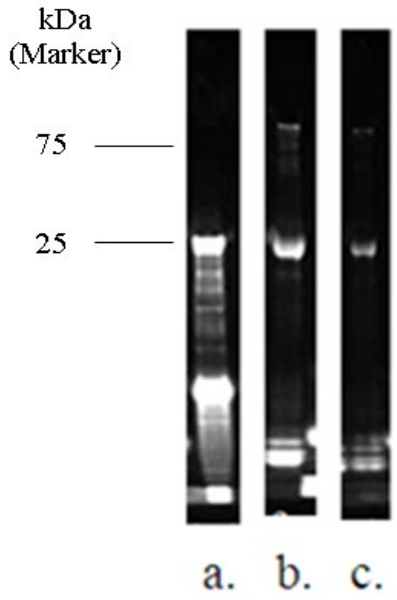

Figure 4.4: **SDS-PAGE gel showing venom profiles between a. standard marker, b. fed individuals and c. unfed individuals of *S. horrida*.**

4.5 Discussion

It was clear that the lack of nutritional supplementation (i.e. starvation) and length of time between successive milkings considerably altered the quantity of venom produced after a four week period. Nevertheless, the SDS-PAGE gel and FPLC profile revealed that although venom volumes were reduced, the components of the venom were not extensively different between the two treatment

groups. Previous research has shown that stonefish are capable of replacing venom stores within a three-week period (Gopalakrishnakone and Gwee, 1993). However, our results suggest that although a full venom profile can be returned regardless of nutritional intake, the speed at which venom is replenished is dependent on food intake by the animals.

It is advantageous to the stonefish that the venom system should continue to function, although at a reduced capacity, during periods of nutritional suspension, since the use of its venom is not associated with the "reward" of a prey item. Although the rate of venom production slowed, its toxic components appeared to be adequately replenished, despite being void of resources. It remains unknown if individuals would be able to produce sufficient amounts of venom to fully supply the glands in the complete absence of food.

4.6 Conclusions

During this study, *S. horrida* easily coped with the month-long suspension of food and it is likely that the animals could have gone longer without feeding, as individual showed no signs of weakness or reduced activity. Although there was a significant decrease in venom production between the fed and unfed treatment groups, the stonefish maintained a healthy outward appearance. Therefore, continued investigations are necessary to identify the nutritional limits influencing venom production. In summary, these data reveal the important influence food has on venom yield in one species of stonefish, *S. horrida*. Comprehending the driving forces responsible for this relationship will require further research that bridges the gap between stonefish ecology and venom toxicology.

Ethics approval: Ethics approval number A2232.

Availability of data and material: The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare no competing interests.

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Authors Contributions: **J.S.** conceived and designed the experiments, as well as contributed to reagents, materials and analysis tools; **J.S. and C.Z.** performed the experiments**; J.S., C.Z. and S.S.** analyzed the data, **S.S.** wrote the paper. All authors read and approved the final manuscript.

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Chapter 5

5 Exploratory investigation of immunomodulation effects of stonefish venoms (*Synanceia verrucosa* and *Synanceia horrida*) in human PBMCs

5.1 Introduction

Animal venoms are known to modulate immune processes, and in particular are associated with inflammation. This modulation has been shown in both experimental models and clinical settings and can involve changes in cytokine and chemokine levels (Ryan et al., 2021). Upregulation of various cytokines or chemokines produced by immune cells is associated with inflammatory states. Increased plasma levels of cytokines such as interleukin (IL)-1ß, IL-4, IL-6, IL-8, IL-10 and tumour necrosis factor (TNF) have been observed in patients with moderate to severe scorpion and snake envenomation (Açikalin and Gökel, 2011; Fukuhara et al., 2003). With the exception of IL-6 in snake bites, cytokine levels were correlated positively with envenomation severity, suggesting that the clinical physio pathologies present upon envenomation might be, at least in part, mediated by cytokines. Venomous fish species such as the Brazilian toadfish *Thalassophryne nattereri* and the scorpionfish *Scorpaena plumieri* also induce inflammatory responses in experimental models, increasing levels of the pro-inflammatory cytokines IL-1ß, IL-6, TNF and monocyte chemoattractant protein (MCP)-1 in mice (Lima et al., 2003; Menezes et al., 2012).

Pro-inflammatory cytokines and chemokines can also be downregulated by venoms. Such downregulation can be associated with anti-inflammatory effects (Rainsford, 2007). For instance, current anti-inflammatory therapeutics aid in the reduction of inflammation by suppressing cytokine levels. This suppression ultimately helps reduce pain and pathologies caused by inflammation (Rainsford, 2007). Venoms and venom components shown to downregulate cytokines include venom from snakes such as the red-bellied black snake (*Pseudechis porphyriacus*) and a protein, called cobrotoxin, extracted from the Chinese cobra (*Naja atra*) suppressed expression of the pro-inflammatory cytokines TNF, IL-1ß and IL-6 in activated purified human T cells in *in vitro* models (Ryan et al., 2020; Zhu et al., 2016). Fish venoms have also been described as possessing potent anti-inflammatory effects, such as shown by natterins from *T. nattereri* in an *in vivo* inflammation model using intravital microscopy in mice (Ferreira et al., 2014). The impressive results led to a deeper investigation of the toadfish venom and the discovery of a peptide, called *Tn*P, with anti-inflammatory and anti-allergic activities that improved the symptoms of asthma and multiple sclerosis in *in vivo* murine models (Komegae et al., 2017). Given these results, studying venom compounds and understanding their immunomodulatory effects could directly impact the livelihood of people who suffer from chronic inflammatory diseases, as well as improve treatment modalities of sting victims.

Analysis of the transcriptome and proteome of the estuarine stonefish *Synanceia horrida* venom showed evidence of homologues to natterins and nattectin (a C-type lectin) from *T. nattereri* (Ziegman et al., 2019). These analyses of *S. horrida* venom also showed the presence of several unique proteins that had never been described in animal venoms and highlighted the need to further investigate the activity of stonefish venoms, particularly for immunomodulatory effects. *S. horrida* is considered to be one of the most venomous fish species in the world, alongside its reef counterpart, *Synanceia verrucosa*. They are medically relevant species, causing high numbers of envenomation and morbidity around the Indo-Pacific region, and stings from the latter led to the death of a boy in 2018 (Maillaud et al., 2020; Saggiomo et al., 2021).

Envenomation from stonefish takes place when one or more dorsal spines are introduced into the flesh of the victim or predator. This allows for the compression of the dual venom glands associated with each spine, releasing venom inside the newly formed wound (Saggiomo et al., 2021). The most remarkable characteristic symptoms of stonefish envenomation are excruciating pain and edema formation in the affected limb (Sutherland and Tibballs, 2001). Serious pathologies may occur following envenomation, affecting cardiovascular, respiratory and neuromuscular systems in both experimental and clinical settings (Saggiomo et al., 2021). Many of these effects are believed to be caused by a few venom components, such as cytolysins, whose ability to form pores in a variety of cell types have been linked to the venom's hemolytic and lethal activities (Church and Hodgson, 2002; Ziegman and Alewood, 2015).

Pathologies and symptoms are well described in the literature following stonefish envenomation in animal models, but detailed clinical reports are lacking. There is currently only one clinical report following *S. verrucosa* envenomation that shows biochemical changes in a victim, with increased numbers of white blood cells, troponin and C-reactive protein, and lowered levels of creatinine (Ghanem et al., 2019). Beyond the knowledge that stonefish venom can cause inflammation in the form of localised or systemic oedema (Saggiomo et al., 2021), to the best of my knowledge, no clinical or experimental evidence of cytokine modulation is currently available.

In this study, I demonstrate that stonefish venom can suppress a range of pro-inflammatory cytokines secreted from human peripheral blood mononuclear cells, indicating the presence of anti-inflammatory molecules in the venom. These findings will help close an important gap in the mechanism of action of these venoms, as well as possibly carve a pathway for future drug discovery, as a number of other venom-derived compounds have shown promising results as therapeutic leads in animal models for the treatment of disorders such as asthma, autoimmune diseases and

rheumatoid arthritis in animal models (Beeton et al., 2005; Hwang et al., 2015; Koshy et al., 2014; Zhao et al., 2015).

5.2 Materials and methods

5.2.1 Animal collection

Two different species of stonefish, *S. verrucosa* and *S. horrida*, were used in the experiments (ethics application number A2572). *S. verrucosa* was bought from a commercial supplier (Cairns Marine) and acclimated for a period of two weeks at James Cook University (JCU) Aquarium in Cairns, Australia prior to milking. *S. horrida* had been housed at JCU Aquarium for a period longer than six months. Individuals were housed together and apart from other fish species. They were kept alive throughout the duration of the experiments and were offered prawns once daily until they rejected additional food. From here on in, *S. verrucosa* venom will be referred to as SvV and *S. horrida* venom will be referred to as ShV.

5.2.2 Venom collection

Venom was collected from either all of the dorsal spines or from only a selected number. Milking was performed with at least a four-week interval between milking events, based on previous studies (Saggiomo et al., 2017). Venom collection was performed using a tube containing a membranous lid as previously reported (Wahsha et al., 2019). The tube was inverted vertically onto the spine and its lid was pressed on both venom sacs until all of the venom was excreted into the tube. Venom was immediately placed in an ice bath and used for experiments as crude fresh venom or divided into aliquots, labelled, frozen or lyophilized (ScanSpeed 40, Serial No LVT22513080042, LabGear, Australia) and stored at -80°C.

5.2.3 Protein concentration determination

Venom protein concentrations were assayed using a bicinchoninic acid (BCA) protein assay kit according to manufacturer's instructions (Pierce Rapid Gold, ThermoFisher Scientific; Cat A53225). Frozen venom was allowed to thaw in an ice bath and lyophilized venom was rehydrated in DPBS (ThermoFisher) prior to testing, where they were serially diluted seven times. Venom was kept in an ice bath throughout the entire process. Venom samples and BSA standards were plated in duplicate. Protein concentrations were calculated by subtracting the average 480 nm blank absorbance measurement from all the other sample and standard measurements. A standard curve was generated (Microsoft Office Excel 2016 or GraphPad Prism Ver 9.0) and used to determine the protein concentration of each sample.

5.2.4 Limulus amebocyte lysate (LAL)

Venom samples were tested for Gram-negative bacterial contamination using a LAL QCL-1000 kit (Lonza, Morristown, NJ, USA), according to manufacturer's instructions. Venoms were run either in duplicate or triplicate, mixed with endotoxin-free water and serially diluted to 1:10, 1:100 and 1:1000.

5.2.5 Human blood collection

Human blood was obtained either from healthy donors and drawn by a trained phlebotomist at James Cook University, Cairns, Australia, or from buffy coats provided by the Australian Red Cross Blood Service. Ethics was approved by the James Cook University Human Research Ethics Committee (ethics application number H6702) and by the QIMRB Berghofer Medical Research Institute (ethics application number H7010). The study was carried out according to the rules of the Declaration of Helsinki of 1975.

5.2.5.1 Media, reagents and buffers

All cells were cultured at 37°C, 5% CO_2, in 96-well U-bottom Falcon plates. The media, reagents and buffers used throughout the experiments are listed in the table below.

Table 5.1: Media, reagents and buffers recipes used for cell culture throughout the experiments in this chapter.

Reagent	Details
R0 medium	ThermoFisher RPMI-1640 medium
R5 medium	Gibco RPMI medium 1640 (1X) [-] L-glutamine
	Bovogen heat inactivated foetal bovine serum 5% (25 mL in 500 mL)
	Gibco 10,000 units/mL of penicillin + 10,000 µg/mL of streptomycin. Used as 1X (5 mL in 500 mL)
	Gibco GlutaMAX 100X. Used as 1X
R10 medium	R0 medium supplemented with 10% ThermoFisher FBS
PBS (1X) (bought or made in-house)	ThermoFisher Dulbecco's PBS (DPBS)
	137 mM sodium chloride (NaCl)
	2.7 mM potassium chloride (KCl)
	10 mM disodium phosphate dibasic (Na_2HPO_4)
	1.8 mM potassium phosphate monobasic (KH_2PO_4)
	Adjusted pH to 7.2-7.4 and sterilized when required
Freezing buffer	90% ThermoFisher FBS
	10% Sigma-Aldrich dimethyl sulfoxide (DMSO)

** All cell culture additives were filtered with a 0.22 µm filter*

5.2.5.2 Human peripheral blood mononuclear cells (hPBMCs) isolation and cryopreservation

Human PBMCs were purified from whole blood or buffy coats using standard procedures (Betsou et al., 2019; Ryan et al., 2020) and were either used immediately or cryopreserved. Cells that were cryopreserved were centrifuged at 400 x *g* for 5 min to remove any medium and were re-suspended in freezing buffer at the desired concentration. The cells were then transferred into 1 mL cryovials (Cryo.s vials, Greiner Bio-One) and frozen using a standard freezing device (Corning CoolCell FTS30 or LX) with a controlled freezing rate to -80°C. Cells were then transferred to liquid nitrogen within 48 h for long term storage.

5.2.5.3 Cell thawing and counting

Cryopreserved cells were thawed in a 37°C water bath until the ice melted using standard procedures (Ryan et al., 2020) and then diluted in R10 medium in a 1:10 ratio, then centrifuged at 400 x *g* for 5 min. The cell pellet was re-suspended in R10 medium and treated with 20 µL of DNase-I (Merck) at 37°C for a minimum of 20 min to ensure DNA digestion and prevention of negative effects from dead cell debris. The cell pellet was washed twice to remove DNase at 500 x *g* for 5 min and re-suspended with R10 medium each time. After re-suspension, an automated cell counter (OLS CASY 2.5, OMNI Life Sciences) was used, where cells were analysed, and viable cell numbers were obtained.

5.2.6 *In vitro* assays

5.2.6.1 Cytotoxicity assays of stonefish venoms

Cytotoxicity assays used hPBMCs exposed to venom at different concentrations (1.25 to 100 µg/mL) for up to 24 h. Treatment conditions were performed in triplicate, plated in a white flat-bottom opaque 96-well plate (BMG Labtech), with fluorescence measured in the FLUOStar Omega (BMG Labtech) plate reader.

The cellular toxicity study was conducted following manufacturer protocols (CellTox Green, Promega). Briefly, thawed cells were centrifuged and seeded at 100,000 per well in triplicate. Lysis buffer (positive control), DPBS (negative control) and venom treatments were then added. Fluorescence was measured using FLUOStar Omega Ver 5.11R4 (BMG Labtech) at different time points depending on the experiment (600 gain, top optic). Each reading was exported to a Microsoft Office Excel 2016 spreadsheet, collated, and then exported into GraphPad Prism Ver 9.0 for further statistical analysis.

5.2.6.2 Cytokine and chemokine quantification assays

Levels of cytokine expression or suppression secreted by hPBMCs were measured using three different assays, namely cytometric bead array (CBA) assay, reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) and enzyme linked immunosorbent assay (ELISA).

Positive controls of well-known immunomodulatory reagents (Ai et al., 2013; Bertani and Ruiz, 2018) were employed in the assays to compare against the immune response caused by stonefish venom. These positive controls were lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA)-ionomycin (P/I). Positive controls for the suppression activity were the anti-inflammatory drugs dexamethasone (DEX) and cyclosporine A (CsA), known to strongly inhibit LPS- and P/I-stimulated inflammation, respectively (Arbabian et al., 2011; Castano et al., 2002).

5.2.6.2.1 Human PBMC stimulation assays

All immunoassays for this chapter were plated and incubated in a 96-well U-bottom Falcon plate, where treatments were plated in triplicate and positive controls in duplicate. Cells (100,000 per well) were incubated overnight for 16 h either alone (negative controls), with LPS (10 ng/mL, Sigma-Aldrich) or P/I (0.5 X, Sigma-Aldrich) (positive controls) or with venom concentrations of 20, 5 or 1.25 µg/mL. As positive controls for immunosuppression, DEX (10 µg/mL, Sigma-Aldrich) and CsA (10 µg/mL, Sigma-Aldrich) were also used. All treatments were plated with final dilutions of 1:10 in R5 medium.

After overnight stimulation with venom, LPS or P/I, plates were removed from the incubator, checked under the microscope, and pelleted by centrifugation. Culture supernatant was then harvested and either used fresh for CBA, RT-qPCR, ELISA, or transferred into 96-well PCR plates in various aliquots, sealed with adhesive alfoil, labelled, and stored at -20°C for future use. Each assay will be described in more detail in the following sections.

5.2.6.2.2 Cytometric bead array (CBA) assay using *S. verrucosa* freshly milked venom

CBA was used to screen both expression and suppression properties of SvV on various cytokines and chemokines. This assay was only performed on SvV due to sample availability. The CBA kit used (CBA Human Soluble Protein Master Buffer Kit, BD Biosciences) measured cytokines produced by Type 1 T helper (Th1) lymphocytes: TNF, IFN-γ, IL-1ß, IL-2, IL-12, by Type 2 (Th2) lymphocytes: IL-4, IL-6, IL-10, IL-13, and also chemokines such as MCP-1 and IL-8 (Austin et al., 1999; Lucey et al., 1996).

Freshly milked SvV was collected, titrated three times from 20 to 1.25 µg/mL and kept on ice until the end of the experiment. Human PBMCs (n = 4 donors) were plated following the stimulation protocol described in the previous section. Supernatant was transferred into a 96-well U-bottom microplate and diluted with assay diluent (1:2). The standard was serially titrated eight times from 2,500 to 10 pg/mL with assay diluent and plated in duplicate, along with blank wells.

Mastermix was added to a 96-well V-bottom plate, followed by the standards and the diluted supernatant (1:10) from cellular stimulation. The microplate was covered with alfoil, mixed for 5 min on a plate shaker at room temperature and incubated in a fridge overnight. On the following day, the microplate was removed from the fridge and incubated on a plate shaker at room temperature for 1

h. After the incubation, detection reagent was added into the V-well plate, which was then covered with alfoil and incubated at room temperature on a plate shaker for 2 h. The microplate was washed twice, and the pellet was re-suspended in wash buffer and placed in the flow cytometer for reading (BD Fortessa X-20 Ver Diva 8.0.1). Data were analysed using the BD FCAP Array v3 Ver 3.0.19.2091 analysis software and exported into a Microsoft Office Excel 2016 spreadsheet and further analysed in GraphPad Prism Ver 9.

5.2.6.2.3 Reverse-transcriptase quantitative polymerase chain reaction assays of *S. verrucosa and S. horrida* venoms

RT-qPCR assay was used to investigate whether the cytokine level changes observed in the CBA (at the protein level) were also evident at the gene level. The materials and methods for this assay were followed as previously described (Browne et al., 2020) and as recommended by the manufacturer (for further information, please refer to Appendix D). RT-qPCR measurements used the QuantStudio 3 Real-Time PCR system running QuantStudio Design and Analysis Software (v1.4.3, Applied Biosystems). Samples were run in technical triplicates, and each plate included a standard curve, a calibration sample and also a negative control. Synthesis of cDNA was performed on a SimpliAmpTM thermocycler (ThermoFisher Scientific).

This assay was performed with both SvV and ShV. The cytokines chosen for these assays were TNF, IFN-γ and IL-10, based on key immunological roles (as stated in the Results section). Human PBMCs (n = 4 donors) were plated following the same protocol previously described for cell thawing (Section 5.2.5.3) and cell stimulation (Section 5.2.6.2.1), but cells were supplemented with 10% human serum instead of FBS, and also received additional 10 mM HEPES (ThermoFisher Scientific) and 1% non-essential amino acids (Gibco). Human PBMCs were stimulated for 6 h at 100,000 cells per well. This assay only used the highest venom concentration (20 µg/mL) as SvV showed greater suppression activity at this concentration in the CBA assay.

5.2.6.2.4 Enzyme linked immunosorbent assay (ELISA)

ELISAs were performed to confirm the results from both CBA and RT-qPCR and to validate the assay's feasibility in venom-driven suppression studies of TNF at the protein level. TNF was chosen for further testing because it is a pro-inflammatory cytokine that plays important roles in immune modulation, being one of the first cytokines to be expressed upon pathogen detection, starting the cascading events that will ultimately lead to the recruitment of other cells and expression of further cytokines to protect the host (Akdis et al., 2016; Cruz et al., 2008; Jaffer et al., 2010). Assays firstly investigated freshly milked SvV and ShV to compare immunological results with the CBA and the results at the gene level from RT-qPCR. Secondly, frozen venoms were tested to investigate whether the venom maintained its immunomodulatory activity following long-term frozen storage. Suppression of LPS-

mediated inflammation was more significant and reproducible compared to P/I with the *Synanceia* spp. venoms used in this thesis, therefore, experiments focused on cells stimulated with LPS only.

To reduce potential confounding factors, the same batch of venom was used across the assays. Freshly milked venom used in the ELISAs was aliquoted and stored frozen at -80°C for a minimum of one year prior to use. Venom samples were diluted three times from 20 to 1.25 µg/mL and kept on ice until the end of the experiment. Incubation and stimulation of hPBMCs was conducted in 96-well half-area flat bottom microplates (Corning Costar) following manufacturer's protocol. Microplates were coated with TNF capture antibody as per kit instructions (human TNF-alpha DuoSet, R&D Systems), sealed, labelled, and incubated overnight at room temperature. During each assay, the ELISA microplate was washed between each step with an automated microplate washer (BioTek 405LS, Millenium Science) using wash buffer and following the manufacturer's instructions. All incubations were performed at room temperature. Reagents and buffers are listed below in Table 5.2:

Table 5.2: Reagents used for ELISA assays were either from a commercial kit or made in-house. *The table below lists the reagents recipes and respective manufacturers used for ELISA assays throughout this chapter.*

Reagent or buffer	Details
Reagent diluent (RD)	1% bovine serum albumin (Sigma-Aldrich) in PBS
Wash buffer	0.05% Tween-20 (Sigma-Aldrich) in PBS
Substrate solution (from kit)	50% hydrogen peroxide (H_2O_2) (colour reagent A)
	50% tetramethylbenzidine (colour reagent B)
Stop solution	2 N sulphuric acid (H_2SO_4), 1 M (Sigma-Aldrich)

Following the overnight incubation, the ELISA half-area microplate was blocked for at least 1 h. During this time, the stimulated cells were removed from the incubator, checked under the microscope, and pelleted by centrifugation. Culture supernatant was harvested and diluted 1:10 in RD. After the blocking period, the ELISA microplate was incubated for at least 2 h with cell supernatant or recombinant human TNF protein standard. The plate then received TNF detection antibody, followed by HRP-conjugated streptavidin, then substrate solution, and finally stop solution. The optical density (OD) of the plate was immediately determined using a plate reader (FLUOStar Omega Ver 5.11R4, BMG Labtech). The final output was transferred to an online data analysis website designed for ELISAs (www.elisaanalysis.com) and the export from the website was multiplied by the dilution factor and the final TNF concentration was used for statistical analysis in GraphPad Prism Ver 9.

5.3 Results

A range of assays were carried out to analyse the cellular effects of stonefish venoms. All *S. verrucosa* and *S. horrida* venom samples were tested for the presence of endotoxin using LAL kits. The results

established the venom samples were not contaminated by endotoxin and, consequently, were appropriate for use in the cellular assays. Additionally, cytotoxicity of the venom was assessed to ensure concentrations used for *in vitro* immunomodulatory assays were not cytotoxic towards hPBMCs.

A cytokine screen using CBA was then carried out to provide insight into venom immunomodulatory activities, and more comprehensive analysis of venom-driven suppression of appropriate cytokines undertaken using RT-qPCR assays and ELISAs. These assays used two distinct immunomodulatory agents, LPS and P/I, and their respective inhibitors, DEX and CsA. LPS is a glycolipid produced by the majority of Gram-negative bacteria that is capable of stimulating and modulating responses by the human immune system, and historically classified as an endotoxin (Bertani and Ruiz, 2018). Human immune systems have evolved to react dramatically in the presence of LPS, as it is toxic to the host, playing major roles in bacterial pathogenesis and cytokine expression (Bertani and Ruiz, 2018). LPS is strongly inhibited by DEX (Castano et al., 2002), which was used as a positive control for suppression in these assays. P/I is also commonly used to stimulate immune cells to produce a variety of cytokines and chemokines. PMA stimulates immune cells through activation of protein kinase C, whereas ionomycin is a calcium ionophore. Together, they play an important role in T cell activation, as well as in signalling transduction cascades and overall production of several types of cytokines and chemokines (Ai et al., 2013). P/I is strongly inhibited by CsA, which was also used as a positive control for suppression in these assays (Arbabian et al., 2011).

5.3.1 Cytotoxicity of stonefish venoms

The cellular toxicity of freshly milked SvV and ShV on hPBMCs was analysed using fluorescence-based cytotoxicity kits (Figure 5.1). No cytotoxic effects were observed for both freshly milked and frozen SvV at concentrations up to 20 μg/mL (Figure 5.1A and 5.1B, respectively). Treatment of hPBMCs with 80 μg/mL of SvV was significantly different from all other treatments (Figures 5.1A and 5.1B), indicating some level of cytotoxicity at this concentration. By contrast, ShV fresh or frozen venoms were not cytotoxic at concentrations up to 100 μg/mL (Figure 5.1C and 5.1D, respectively), suggesting a potential difference in venom composition or mode of action compared to SvV. Cytotoxicity did not seem to be impacted by freezing the venom and indicates that, at least for SvV at a concentration of 80 μg/mL, the activity was maintained through one freeze/thaw cycle.

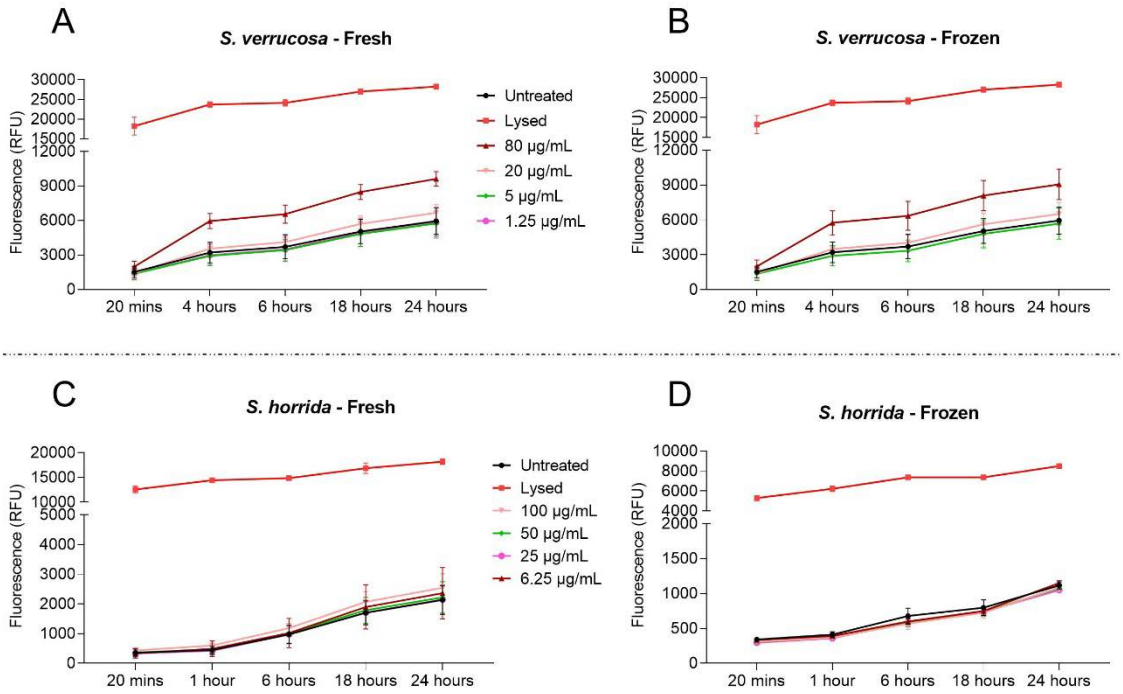

Figure 5.1: Cellular toxicity of freshly milked and frozen stonefish venoms on hPBMCs. *(A) Fresh SvV for n = 4 donors, (B) Frozen SvV for n = 4 donors, (C) Fresh ShV for n = 3 donors, and (D) Frozen ShV for n = 2 donors. Results represent the cellular response of hPBMCs to crude venom treatments, where data are shown as mean fluorescence ± SD. Untreated cells are negative controls; lysed cells are positive controls; venom dilution treatments are shown in different colours. A two-way ANOVA with multiple comparisons was performed for each venom separately and showed a significant difference between untreated and venom-treated cells for SvV at 80 µg/mL ($p < 0.0001$), but no significant differences for ShV ($p = 0.9990$).*

5.3.2 Cytometric bead array (CBA) assay

To investigate venom-induced expression or suppression of various cytokines on hPBMCs, a CBA assay was performed using only SvV due to sample availability, at three concentrations up to 20 µg/mL. A total of 12 different cytokines were measured, namely TNF, IFN-γ, MCP-1, IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, and IL-13. Four cytokines (IL-2, IL-4, IL-5, and IL-13) did not show sufficient expression levels to be measured in the assay following cell stimulation with LPS or venom. Likewise, five cytokines (IL-1ß, IL-4, IL-10, IL-12, and IL-13) did not express measurable levels following cell stimulation with P/I or venom. Data for these results are not shown.

Incubation of venom alone did not cause significant secretion of the cytokines or chemokines tested, regardless of the concentrations used (Figures 5.2, 5.3 and 5.4). Figure 5.2 shows the cytokines where secretion levels were significantly reduced after venom treatment. Figures 5.3 and 5.4 show the cytokines where secretion levels were not diminished after venom treatment following LPS and P/I stimulation, respectively. Following LPS stimulation, SvV suppressed expression levels of TNF, IL-12, IFN-γ (Th1) and MCP-1 in a dose-dependent manner and IL-6 (Th1) following P/I stimulation (Figure 5.2). Levels of TNF secretion were significantly suppressed after SvV treatment with 20, 5 and 1.25

µg/mL (83.2%, 61.2%, and 40.1%, respectively) or with DEX (87.9%). Levels of IL-12 followed a similar pattern (reduction of levels by 100%, 94.6%, 90.2%, and 99.5% for SvV concentrations of 20, 5 and 1.25 µg/mL, and DEX, respectively). IFN-γ and IL-6 secretion levels were only significantly reduced by SvV at 20 µg/mL (88% and 73.5%, respectively) and DEX or CsA (99.5% and 77.5%, respectively). MCP-1 levels were suppressed by SvV at all concentrations tested (79.3%, 75.7%, and 62.5% for 20, 5 and 1.25 µg/mL, respectively), and demonstrated a greater level of suppression activity compared to DEX (Figure 2). SvV, therefore, potently suppresses TNF, IL-12, IFN-γ, IL-6 and MCP-1 secretion levels from hPBMCs. This suppression is comparable to the anti-inflammatory drugs used, and it appears that SvV has selectivity towards cells incubated with LPS rather than P/I.

In contrast, SvV did not suppress levels of IL-1ß, IL-6, IL-8 and IL-10 in LPS-stimulated hPBMCs (Figure 5.3), where IL-1ß secretion was increased in a dose-dependent manner, reaching significance at the highest dose. In P/I-stimulated cells SvV did not show significant suppression of TNF, IFN-γ, MCP-1, IL-1ß, IL-2, IL-4, IL-5, IL-8 and IL-13 (Figure 5.4).

These results indicate that venom from *S. verrucosa* has a suppression activity bias towards LPS-stimulated cells and towards Th1 cytokine levels. The implications for this bias are discussed later in this chapter.

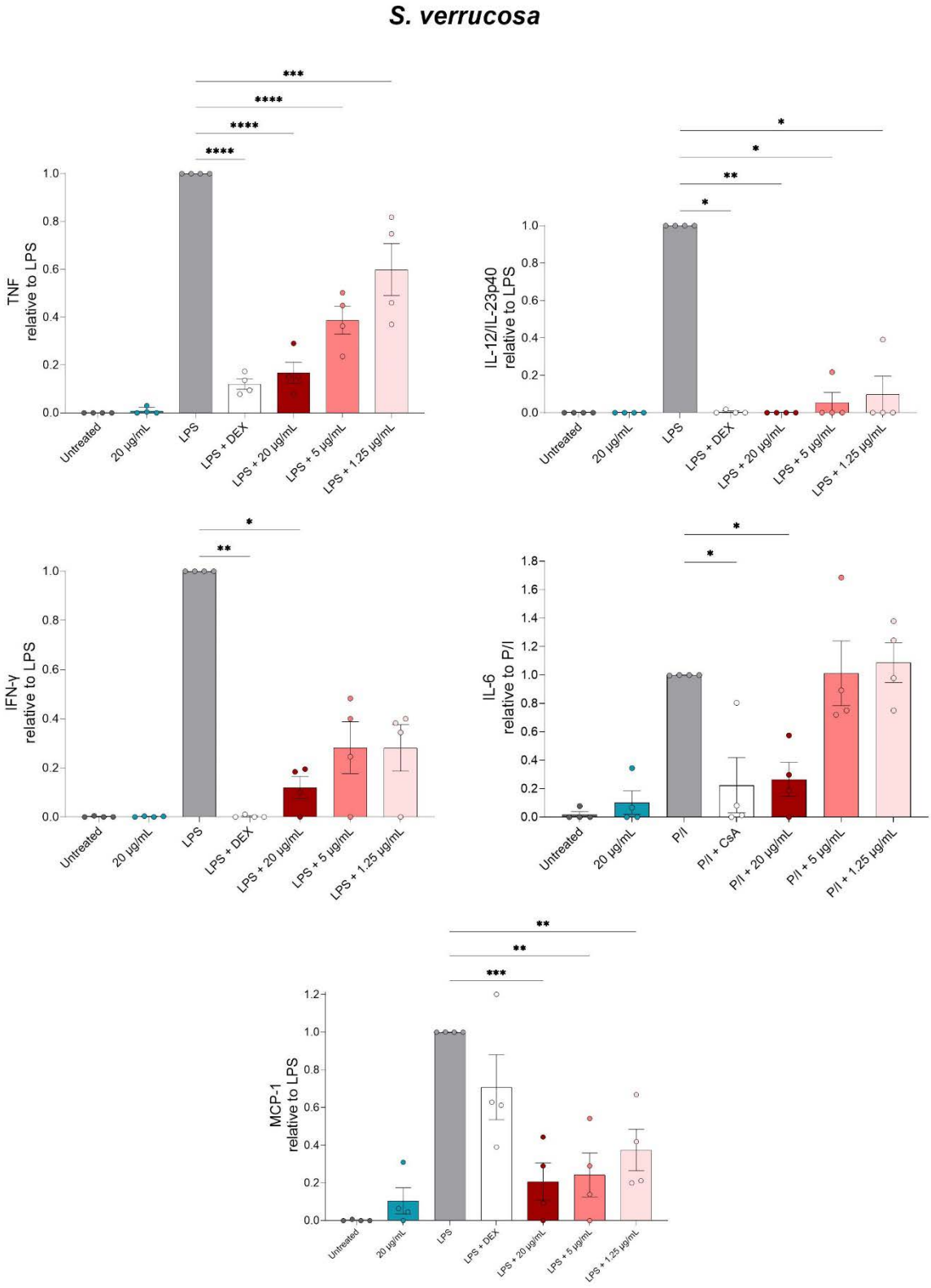

Figure 5.2: CBA screening showing that SvV treatment significantly reduced cytokine secretion levels from hPBMCs stimulated with LPS or P/I. *Cells were incubated alone, with 20 µg/mL of SvV, LPS, P/I or LPS or P/I + 20, 5 or 1.25 µg/mL of SvV. Twelve cytokines, representing a range of Type 1, Type 2 and regulatory cytokines, were measured and the graphs show only those with significant suppression levels: TNF, IL-12, IFN-γ, IL-6 or MCP-1. All cytokines were secreted after LPS-stimulation, with the exception of IL-6, which was secreted following P/I stimulation. Histograms illustrate soluble cytokine levels released from hPBMCs, where error bars are mean ±*

*SEM (n = 4 donors). Untreated cells are negative controls, LPS, LPS + DEX, P/I, and P/I + CsA are positive controls. Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). * p < 0.05, ** p < 0.006, *** p < 0.009, **** p < 0.0001.*

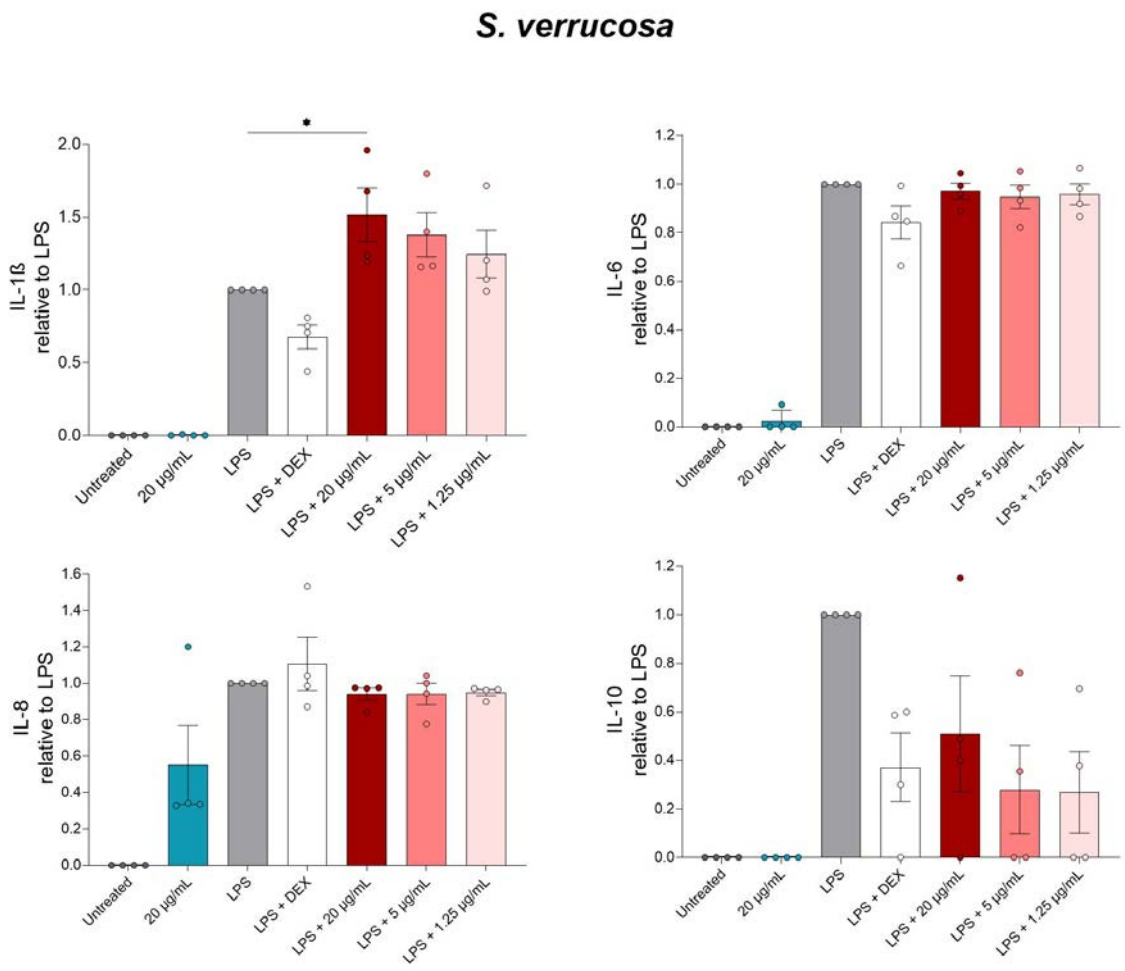

Figure 5.3: CBA screening showing that SvV or DEX treatments did not cause a significant reduction in cytokine secretion levels from hPBMCs stimulated with LPS. *Cells were incubated alone, with 20 µg/mL of SvV, with LPS or with LPS + 20, 5 or 1.25 µg/mL of SvV. Twelve cytokines, representing a range of Type 1, Type 2 and regulatory cytokines, were measured and the graphs show the cytokines where levels were not significantly reduced by venom treatment following LPS stimulation: IL-1ß, IL-6, IL-8 or IL-10. Histograms illustrate soluble cytokine levels released from hPBMCs, where error bars are mean ± SEM (n = 4 donors). Untreated cells are negative controls, LPS and LPS + DEX are positive controls. Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). * p < 0.05, ** p < 0.006, *** p < 0.009, **** p < 0.0001.*

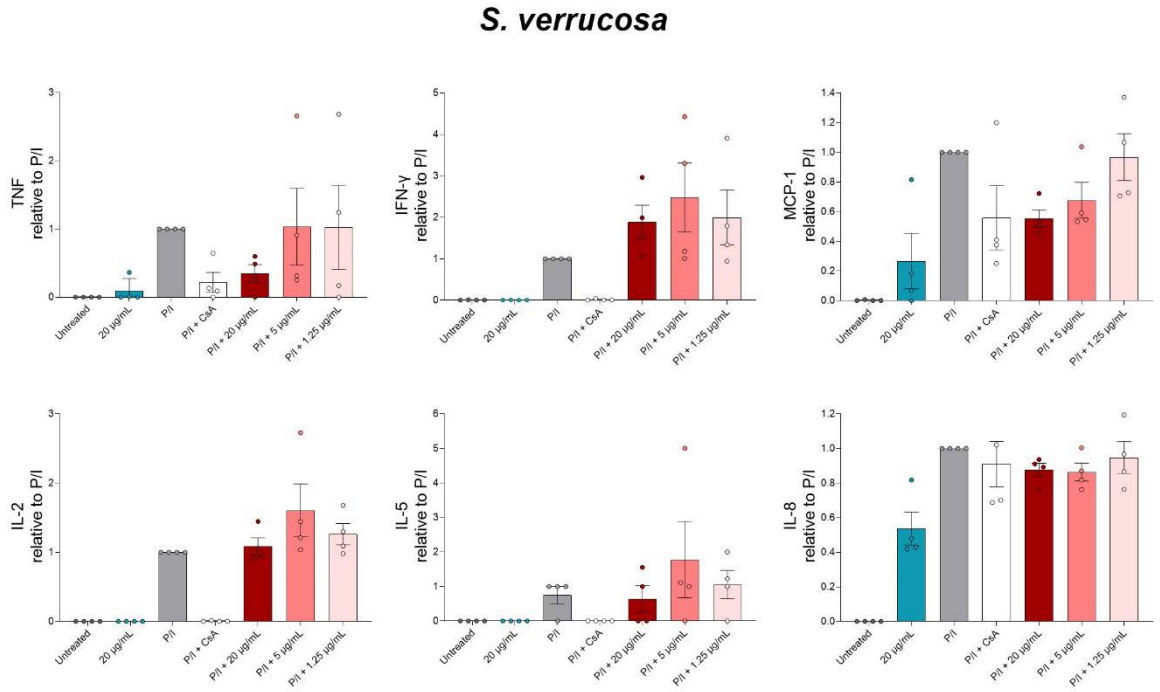

Figure 5.4: CBA screening showing that SvV or CsA treatments did not cause a significant reduction in cytokine secretion from hPBMCs stimulated with P/I. *Cells were incubated alone, with 20 µg/mL of SvV, with P/I or with P/I + 20, 5 or 1.25 µg/mL of SvV. Twelve cytokines, representing a range of Type 1, Type 2 and regulatory cytokines, were measured and the graphs show the cytokines where levels were not significantly reduced by venom treatment following P/I stimulation: TNF, IFN-γ, MCP-1, IL-2, IL-5, or IL-8. Histograms illustrate soluble cytokine levels released from hPBMCs, where error bars are mean ± SEM (n = 4 donors). Untreated cells are negative controls, P/I, and P/I + CsA are positive controls. Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). * p < 0.05, ** p < 0.006, *** p < 0.009, **** p < 0.0001.*

5.3.3 Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR allows for the investigation of gene modulation, revealing up- or down-regulation of targeted gene expression in response to a stimulus. RT-qPCR was used to study gene regulation of three cytokines (Th1: TNF, IFN-γ and Th2: IL-10) from hPBMCs stimulated with either LPS or P/I. These cytokines were chosen based on the CBA results, and the distinct roles they play in immunomodulation. TNF is one of the first pro-inflammatory cytokines to be released from immune cells. IFN-γ is known to inhibit replication of viruses and activate macrophages to kill intracellular pathogens. IL-10 is anti-inflammatory, inhibiting cytokine secretion (Cohen and Cohen, 1996). Both SvV and ShV were analysed in the RT-qPCR experiments.

Application of SvV and ShV alone did not cause significant changes in cytokine secretion levels from hPBMCs. For LPS-induced secretion of cytokines from hPBMCs, SvV caused significant down-regulation of mRNA levels of all three cytokines tested (Figure 5.5A). Treatment with 20 µg/mL of SvV caused TNF secretion levels to significantly reduce by 48.2% and IL-10 levels by 61.1%, whereas DEX

did not show a significant reduction in either, compared to LPS. By contrast, IFN-γ secretion levels were reduced by 91.6% after SvV addition and by 96.9% by DEX (Figure 5.5A).

ShV showed significant down-regulation of mRNA TNF and IL-10 levels (34.9% and 54.9%, respectively) compared to LPS, whereas DEX did not (Figure 5.5B). IFN-γ levels were not significantly reduced by ShV, only by DEX (84.6%) (Figure 5.5B). For P/I-induced cells, neither SvV (Figure 5.6A) nor ShV (Figure 5.6B) caused significant down-regulation of mRNA levels for any cytokine tested.

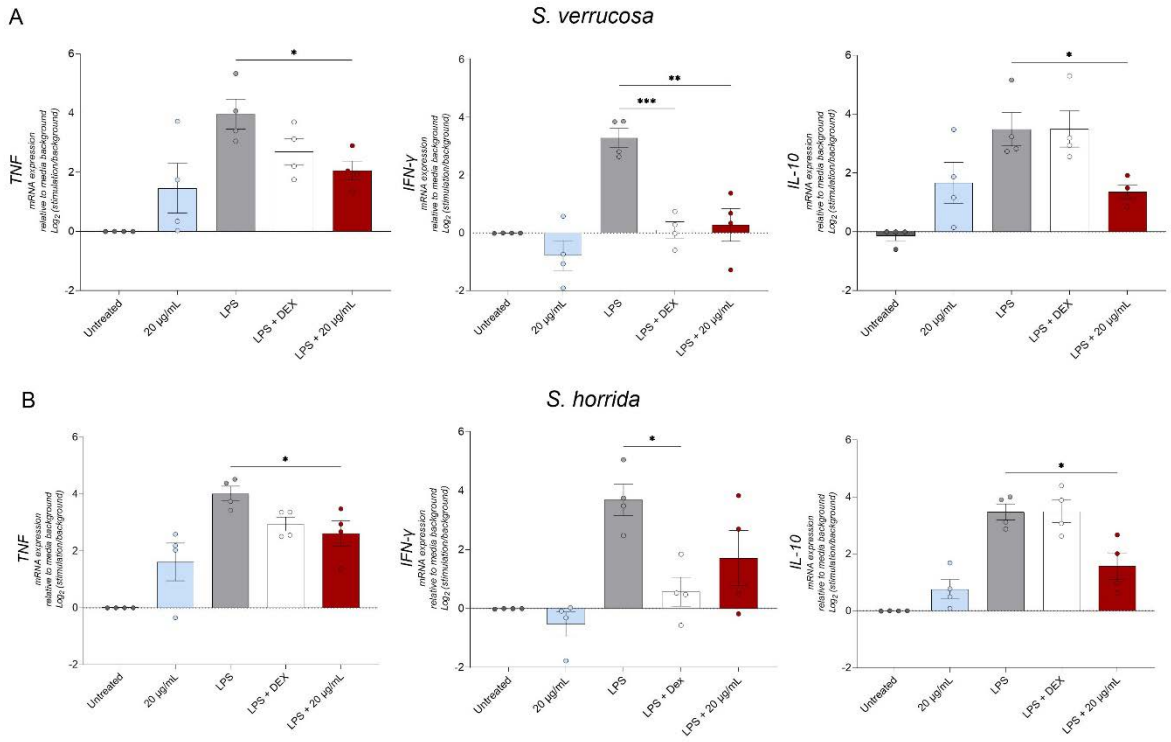

Figure 5.5: RT-qPCR screening assay showing that (A) SvV or (B) ShV treatment significantly reduced cytokine secretion from hPBMCs stimulated with LPS. *Histograms illustrate levels of mRNA expression relative to media background Log_2 (stimulation/background) of LPS-induced hPBMCs: TNF, IFN-γ or IL-10. Cells were incubated alone, with 20 µg/mL of either (A) SvV or (B) ShV, with LPS only, or LPS + 20 µg/mL of (A) SvV or (B) ShV. Histograms illustrate soluble cytokine levels released from hPBMCs, where error bars are mean ± SEM (n = 4 donors). Untreated cells are negative controls, LPS and LPS + DEX are positive controls. Results were quantified through a one-way ANOVA followed by Dunnett's multiple comparison tests or their equivalent non-parametric tests (Kruskal-Wallis followed by Dunn's tests). Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). * p < 0.05, ** p < 0.006, *** p < 0.009, **** p < 0.0001.*

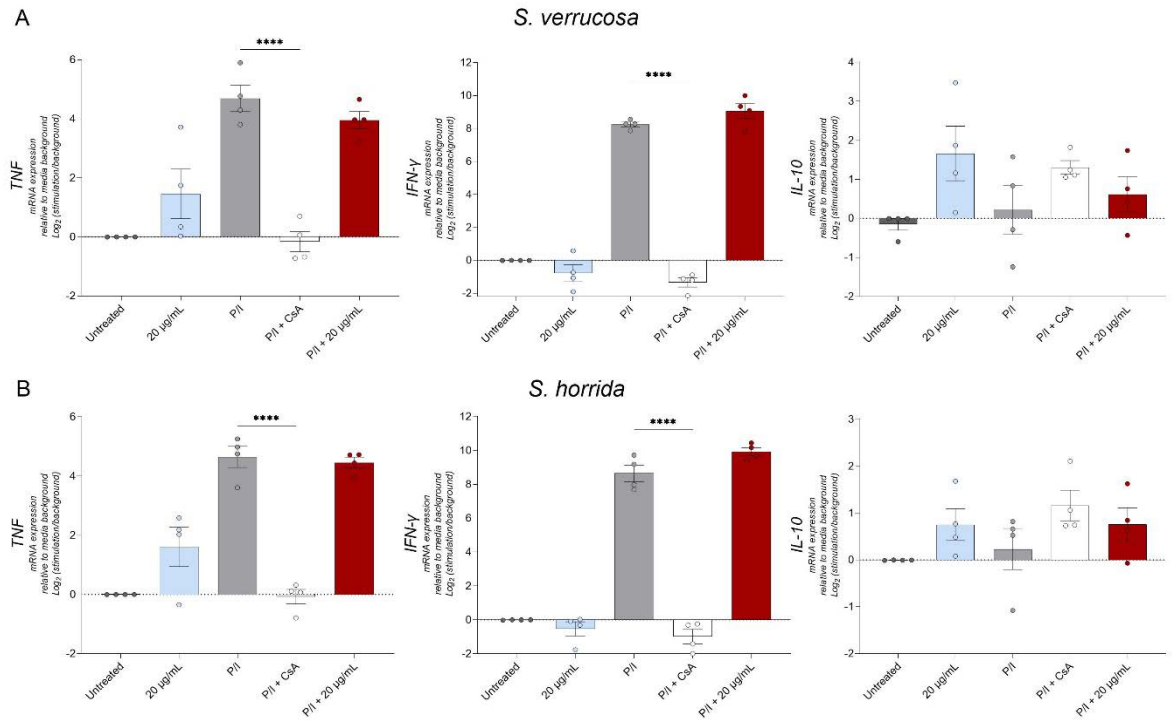

Figure 5.6: RT-qPCR screening assay showing that SvV (A) or ShV (B) treatment did not reduce cytokine secretion levels from hPBMCs stimulated with P/I. *Histograms illustrate levels of mRNA expression relative to media background Log_2 (stimulation/background) of P/I-induced hPBMCs: TNF, IFN-γ or IL-10. Cells were incubated alone, with 20 µg/mL of either (A) SvV or (B) ShV, with P/I only, or P/I + 20 µg/mL of (A) SvV or (B) ShV. Histograms illustrate soluble cytokine levels released from hPBMCs, where error bars are mean ± SEM (n = 4 donors). Untreated cells are negative controls, P/I and P/I + CsA are positive controls. Results were quantified through one-way ANOVAs followed by multiple comparison tests or their equivalent non-parametric tests (Kruskal-Wallis followed by Dunn's tests). Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). * p < 0.05, ** p < 0.006, *** p < 0.009, **** p < 0.0001.*

5.3.4 ELISA screenings of tumour necrotic factor (TNF) of fresh and frozen stonefish venoms

Since TNF was dramatically suppressed in both the CBA and RT-qPCR screenings for both stonefish species, TNF was further explored for suppression activity of both crude fresh and frozen venoms using ELISA methods.

Consistent with the CBA and RT-qPCR results, freshly milked stonefish venoms from both species did not produce a significant effect on TNF levels compared to untreated cells (Figure 5.7). However, SvV suppressed TNF secretion levels from LPS-stimulated cells by 60.4% and 30.3% when applied at 20 and 5 µg/mL, respectively (Figure 5.7A). Data obtained for P/I-stimulated cells showed a significant decrease of TNF levels for 20 µg/mL venom sample (reduction of 36.1%) (Figure 5.7B). These results confirmed both CBA and RT-qPCR assay results.

In the case of ShV, suppression activity was only seen for LPS-stimulated hPBMCs, where TNF secretion reduction was only observed for venom at 20 µg/mL (29.4%), whereas DEX had a reduction of 67.3%

(Figure 5.7C). Following P/I stimulation, ShV increased expression of TNF levels, although not significantly (Figure 5.7D). These results also confirmed the RT-qPCR assay results.

Overall, SvV reduced secretion levels of the tested cytokines to a greater degree than ShV. SvV suppressed both LPS- and P/I-stimulated TNF secretion at levels comparable to the positive control anti-inflammatory drugs, DEX and CsA. ShV only showed suppression of LPS-mediated inflammation, and only at the highest concentration tested. Both venoms appeared to suppress LPS-stimulated inflammation more strongly than P/I-stimulated levels. Importantly, these results indicate that ELISAs are a reliable assay to investigate venom-driven suppression at the protein level. Due to reduced cost and reliable reproducibility, ELISAs were used to further investigate the effect of venom storage (and fractionated venom in Chapter 6) on the immunosuppressive activity of LPS-stimulated hPBMCs.

Storage of venom is an essential safeguard to ensure adequate venom supplies, as animals can stop producing sufficient venom amounts for assays, die unexpectedly or venom profiles can change depending on the age of the animal or season in which the milking took place. It is important, therefore, to establish whether long-term storage affects the bioactivity of the venom. The results of ELISAs comparing fresh venom to frozen stored venom showed that both SvV and ShV at 20 µg/mL maintained suppression activity of TNF secretion levels in LPS-stimulated hPBMCs (Figure 5.7E and 5.7F, respectively).

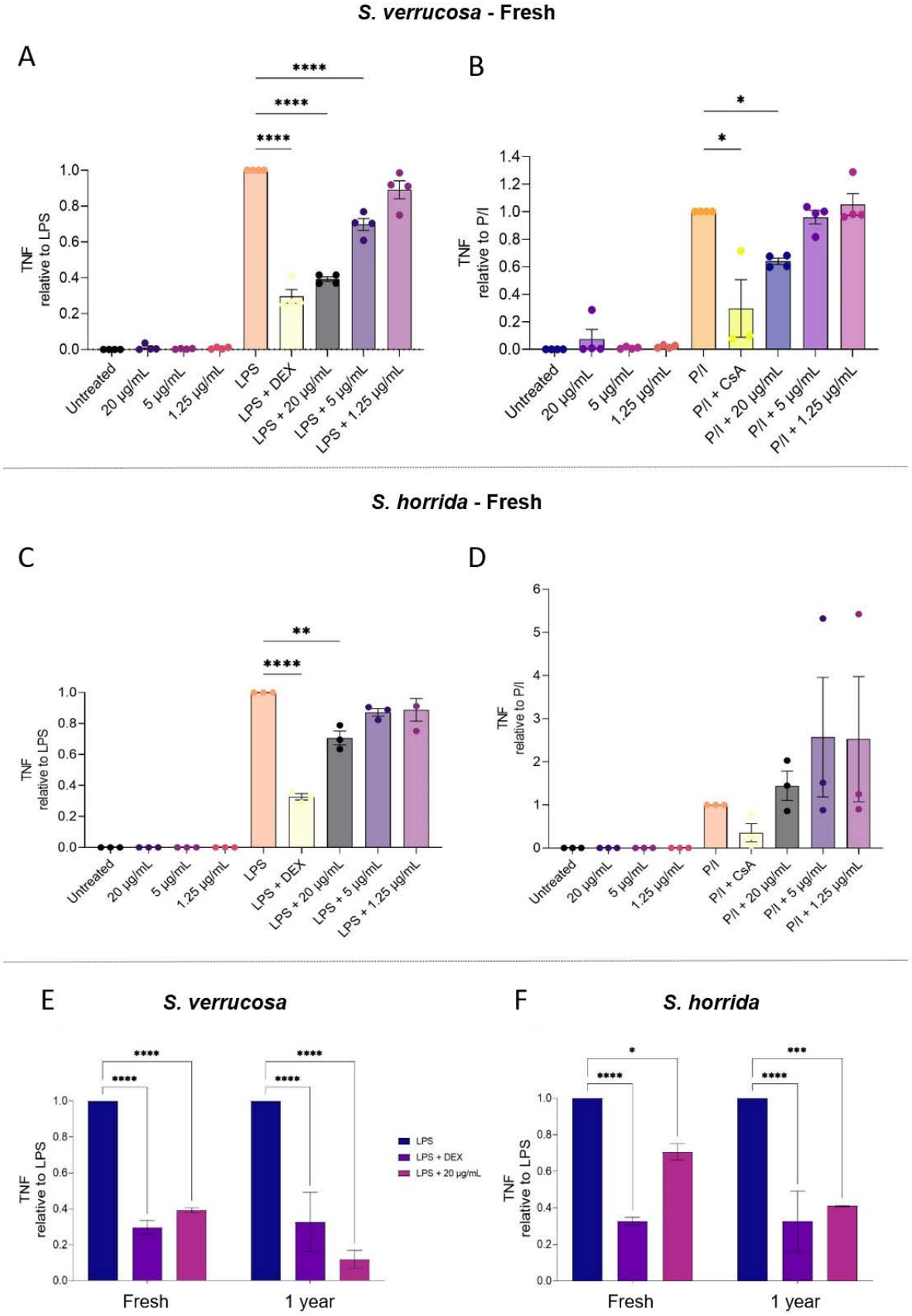

Figure 5.7: Stonefish venoms reduced TNF secretion levels from hPBMCs stimulated with either LPS or P/I. *Fresh SvV treatment effects on hPBMCs (n = 4 donors) stimulated with (A) LPS or (B) P/I; Fresh ShV treatment effects on hPBMCs (n = 3 donors) stimulated with (C) LPS or (D) P/I; (E) Comparison between fresh SvV (n = 4*

*donors) and SvV kept at -80°C for 1 year (n = 3 donors); (F) Comparison between fresh ShV and ShV kept at -80°C for 1 year (n = 3 donors). Histograms display untreated cells, cells treated with 20, 5 or 1.25 µg/mL of venom, cells treated with LPS or P/I (positive control for cytokine expression), cells treated with LPS + DEX or P/I + CsA (positive suppression control), and cells incubated with LPS or P/I + 20, 5 or 1.25 µg/mL of venom. Error bars indicate mean ± SEM. Results were quantified through one-way ANOVAs followed by multiple comparison tests or their equivalent non-parametric tests (Kruskal-Wallis followed by Dunn's tests). Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). ** $p < 0.05$, *** $p < 0.006$, **** $p < 0.009$, ***** $p < 0.0001$.

5.4 Discussion

The mechanisms associated with stonefish envenomation are poorly understood, and the effects on the immune system have not been well documented. Here we examined the immunological properties of two medically important stonefish species, *S. verrucosa* and *S. horrida*, using a range of assays including CBA, RT-qPCR and ELISAs. To the best of my knowledge, this is the first study to effectively identify immunological effects of *Synanceia* spp. venoms.

Synanceia spp. are reported in both experimental models and clinical reports to cause cardio-respiratory pathologies, which may lead to morbidity and mortality (Saggiomo et al., 2021). Both *S. verrucosa* and *S. horrida* venoms have been shown to possess cardiotoxic effects (Austin et al., 1961; Barnett et al., 2017), which could play a role in the cardiac pathologies upon envenomation. This cytotoxicity is caused by specific proteins found within the venoms that form pores in cellular membranes, called verrucotoxin and neoverrucotoxin from *S. verrucosa* venom, and stonustoxin from *S. horrida* venom (Ellisdon et al., 2015; Garnier et al., 1997; Wahsha et al., 2019). Interestingly, the cytotoxicity appears to show selectivity across cell types, as stonefish venom demonstrated weak toxicity towards HeLa cells (only at 420 µg/mL after 48 h of incubation) or human erythrocytes (Garnier et al., 1997; Khoo et al., 1992; Kreger, 1991), and the current results indicated that ShV is not cytotoxic to hPBMCs, even at concentrations of 100 µg/mL, and only at higher concentrations (80 µg/mL) for SvV.

To date, no research has been published differentiating stonefish species based on clinical presentation. As such, the extent to which venom effects differ regarding the clinical pathologies each species might produce remains unknown. In addition, although there is a consensus that stonefish venoms give rise to hypotension in experimental models (Church and Hodgson, 2002), there is divergence of opinions and results in the field, depending on the study design and the venom used. Based on my findings, comparing and contrasting venoms from species could be useful to distinguish pathologies and symptoms experienced by victims, ultimately improving treatment modalities and patient outcome.

5.4.1 Immunomodulatory effects of *S. verrucosa* and *S. horrida* venoms on cytokines and chemokines

Despite stonefish venoms causing acute inflammation in both clinical reports and experimental models, the venoms did not significantly enhance the levels of pro-inflammatory cytokines or chemokines secreted from hPBMCs at the concentrations used in the cell assays. This apparent discrepancy between the clinical symptoms and effects on cytokines could be related to the venom concentration used, as venom concentrations were selected to avoid cytotoxicity towards hPBMCs rather than based on relevant clinical doses. Previous research with *S. horrida* lyophilized venom showed that 18 mg (or 6 spines) would be sufficient to cause the death of a 60 kg human, given the LD_{50} in mice (Khoo et al. 1992). A review of the literature that included clinical reports of *Synanceia* spp. stings showed that patients survived after being stung by up to three spines (Saggiomo et al. 2021), and unfortunately the latest report of a death caused by a stonefish (*S. verrucosa*) did not include the number of puncture wounds seen in the patient (Maillaud et al. 2020). The highest dose used (20 µg/mL) in this study was around 0.024 µg of protein, which would probably be too low to cause serious deleterious effects in humans.

The incubation time for the assays of 16 h for the ELISA and CBA assays, and 6 h for the RT-qPCR assays may have also been insufficient for cells to release significant levels of cytokines. This is unlikely, since many cytokines are released early on in both envenomation models and clinical settings, precisely to help the body fight infections or envenomation events. For instance, levels of TNF peak within 2 h in plasma after endotoxin stimulation, facilitating the activation of other inflammatory mediators during the early stages of a clinical inflammatory event (Cruz et al., 2008; Jaffer et al., 2010). Moreover, TNF, IL-4, IL-6, IL-10 and IFN-γ are known to play important roles in the initial stages of the South American rattlesnake (*Crotalus durissus terrificus*) envenomation, peaking in mice sera from 15 min to 4 h post-envenomation (Cruz et al., 2008).

Although stonefish venom did not increase secretion levels of the various cytokines tested, these venoms were able to supress secretion levels of several cytokines. Initial screening of SvV measured some of the most important cytokines as well as chemokines that are involved in viral growth inhibition, are pro-inflammatory, and are derived from both lymphocytes and macrophages (Cohen and Cohen, 1996). This screening showed the potent immunological suppression potential found within stonefish venoms, as secretion of five different cytokines were significantly reduced by SvV, namely TNF, IFN-γ, MCP-1, IL-6 and IL-12. Following this initial screen at the protein level, an analysis was performed at the gene level through RT-qPCR to investigate both SvV and ShV immunosuppressive properties. For SvV, RT-qPCR assay results were consistent with CBA for TNF and IFN-γ, but the RT-qPCR results also showed down-regulation of IL-10, which was not apparent by CBA.

Additionally, the RT-qPCR also provided results for ShV immunosuppression activity, which were consistent between the two stonefish species. Interestingly, SvV downregulated mRNA expression levels of all cytokines to a greater extent than ShV in absolute terms, and SvV also caused significant downregulation of IFN-γ in LPS-stimulated cells, suggesting a possible difference in the immunological potential of these venoms.

Furthermore, the venoms did not show strong cytokine modulation at protein or gene levels in P/I-stimulated cells. The bias demonstrated by both SvV and ShV to suppress LPS-stimulated cytokine secretion levels, but not P/I-stimulated levels, suggests the venom's mechanism of action is through a T cell specific regulatory pathway. LPS-induced inflammation begins with stimulation of immune cell surface receptors, such as toll-like receptor 4 (TLR4), which leads to the recruitment of transcription factors such as NF-κB in the cellular nucleus, ultimately leading to the secretion of cytokines and chemokines (Heinbockel et al., 2018). Natterins, a new class of fish-derived proteins, sourced from the Brazilian toadfish *T. nattereri* were shown to possess anti-inflammatory activities through a TLR2-TLR4/MyD88 mediated signalling cascade in a mouse model of leukocyte-endothelium cell interaction (Ferreira et al., 2014). These fish proteins demonstrated potent inhibition of leukocyte interactions with the microvascular endothelium *in vivo*, which led to the down-regulation of leukocyte adhesion receptor expression, ultimately down-regulating inflammation (Ferreira et al., 2014). It is possible that stonefish venom could act in a similar manner; however, further research is required to precisely determine which receptors and/or pathways stonefish venom can modulate.

In addition, the majority of cytokines significantly suppressed by the venoms were Th1 cytokines (TNF, IFN-γ, IL-6 and IL-12), with the exception of IL-10 (Th2) and MCP-1. This indicates that certain T cell populations are being inhibited (Th1) more than others (Th2). Modulation of the relative role played by each T cell subset (Th1 or Th2) regulates the balance between immunopathologies, host protection and the severity of immunologic disorders (Del Prete, 1998). Of note, various diseases are mediated by Th1 cell subsets, such as multiple sclerosis and autoimmune thyroiditis (Del Prete, 1998). In addition, SvV showed potent inhibition of MCP-1 release compared to the positive control dexamethasone. MCP-1 prompts the initiation of distinct signaling cascades such as tyrosine kinase, cAMP, and mitogen-activated protein kinase (MAPK), which give rise to pathologies (reviewed by Singh et al. 2021). Overexpression of MCP-1 has been linked, either directly or indirectly, to pathologies such as multiple sclerosis, inflammatory bowel disease, several different types of cancers, rheumatoid arthritis, cardiovascular diseases, diabetes, and has been flagged as a marker of severity in COVID-19 patients (reviewed by Singh et al. 2021). Interestingly, in COVID-19 patients, high levels of MCP-1 seemed to activate Th1 cells, which might play a role in the bias showed by SvV in suppressing Th1 cytokine expression, although this can only be hypothesized at present. Precise

downregulation of this chemokine, therefore, might lead to optimized host protection and improved outcomes for patients suffering from the aforementioned pathologies. Further understanding and study of the molecules present in stonefish venoms might prove useful for the development of novel immunotherapeutic agents, especially for pathologies that are mediated by Th1 cell subsets and MCP-1, in particular.

5.4.2 Stability of stonefish venoms

The stonefish venom appeared to be stable based on maintenance of TNF suppression following storage at -20°C. Previous studies have indicated that fish venoms tend to be extremely labile, where changes in temperature, storage conditions, lyophilisation and repeated freeze-thaw cycles have significantly decreased venom activity (Church and Hodgson, 2000; Saunders and Tokes, 1961; Wiener, 1959). For instance, the pharmacological effects of lyophilized ShV could not be compared to fresh venom in cardiac *ex vivo* and anaesthetised models, because it was many times weaker, although qualitative similarities were seen between the two forms (Church and Hodgson, 2000). Although none of these studies investigated immunological effects of these venoms, and the different assays used might account for this inconsistency in bioactivity stability, it is likely that characterisation of the compounds involved in the various activities will be required to resolve this discrepancy between our studies and previous studies. Additionally, further investigation is required to establish the relationship between venom composition and immunosuppression. The experiments in the next chapter shed light into the compositional and immunological activities of these venoms.

5.5 Conclusion

In this chapter we revealed that both *S. verrucosa* and *S. horrida* have immunosuppressive properties on hPBMCs, particularly towards Th1 cell subsets stimulated with LPS. Both venoms strongly suppressed TNF and IL-10, where SvV also showed strong suppression of IL-12, MCP-1, IFN-γ and IL-6. Importantly, the immunosuppression was observed without cytotoxic effects towards the cells. This bias towards Th1 cell subsets and LPS suggests a specific regulatory pathway in these venoms, and the characterisation of the bioactive venom components might lead to the discovery of potential novel immunotherapeutic agents. Furthermore, the venom components responsible for suppressing TNF secretion levels from hPBMCs appear to be stable over time. Further characterisation of the venom components is given in the next chapter.

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Chapter 6

6 Molecular characterisation of *S. verrucosa* and *S. horrida* venoms and consequent immunomodulation effects in human PBMCs

6.1 Introduction

Venoms are generally composed of many different proteins, peptides, salts, and small molecules, which can act in synergy to produce toxic effects (Minutti-Zanella et al., 2021). Characterization of these molecules can provide insight into the ecology of the animals, envenomation treatment, but also inspiration for drug development with several examples of venom derived compounds being used as drugs or in pre-clinical or clinical development (Herzig et al., 2020; King, 2011; Wilson and Daly, 2018).

Despite the medical importance of stonefish, there is still much to be characterized in terms of venom composition. Several compounds have been identified, which include large proteinaceous toxins responsible for some of the lethal, haemolytic, neurotoxic, cardiotoxic, vasorelaxant, and inflammatory activities, such as verrucotoxin and stonustoxin (Church and Hodgson, 2002; Garnier et al., 1997; Ghadessy et al., 1996; Sivan, 2009), cardioleputin, a 46 kDa protein that causes chronotropic and inotropic effects (Abe et al., 1996), a 45 kDa lectin that causes agglutination in erythrocytes isolated from rabbits (Kato et al., 2016), a cytolysin (Kreger, 1991), and enzymes such as hyaluronidases, acetylcholinesterase, and phosphodiesterase have also been identified (Austin et al., 1965; Hopkins and Hodgson, 1998; Madokoro et al., 2011; Poh et al., 1992). In terms of small molecules, dopamine, norepinephrine, tryptophan, and negligible amounts of histamine have also been reported in stonefish venom (Garnier et al., 1996; Hopkins et al., 1994). However, a recent proteomic and transcriptomic analysis into venom compounds in venom from *S. horrida* also indicated the presence of numerous uncharacterised and unique toxins (Ziegman et al., 2019). The lack of understanding of venom composition and the bioactivity of these molecules highlights the need for further investigation into stonefish venoms.

This chapter explores components found within *S. verrucosa* and *S. horrida* venoms to further understand the biological immunosuppression activity observed in the previous chapter. This is accomplished using analytical techniques such as nuclear magnetic resonance spectroscopy (NMR), liquid chromatography-mass spectrometry (LCMS) and venom fractionation. Molecules that had never been reported in fish venoms were discovered and identified, and their respective immunological activities investigated. The implications of these findings are discussed.

6.2 Materials and methods

Venom collection and protein determination followed protocols described in Chapter 5.

6.2.1 Analytical assessment of crude stonefish venoms

6.2.1.1 SDS-PAGE gel

Frozen SvV and lyophilized ShV were used for this experiment, where frozen SvV was allowed to thaw in an ice bath, and lyophilized ShV was re-suspended with DPBS (ThermoFisher). Samples were kept in an ice bath until the end of the experiment. SDS-PAGE gel was run on venoms mixed with Laemmli sample buffer (ThermoFisher) (1:1) to give a final volume of 20 µL, added into individual tubes and heated at 95°C for 5 min. Gel, running buffer (NuPAGE Novex MOPS SDS, ThermoFisher), molecular weight standards (BioRad Laboratories), and replicated samples underwent electrophoresis at 150 V for approximately 60 min.

6.2.1.2 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy was used to observe the presence of small molecules in crude SvV and ShV. Crude venoms were pooled from multiple spines of each individual fish for analysis. In addition, seven individual spines from SvV were also analyzed to investigate any differences in venom composition between spines.

NMR data were obtained as previously reported following standard procedures (Wilson et al., 2017). Spectra were recorded at 290 and 298 K on a Bruker Avance III 600 MHz spectrometer (Bruker, Billerica, MA, USA) equipped with a cryoprobe. Crude venom samples were prepared in 90% H_2O/10% D_2O (*v*/*v*) (99.9%, Cambridge Isotope Labs), vortexed and centrifuged prior to transfer to an NMR tube (Wilmad, 5 mm). One-dimensional (1D) and two-dimensional (2D) spectra were collected using standard Bruker pulse programs and referenced to external 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS; Cambridge Isotope Laboratories). The 1D data included a cpmgpr1d experiment to supress the larger peptide and protein signals. The 2D spectra included TOCSY, NOESY, COSY, HMBC, ^{13}C-HSQC and ^{15}N-HSQC experiments with TOCSY and NOESY mixing times of 80 ms and 500 ms, respectively. Spectra of crude SvV were also collected in 100% MeOD (Cambridge Isotope Labs, methanol-d_4) to observe peaks otherwise obscured by the water suppression. Spectra were analysed with TopSpin v3.6.3 (Bruker, Billerica, MA, USA).

6.2.1.3 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS experiments were performed to further examine the molecular weights and venom compositions using standard procedures for LC-MS. Samples were mixed with LC-MS solvent A (H_2O / 0.1% formic acid (FA, Sigma-Aldrich) (1:2), centrifuged and the supernatant analysed using a Shimadzu

LCMS-2020 mass spectrometer coupled to a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan). Solvent B contained 90% acetonitrile (ACN, Sigma-Adrich)/10% H_2O/0.09% FA.

Crude venom samples were injected via an autosampler (Shimadzu SIL-20ACHT) onto a RP-HPLC column (Phenomenex Kinetex 5µ C8 100 Å 50 x 2.1 mm; Phenomenex, Torrence, CA, USA) kept at 35°C. Solvent was delivered by Shimadzu LC-20AD pumps at a flow rate of 0.250 mL/min, and the absorbance monitored at 214 nm and 280 nm (Shimadzu SPD-20A detector). Mass spectra were collected in positive and negative ion modes (scan range m/z 200–2000), with a detector voltage of 1.15 kV, and nebulizing and drying gas flows set to 1.5 L/min and 3.0 L/min, respectively. Crude venom samples were eluted with a 1% gradient (Solvent B: 0-80% 80 min; 60-90% 5 min; 90% 5 min; 90-0% 5 min; 0% 10 min). Data were then analysed with the Shimadzu LabSolutions v5.96 software (Shimadzu, Kyoto, Japan) and GraphPad Prism Ver 9.

6.2.2 Fractionation and partial purification of small molecules from *S. verrucosa* and *S. horrida* venoms

Venoms were fractionated using a range of chromatography techniques to investigate venom composition and to further analyze the immunosuppression observed in Chapter 5.

6.2.2.1 Reversed-phase high performance liquid chromatography (RP-HPLC)

Crude SvV was fractionated using RP-HPLC on an Agilent 1260 Infinity HPLC (Agilent Technologies, Hanover) and a Phenomenex Kinetex 5 µm, C8, 100 Å, 50 x 2.1 mm (Phenomenex, Torrence, CA, USA). Frozen SvV (20-30 µL) was mixed with 20 µL of solvent A (H_2O/0.05% trifluoroacetic acid (TFA, Auspep)) and centrifuged at 4°C for 10 min at 15,000 rpm (Mikro 200R, Hettich Zentrifugen, LabGear). The supernatant (~ 60 µL) was injected through an autosampler and run with a 0.5% gradient solvent B (90% ACN (Sigma-Aldrich)/10% H_2O/0.045% TFA) (0-60% B, 60 min; 60-90% B, 5 min; 90% B, 10 min; 90-0% B, 5 min) at a flow rate of 1 mL/min. The column oven was set to 35°C and the absorbance monitored at 214 and 280 nm.

Fractions were manually collected. Aliquots from each fraction were re-combined to investigate whether immunosuppression activity was lost after the venom was passed through a RP-HPLC column. The combined fractions were subsequently aliquoted, labelled, freeze-dried, and kept in -80°C until they were required.

6.2.2.2 Size-exclusion chromatography (SEC)

SEC was performed with both SvV and ShV using standard procedures with the aim of fractionating the protein components of the venom. Crude venom samples were fractionated using an Agilent 1260 Infinity HPLC (Agilent Technologies, Hanover) and a Phenomenex Yarra 3 µm SEC-2000, 300 x 7.8 mm column (Phenomenex, Torrence, CA, USA). Venoms were diluted (1:1) with DPBS and centrifuged prior

to loading. An isocratic gradient of DPBS at a flow rate of 0.8 mL/min for 60 min was used. Fractions were collected either manually in individual Eppendorf tubes or as 30 s fractions via the fraction collector into 1.1 mL 96-deep well plates (Axygen, Corning). SEC fractions were pooled from repeat experiments, freeze-dried, and kept in -80°C until they were required. SEC fractions were also re-combined, where equal volumes of each fraction were pooled to test the bioactivity (referred to as "pooled fractions" in the assays) following chromatography.

6.2.2.3 Solid-phase extraction (SPE)

Solid-phase extraction columns (Phenomenex Strata C18-U 55 µm 70 Å) were used to partially purify the small molecules found in both SvV and ShV. The column was first equilibrated twice with 1 mL 100% methanol and washed twice with 1 mL 100% H_2O prior to loading the venom samples. The column was then washed with 1 mL 5% methanol/H_2O and the partially purified small molecules were eluted with 100% methanol prior to freeze-drying, and later rehydrated and analysed using NMR spectroscopy.

6.2.3 Analytical assessment of fractions and partially purified small molecules

Individual SEC fractions from SvV and ShV were analysed with NMR spectroscopy and LC-MS. NMR spectra were recorded as previously described (Section 6.2.1.2), and LC-MS samples were run as described in Section 6.2.1.3, with the inclusion of a desalting step (0% B, 10 min) followed by a 1% gradient solvent B (0-60% B, 60 min; 60-90% B, 5 min; 90% B, 5 min; 90-0% B, 5 min) at 90-2000 m/z scan range in positive and negative modes. Data were then analysed with TopSpin NMR software (Bruker, Billerica, MA, USA), Shimadzu LabSolutions v5.96 software (Shimadzu, Kyoto, Japan) and GraphPad Prism Ver 9.

6.2.4 Bioactivity assessment of fractions and partially purified small molecules

6.2.4.1 Electrophysiology

The effects of SEC fractions from crude SvV on sodium channels were analysed following standard protocols previously described (Peigneur et al., 2019a, 2019b). Expression of Na_V channels ($rNa_V1.3$, $mNa_V1.6$, $hNa_V1.7$, $hNa_V1.8$ and $hNa_V1.9$_C4) in *Xenopus laevis* oocytes utilised T7 or SP6 mMESSAGE-mMACHINE transcription kits (Ambion). Oocytes were injected with 50 nL of cRNA (1 ng/mL) and incubated in ND96 solution with 96 mM NaCl, 1.8 mM $CaCl_2$, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH = 7.4), supplemented with 50 mg/L gentamycin sulphate. Recordings were achieved using a Geneclamp 500 amplifier (Molecular Devices, Sunnyvale, CA) and controlled by a pClamp data acquisition system (Axon Instruments, Foster City, CA), where bath solution was ND96. Voltage and current electrodes were then filled with 3 M KCl, where resistance was kept between 0.7 and 1.5 MΩ. Elicited currents were sampled at 20 kHz and filtered at 2 kHz with the use of a four-pole low-pass Bessel filter. Leak subtraction was performed using a-P/4 protocol, and currents were evoked by a 100

ms depolarization to the voltage corresponding to the maximal activation of the channels in control conditions from a holding potential of −90 mV. Data were obtained for three or more independent experiments.

6.2.4.2 Immunosuppression assays

Human blood, ethics, cellular experimental set up and cytotoxicity assays followed protocols described in Chapter 5.

6.2.4.2.1 Cytotoxicity assessment of stonefish venom components

Crude venom and fractions from SEC and SPE were reconstituted with DPBS, and the cytotoxicity tested at two different concentrations. Some fractions could not be tested due to insufficient sample amounts (fraction 1 and pooled fractions from SvV and fraction 4 and pooled fractions from ShV).

Cytotoxicity was investigated by exposing hPBMCs to each treatment at different concentrations for up to 24 h (CellTox Green, Promega). Treatment conditions were performed in triplicate, plated in a white flat-bottom 96-well plate (Costar), with fluorescence measured on a FLUOStar Omega (BMG Labtech) plate reader (600 gain, bottom optic).

6.2.4.2.2 Immunological assessment of venom components on TNF secretion from LPS-stimulated hPBMCs

6.2.4.2.2.1 ELISA

ELISAs were performed to assess whether venom components suppressed TNF secretion levels from LPS-stimulated hPBMCs. Experiments were run with the same samples from the cytotoxicity assay. Human PBMCs (n = 3 donors) were seeded at 100,000 per well and incubated overnight for 16 h either alone (negative control), with each SEC and SPE fraction, with each SEC and SPE fraction + LPS (10 ng/mL), or with LPS (positive control). Controls for suppression were incubated with LPS + dexamethasone (10 µg/mL). All treatment conditions were performed in triplicate, with final dilutions of 1:10 in R10 medium, except for the suppression controls, which were plated in duplicate. Supernatants were then transferred into an ELISA plate, where the assay was developed as per Chapter 5 (Section 5.2.6.2.4).

6.2.4.2.2.2 RT-qPCR

To confirm the results observed with ELISAs (at the protein level), RT-qPCR was performed (at the gene level) using the same samples and donors from the cytotoxicity and ELISA assays. The materials and methods used for this assay were described in Chapter 5 (Section 5.2.6.2.3, Appendix D), which followed standard protocols (Browne et al., 2020) and recommended guidelines by the manufacturer. Both venoms showed potent suppression activity at the highest concentration tested (20 µg/mL) in previous ELISAs (see Chapter 5), so only this concentration was used for this assay. Human PBMCs were stimulated for 6 h and seeded at 100,000 cells per well.

6.3 Results

Venom samples from *S. verrucosa* and *S. horrida* were investigated using SDS-PAGE, RP-HPLC, SEC, SPE, LC-MS and NMR spectroscopy to provide insight into their molecular composition. Activity profiles of samples were assessed through cytotoxicity, electrophysiology, and cell-based immunoassays using ELISA and RT-qPCR protocols.

6.3.1 Molecular analysis of crude stonefish venoms

Analysis of the stonefish venoms using SDS-PAGE gel electrophoresis indicated that the venom is dominated by components with a molecular weight of approximately 75 kDa (Figure 6.1). There were also several proteins with molecular weights between 10-15 kDa. Inter-species differences were evident, with some components at around 250 kDa present in ShV, but not in SvV, whereas SvV had darker bands around 17 kDa and 47 kDa (Figure 6.1).

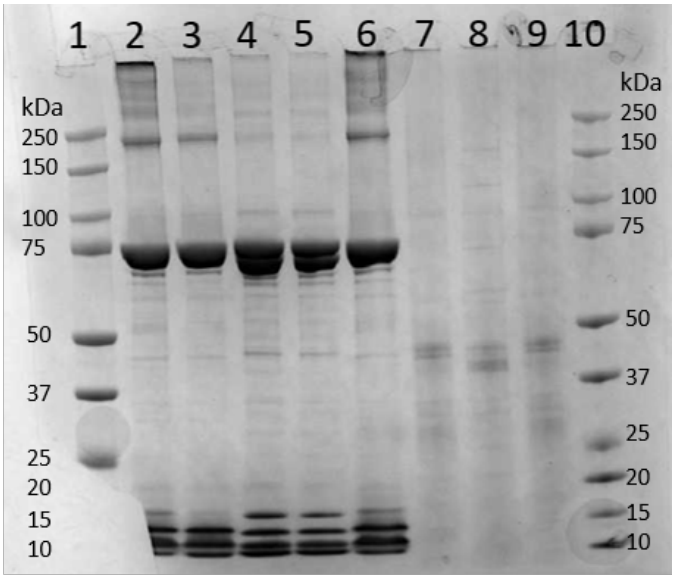

Figure 6.1: SDS-PAGE gel of stonefish venoms. *Molecular weight standards are in lanes 1 and 10 with respective sizes in kDa highlighted. ShV is in lanes 2, 3 and 6 and SvV is in lanes 4 and 5.*

LC-MS analyses were carried out to investigate the molecular weights of the various components found in the venoms. Representative LC-MS total ion current (TIC) and $UV_{280\ nm}$ chromatograms of the SvV and ShV samples are shown in Figure 6.2. Crude venoms contained masses between 12,069 and 17,376 Da, consistent with the SDS-PAGE data, and also contained several small molecules ranging in molecular weight from 103 to 564 Da (Appendix E).

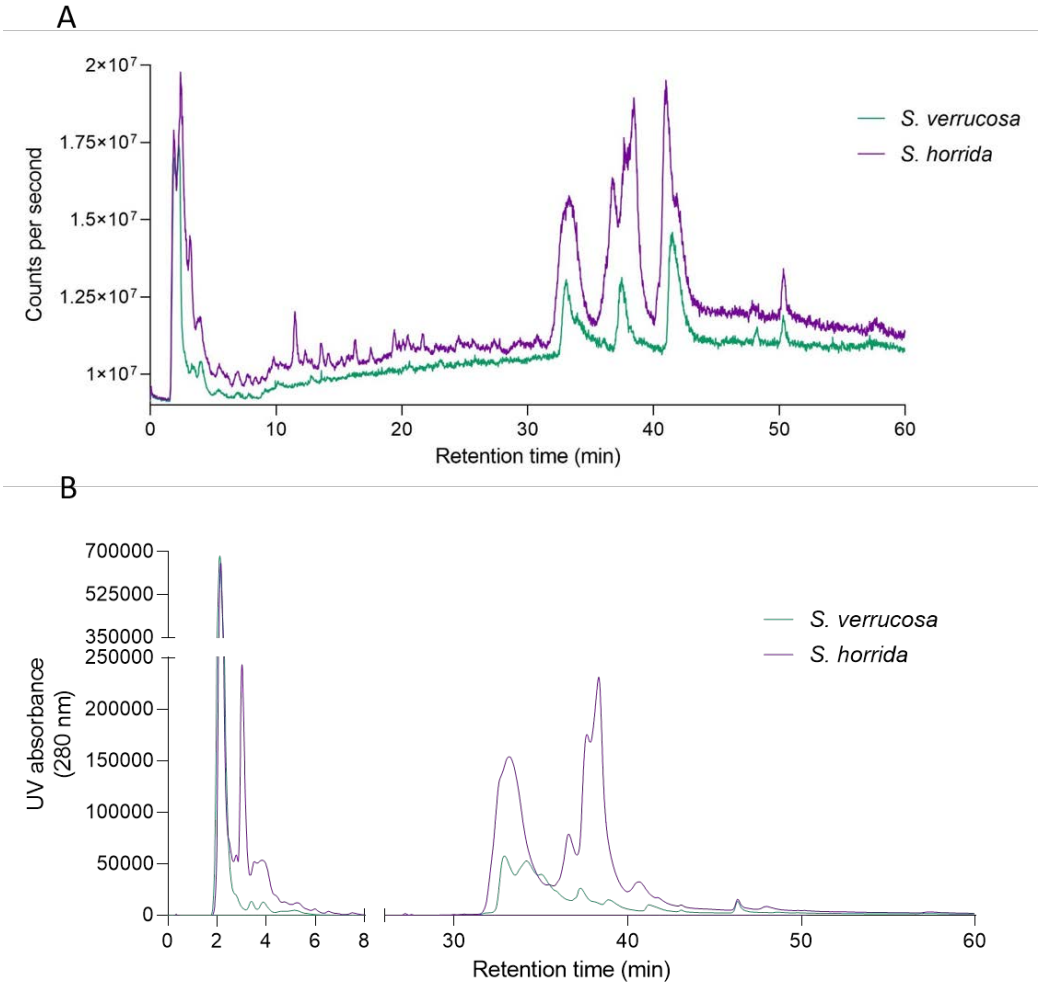

Figure 6.2: Liquid chromatography-mass spectrometry TIC and $UV_{280\ nm}$ chromatograms of* S. verrucosa *and* S. horrida *venoms. *(A) TIC counts per second and (B) UV absorbance. LC-MS chromatograms run on a Phenomenex Kinetex (5µ, C8, 100 Å, 50 x 2.1 mm) column.*

NMR analysis of crude venoms highlighted the presence of several small molecules (MW 103-564 Da), consistent with the LC-MS data. Identification of the predominant small molecules within the venoms was possible based on characteristic chemical shifts and sharp peaks in the 1D spectra, and interactions observed in the 2D spectra, but some less abundant components remain unidentified in both venoms.

Crude ShV NMR analysis showed a more complex ^{1}H NMR spectrum compared to SvV. Norepinephrine and gamma-aminobutyric acid were identified in both venoms (Figure 6.3), whereas dopamine (Figure 3B), choline and *O*-acetylcholine were only present in ShV (Figure 6.4). The presence of these molecules was confirmed by the presence of their molecular weights in the LC-MS data (Figure 6.5 and Appendix E). NMR analysis of the individual spines from SvV did not show any differences in small molecule composition between each spine.

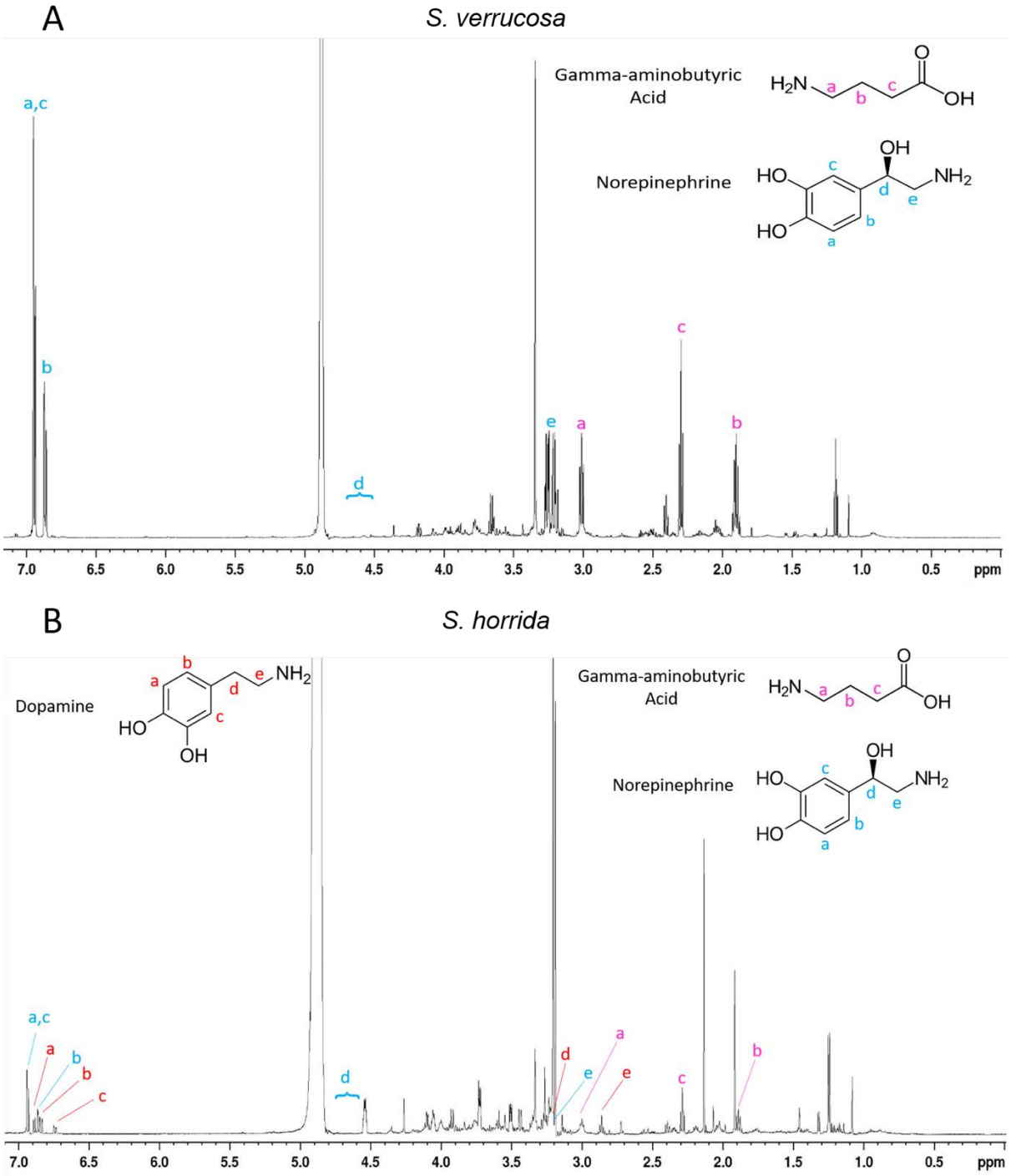

Figure 6.3: One-dimensional NMR spectra of stonefish venoms*. (A)* S. verrucosa *and (B)* S. horrida *frozen crude venoms showing the presence and chemical structures of norepinephrine, gamma-aminobutyric acid, and dopamine. Peak "d" for norepinephrine at around 4.6 ppm is not evident due to water suppression, but its presence was confirmed by running the SvV sample in methanol-d_4. The assignments were derived based on ^{1}H NMR spectra recorded at 600 MHz.*

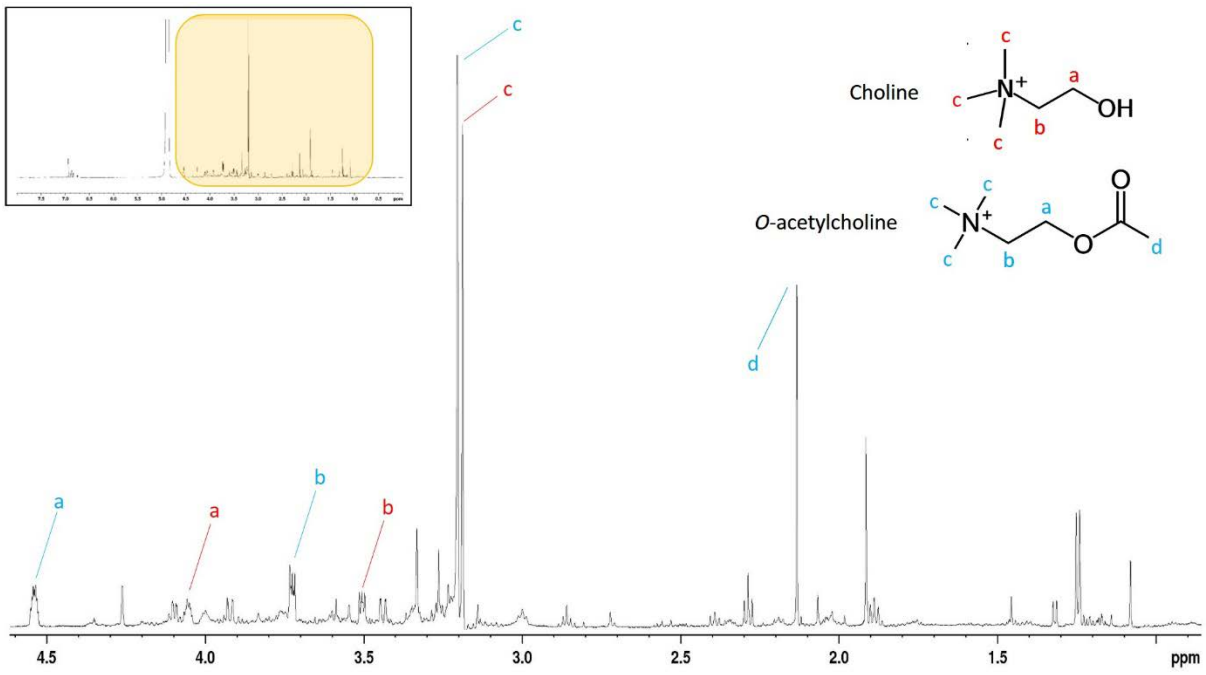

Figure 6.4: One-dimensional NMR spectra of additional small molecules from* S. horrida *venom*. Yellow highlighted area of inset shows from which portion the spectrum was taken. Choline and O-acetylcholine were only found in samples from ShV. The assignments were derived based on ^{1}H NMR spectrum recorded at 600 MHz.***

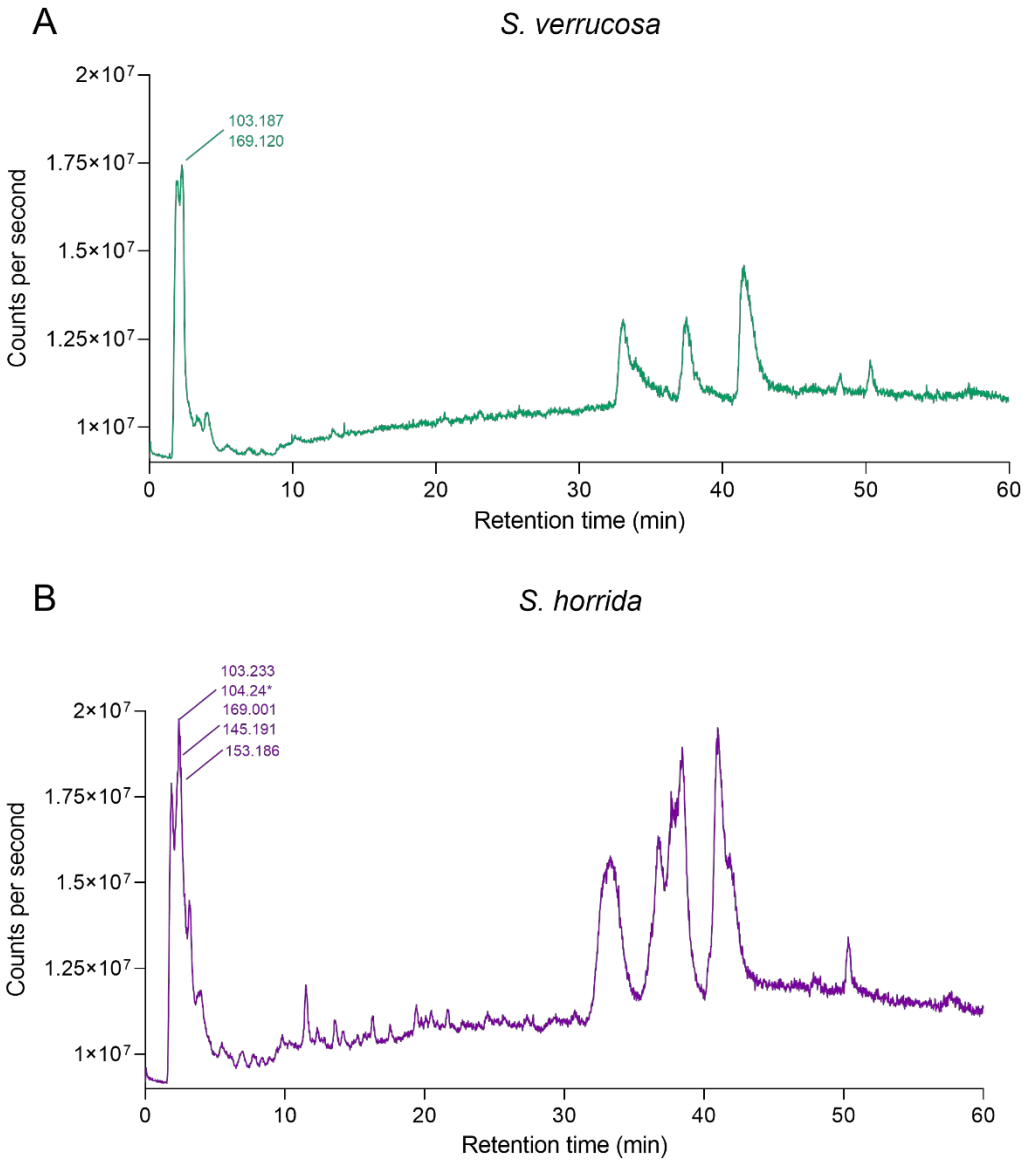

Figure 6.5: TIC counts per second liquid chromatography-mass spectrometry chromatograms of* S. verrucosa *and* S. horrida *venoms in positive ion mode. *(A)* S. verrucosa *venom: The molecular weights of gamma-aminobutyric acid and norepinephrine have been emphasized at 2.3 min (103.187 and 169.120 Da, respectively), and (B)* S. horrida *venom: gamma-aminobutyric acid and choline have been emphasized at 1.7 min (103.233 Da and 104.24 m/z* for choline), O-acetylcholine at 2.4 min (145.191 Da), dopamine at 3.2 min (153.186 Da), and norepinephrine at 2.3 min. LC-MS chromatograms run on a Phenomenex Kinetex (5µ, C8, 100 Å, 50 x 2.1 mm) column.*

6.3.2 Fractionation of stonefish venom

To provide insight into the molecules contributing to the bioactivity of stonefish venom (Chapter 5), crude SvV and ShV were fractionated, and the TNF suppressive effects of the fractions analysed. Preliminary experiments were carried out to determine whether small molecules, peptides or proteins were likely to be responsible for the cytokine suppression effects of stonefish venom. Crude fresh SvV and samples from RP-HPLC (i.e. re-combined fractions eluted from a RP-HPLC column) were tested and compared. The re-combined fractions from RP-HPLC did not suppress TNF secretion levels from

hPBMCs treated with LPS, independently of the concentration tested (Figure 6.6). The fresh venom maintained a similar level of suppression to that observed in previous experiments. Overall, it appeared that fractions collected from RP-HPLC lost their suppression activity, therefore, RP-HPLC fractionation was not applied to ShV.

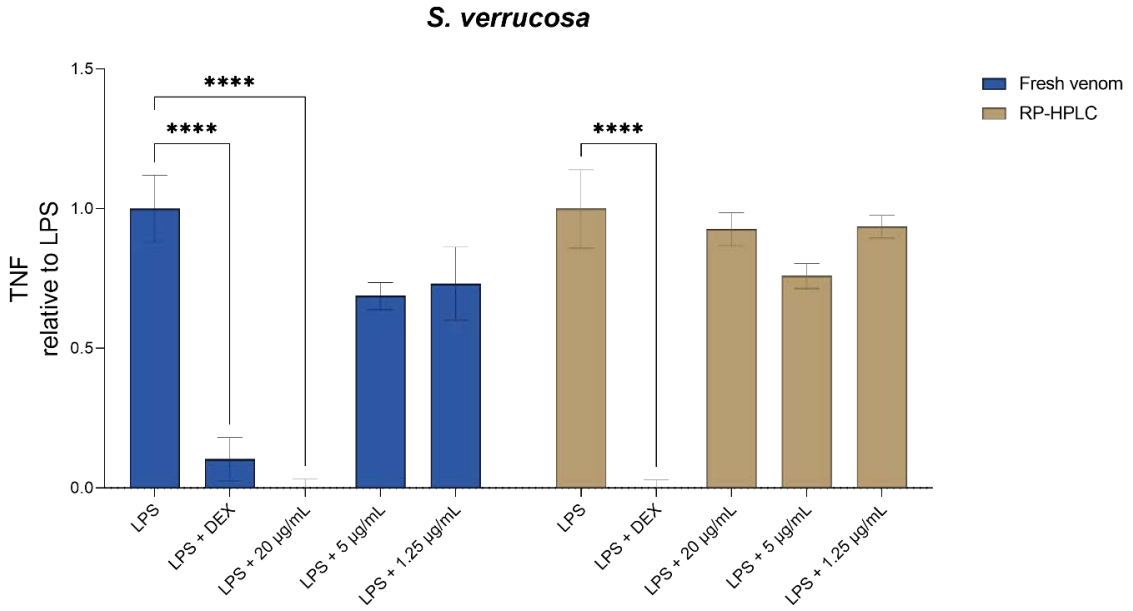

Figure 6.6: Venom eluted from a RP-HPLC column changed the suppression properties of SvV in LPS-stimulated hPBMCs. *Histograms display cells treated with LPS, LPS + DEX, and cells incubated with LPS + 20, 5 or 1.25 µg/mL of SvV or RP-HPLC re-combined fractions. Error bars are mean ± SEM (n = 1 donor). Statistical differences were quantified with a two-way ANOVA followed by Dunnett's multiple comparison tests. ***** p *< 0.0001.*

Crude venoms were fractionated with SEC in DPBS to mimic physiological conditions and to separate venom components by size. The profiles were similar for the two stonefish species as shown in Figure 6.7. SvV was separated into five different fractions (Figure 6.7A), whereas ShV was separated into four fractions (Figure 6.7B). In addition, SPE was used to crudely fractionate the small molecules from the small proteins and salt of the crude venom samples.

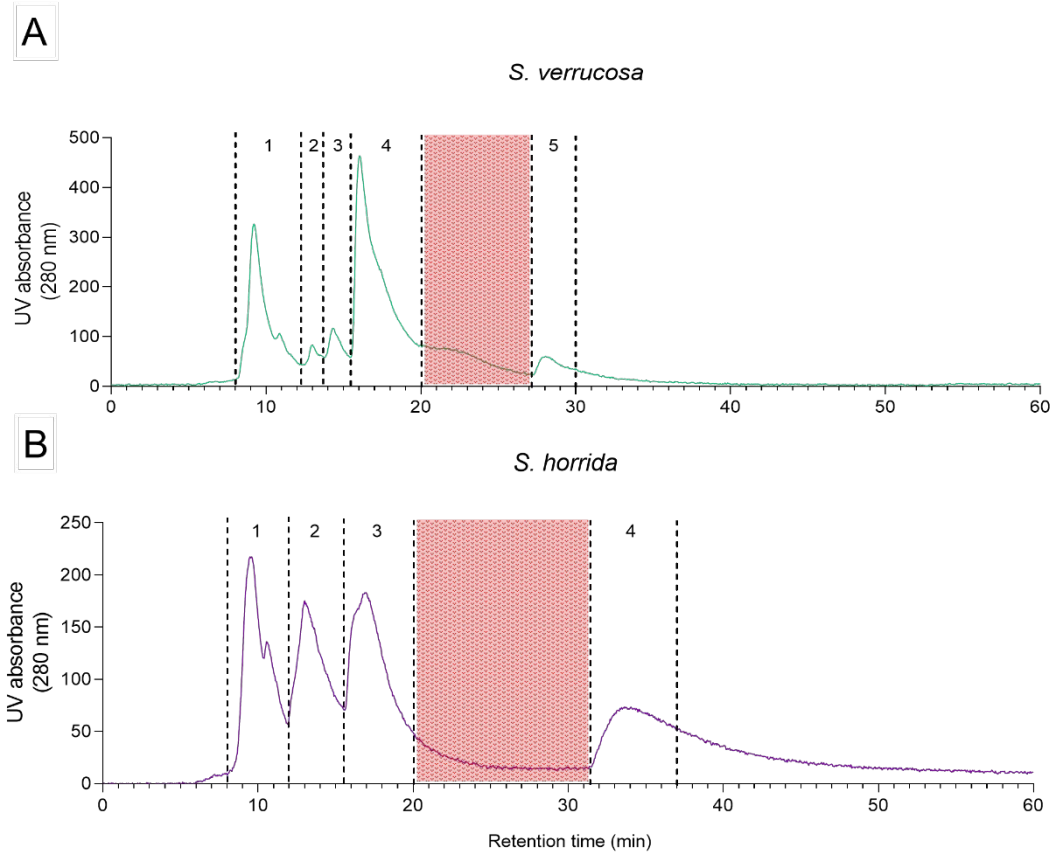

Figure 6.7: Size-exclusion chromatography of crude stonefish venoms. *(A)* S. verrucosa *and (B)* S. horrida *venom SEC chromatograms at 280 nm (Phenomenex Yarra 3 µm SEC-2000 300 x 7.8 mm column). Individual fractions are numbered. Shaded areas were only incorporated in the re-combined sample of "pooled fractions" for immunological assays. Fraction collection stopped at the last vertical dotted lines.*

LC-MS and NMR analyses were used to investigate the composition of the individual SEC fractions of SvV and ShV, and the SPE column fraction. LC-MS data revealed that SEC fractions primarily contained small proteins around 11,892 to 17,375 Da (Appendix E). The detection of small molecules with poor RP-HPLC column retention was not possible by LC-MS due to the desalting step at the beginning of the LC-MS method to remove the DPBS.

NMR and LC-MS analysis of the five SEC fractions from SvV showed that fraction 1 was mainly composed of a small protein of 13,960 Da (Appendix E). Gamma-aminobutyric acid was found in fraction 2 together with the 13,960 Da protein. Norepinephrine was found in fraction 3 with a 17,375 Da small protein (Appendix E). Norepinephrine was also present in fraction 4, which also contained three other major small proteins (13,232 Da, 13,260 Da and 12,365 Da). Fraction 5 was mainly composed of the 12,365 Da small protein (Appendix E).

Analysis of ShV SEC fractions revealed that fraction 1 was mainly composed of a 14,007 Da small protein, which was also detectable in all fractions in lesser amounts (Appendix E). Gamma-aminobutyric acid was observed in fraction 2 with two other major small proteins (11,892 Da and

11,920 Da). These two small proteins were also the main constituents of fraction 3 along with norepinephrine, dopamine, choline, and *O*-acetylcholine. Fraction 4 contained a 12,068 Da small protein, another molecule of 13,776 Da and detectable levels of the 14,007 Da protein. Other small molecules or proteins remain to be identified in both venoms.

NMR and LC-MS analyses of the SPE fraction showed a less complex venom composition for SvV compared to ShV. Therefore, only the SPE small molecule fraction from SvV was used for further cytotoxic and immunological analysis. Further analysis of the SPE fraction NMR spectrum indicated the presence of norepinephrine and GABA.

6.3.2.1 Bioactivity assessment of stonefish venoms

Analysis of the cytotoxicity of the SEC and SPE fractions was performed using a fluorescence-based kit. Consistent with the lack of cytotoxicity for the crude venoms, SEC fractions and SvV small molecules were also not significantly cytotoxic towards hPBMCs at the concentrations tested (Figure 6.8).

Further biological activity of the stonefish venom components was assessed after ensuring fractions were not toxic to hPBMCs. Due to the significant pain experienced in clinical symptoms of stonefish envenomation, SEC fractions from SvV were tested on voltage-gated sodium channels associated with pain transmission (De Lera Ruiz and Kraus, 2015). Electrophysiology assays of the SvV SEC fractions on rNa_V1.3, mNa_V1.6, hNa_V1.7, hNa_V1.8 and hNa_V1.9_C4 expressed in *Xenopus laevis* oocytes did not show any activity.

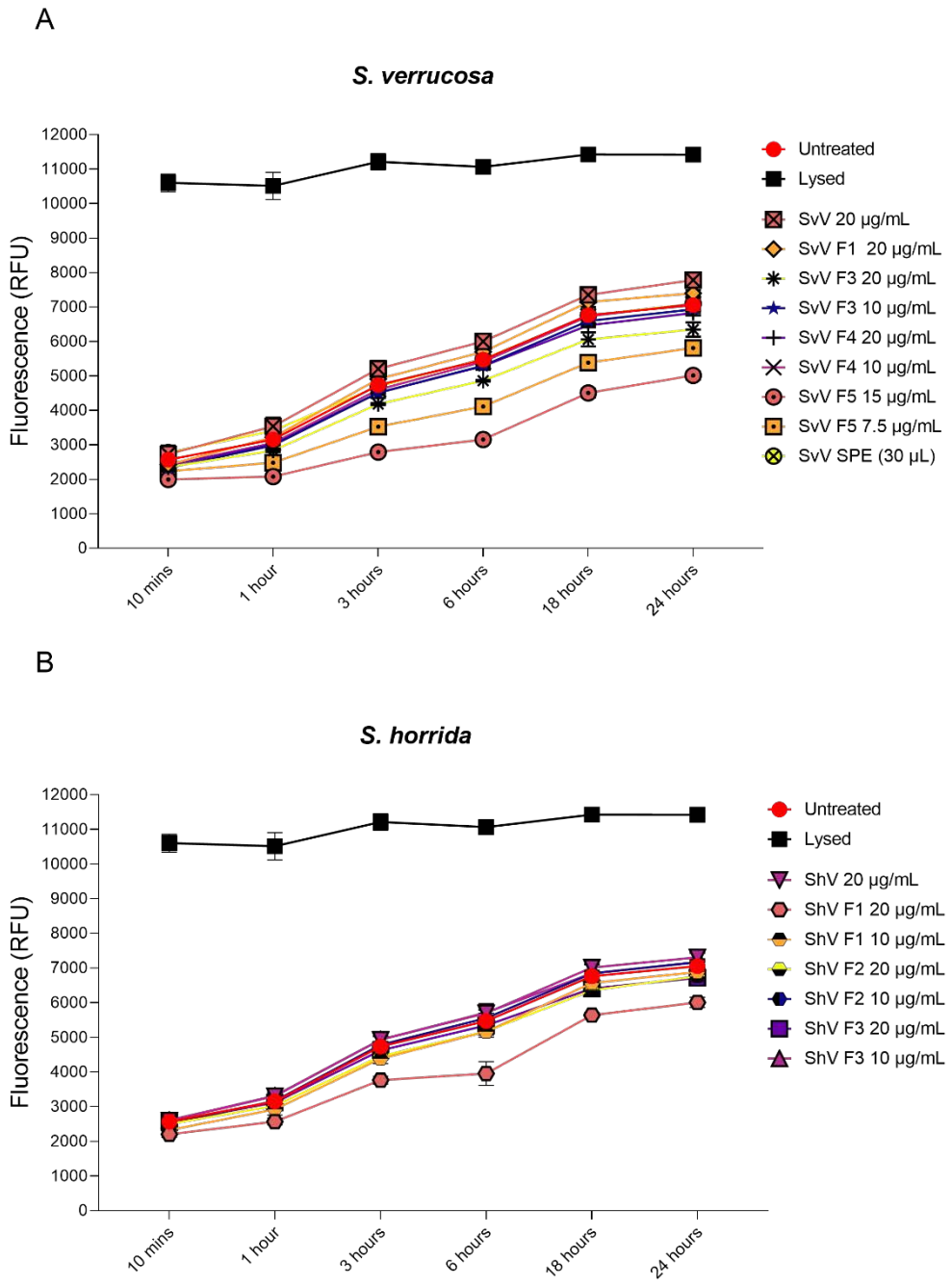

Figure 6.8: Cellular toxicity of crude stonefish venoms on hPBMCs, their SEC and SPE fractions. *Results are the cellular response of human PBMCs to either (A) crude* S. verrucosa*, SEC or SPE fractions (B) crude* S. horrida *venoms or SEC fractions. Concentrations of each fraction tested are given, but not all fractions were tested due to insufficient sample availability. Data shown as mean fluorescence ± SD (n = 1 donor). A two-way ANOVA showed a significant effect (*p *< 0.0001), where Dunnett's multiple comparison tests showed that these were between untreated cells and SvV Fractions 3 (20 µg/mL) and 5 (both 15 and 7.5 µg/mL), and ShV Fraction 1 (20 µg/mL). Lines illustrate time course of cellular toxicity measured for 24 hours according to fluorescence (RFU) levels. Untreated cells are negative controls; lysed cells are positive controls; venom treatments are shown in different colors.*

To test the immunological effects of the SEC and SPE fractions, ELISAs were performed on hPBMCs stimulated with LPS (Figure 6.9). Fractions 1, 2, 5 and small molecules (from the SPE fraction) from SvV

significantly suppressed TNF secretion levels by 97.5, 45.2, 85.3 and 45.9%, respectively, whereas DEX significantly reduced them by 53.7% (Figure 6.9A). Similarly, for ShV, fractions 1, 4 and pooled fractions significantly reduced TNF secretion levels by 92.6%, 98.4% and 84.6%, respectively, whereas DEX significantly reduced them by 48.9% (Figure 6.9B).

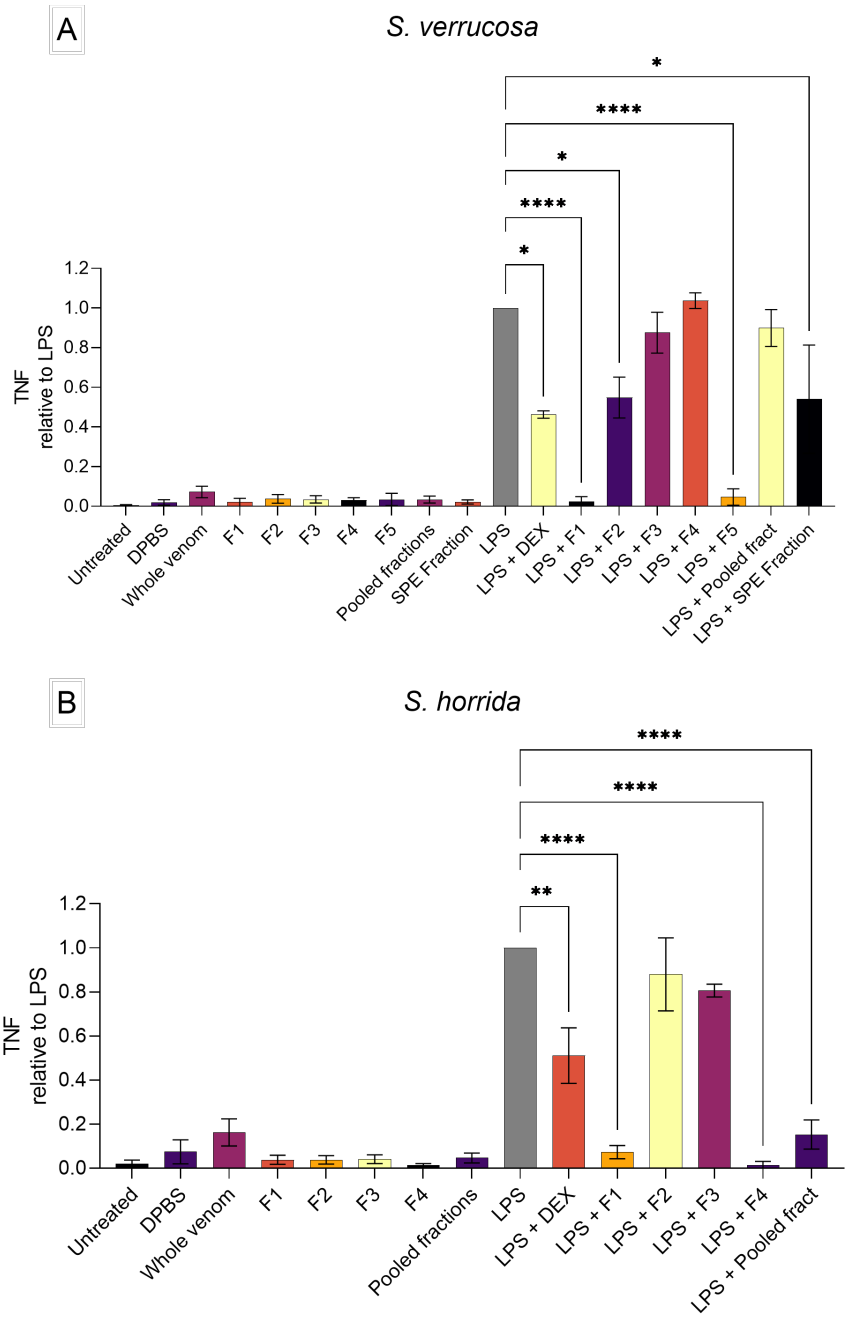

Figure 6.9: Identification of immunosuppressive components from stonefish venoms at the protein level. *ELISAs performed to observe the suppression effect of SEC fractions and the small molecules from the SPE fraction on TNF secretion levels from hPBMCs post LPS stimulation. Bars display untreated cells, cells envenomed with crude venoms, their respective SEC fractions, small molecules from SPE fraction from SvV, cells and LPS, cells and LPS + DEX, and cells incubated with LPS and the various treatments. Error bars indicate mean ± SEM (n = 3 donors). (A) One-way ANOVA showed that SvV Fractions 1, 2, 5 and small molecules from SPE fraction showed significant suppression effects; (B) For ShV, one-way ANOVA showed that Fractions 1, 4 and pooled fractions showed*

*significant suppression effects. Additionally, whole venom showed statistically significant expression of TNF levels compared to untreated cells (*p *< 0.0001). ** p *< 0.05, *** p *< 0.006, **** p *< 0.009, ***** p *< 0.0001.*

To compare the immunosuppression observed in the ELISA assays with the cytokine expression at the gene level, RT-qPCR was performed (Figure 6.10). Similar results to the ELISAs were seen, albeit with less significance in the RT-qPCR. TNF gene expression levels mediated by venom were significantly downregulated by SvV SEC fraction 5 and ShV SEC fraction 4 (Figure 6.10A and 6.10B, respectively). These significant results were consistent with the ELISA results for these two fractions. Although fraction 1 from SvV did not significantly down-regulate gene expression, a sharp decrease in the regulation of TNF secretion levels was observed (Figure 6.10A).

Both the ELISA and RT-qPCR results highlighted it was possible to separate out bioactive components using SEC and SPE. Furthermore, it appears that only the last fraction of each venom, which primarily contained a 12,365 Da in SvV and a 12,068 Da in ShV, supressed TNF at both protein and gene levels.

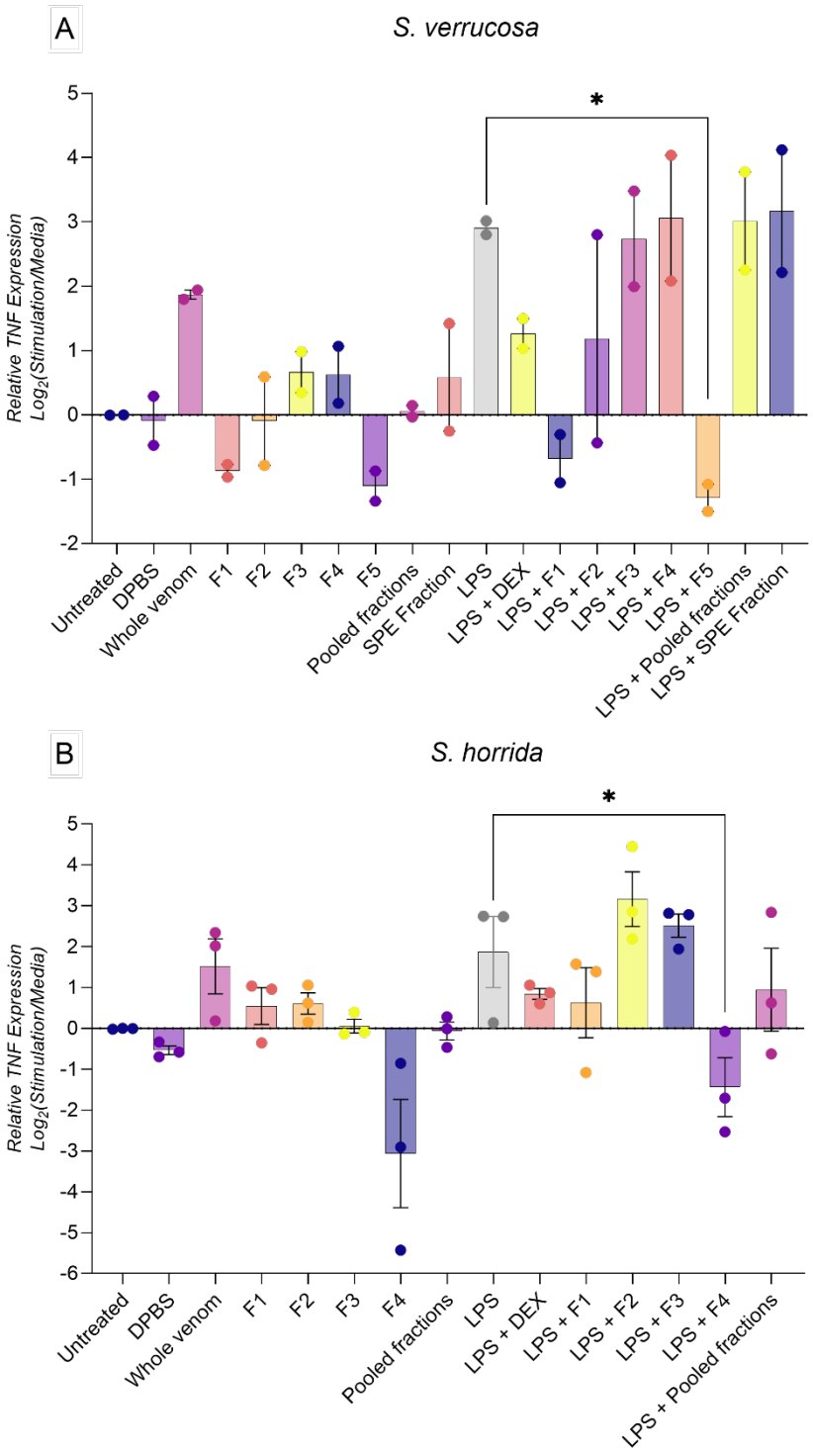

Figure 6.10: Identification of immunosuppressive components from stonefish venoms at the gene level. *RT-qPCR assay performed to observe the suppression effect of SEC fractions and the small molecules from the SPE fraction on TNF secretion levels from hPBMCs post LPS stimulation. Error bars indicate mean ± SEM (A: n = 2, B: n = 3). (A) One-way ANOVA showed that only SvV Fraction 5 had a significant suppression effect; (B) For ShV, one-way ANOVA showed that only Fraction 4 had a significant suppression effect. ** $p < 0.02$.

6.4 Discussion

Analysis of stonefish venom using a range of analytical techniques indicated the presence of several proteins, as well as small molecules not previously identified. Fractionation of the venom indicated that proteins are involved with the immunomodulatory activity observed for these venoms, but the roles of the small molecules remain to be elucidated.

SDS-PAGE analysis of the stonefish venoms was consistent with previous proteomics studies. For instance, the protein bands at around 75 kDa are consistent with the size of the toxic and lethal α- and ß- subunits found within both *S. verrucosa* (Garnier et al., 1997; Ueda et al., 2006) and *S. horrida* (Ghadessy et al., 1996) venoms. Furthermore, bands around 46 kDa are consistent with cardioleputin and a 45 kDa lectin found in SvV (Abe et al., 1996; Kato et al., 2016), and bands around 9 to 17 kDa are also consistent with various lectin and protein sequences found in the proteome of ShV (Ziegman et al., 2019). Additionally, the LC-MS analysis in the present study supported the results of the SDS-PAGE gel for both species, where various components ranged from around 11 to 17 kDa (Appendix E).

6.4.1 Small molecules in stonefish venoms

This chapter elucidated some of the small molecules present in stonefish venoms, which are often overlooked. Peptides tend to be the focal points in studies for drug discovery purposes due to their selectivity, high potency, and stability (Bajaj and Han, 2019; Herzig et al., 2020; Sivan, 2009). Small molecules, however, have some advantages over their peptide counterparts, such as being less complex, less resource-intensive to be synthesized and are often orally bioavailable, all of which facilitate the drug discovery and development pipelines (Shultz, 2019). Technological advances, such as the use of NMR, have made it possible to observe the presence of small molecules in complex mixtures of proteins as well as the large molecular size difference between the small molecules and proteins. NMR allows for the protein signal to be suppressed, so the small molecule signals are evident as prominent sharp peaks, enabling their discovery. Therefore, the use of NMR in this regard is novel, and provides a pathway to observe the molecular diversity and identify novel compounds in venoms (Daly and Wilson, 2018), and it is encouraged.

Here we show that both *S. verrucosa* and *S. horrida* contain gamma-aminobutyric acid (GABA) in their venom. In addition, ShV also contained choline and acetylcholine (ACh). None of these small molecules have previously been identified in these venoms. Previously, NE, DA, tryptophan (Trp) and histamine were reported in stonefish venoms (Garnier et al., 1996; Hopkins et al., 1994). In this chapter, the presence of NE was confirmed in the venoms from both stonefish species, but Trp and histamine were not evident, and DA was only evident in ShV.

Previously in SvV, NE was reported to be by far the most dominant molecule, followed by Trp and DA, respectively (Garnier et al., 1996). In this chapter, NMR spectroscopy analysis of crude venom indicated that NE and GABA are found in approximately the same quantities (~ 1:1). In ShV, DA was previously reported as the most dominant molecule, followed by NE and finally Trp (Garnier et al., 1996). NMR analysis in this chapter indicated that NE, GABA, and DA are present in similar quantities (~ 1:1:1). This discrepancy in venom components could be a result of several factors, including the different techniques and technologies used in the previous study (Garnier et al., 1996), and geographical and individual variation.

These molecules, apart from being novel in stonefish venoms, have been previously reported in the venoms of other creatures. NE and DA have been previously found in arthropod venoms such as bee, wasp, spider, and scorpion (Banks et al., 1976; Frew et al., 1994; Gwee et al., 1993; Owen, 1971), and NE has also been reported in the weeverfish *Trachinus draco* (Haavaldsen and Fonnum, 1963). GABA has been found primarily in the venom of spiders and later reported in snakes and the spider wasp (Hisada et al., 2005; Lange et al., 1992; Savel-Niemann and Roth, 1989; Schanbacher et al., 1973; Villar-Briones and Aird, 2018). The presence of ACh has been previously reported in the venom of the lionfish *Pterois volitans* (Cohen and Olek, 1989) and in the hornet *Vespa crabro* (Habermann, 1972). As different molecules may play different roles within venoms (Daly and Wilson, 2018; Villar-Briones and Aird, 2018; Weisel-Eichler and Libersat, 2004), this dissertation focused on the immunomodulatory activities these small molecules might play on LPS-stimulated hPBMCs and also hypothesized on the biological roles they might play upon envenomation.

It was noted that the molecules separated by SEC eluted in an unusual manner. SEC is a mild chromatography technique that separates molecules according to their size, where larger molecules have shorter retention times and elute earlier, and salts, small molecules and other components are eluted later (Mori and Barth, 2010). However, the results presented in this chapter showed larger molecules eluting later than smaller molecules. The SEC column used has a reported resolution range of 1 kDa-300 kDa and the size range of the venom components separated are skewed heavily towards the lower end of the range which might have contributed to the results observed. In addition, mobility in SEC relies on the hydrodynamic size of the molecules (a function of mass and shape) (Bowman et al., 2013), with the general assumption that the sample molecules all share similar hydrodynamic properties. Linear or elongated structures will behave differently to tightly folded, compact molecules. Variation in hydrodynamic properties between the venom component molecules might explain the results observed. The presence of small molecules in early eluting fractions with larger proteins might result from binding interactions between the small molecules and the proteins. The SEC experiments were repeated several times and provided consistent and reproducible results.

6.4.2 Immunomodulation of stonefish venom components

Given the diversity of components in stonefish venom, it was of interest to fractionate the venom to provide insight into bioactive components. The previous chapter examined immunomodulation in both crude SvV and ShV. Further immunological assessment of both venoms indicated that some fractions demonstrated potent immunosuppression effects of TNF secretion levels, whose significance is explained below for each fish.

6.4.2.1 S. verrucosa

Fractions 1 and 5 had the most potent immunosuppression activity of TNF secretion levels. LC-MS data revealed that a protein of around 13,960 Da was one of the main molecules found in fraction 1, and fraction 5 had a protein of 12,365 Da as its main constituent. These two molecules could potentially be the sole drivers of the strong suppression observed for each fraction, and further emphasis should be placed on the 12,365 Da molecule, as it demonstrated suppression at both the protein and gene levels. Fraction 2 also had significant effects on TNF suppression, with the 13,960 Da molecule as one of its major constituents, as well as a 17,375 Da protein, and GABA. It is possible that the 13,960 Da found in fraction 1 might aid in the weaker immunosuppression observed for fraction 2.

Furthermore, the immunological results acquired after the venom had been subjected to the RP-HPLC conditions has implications for the type of molecule involved in the bioactivity. The RP-HPLC conditions seemed to significantly affect the ability of the venom to suppress TNF levels by completely inhibiting the activity. As small molecules and peptides are generally stable under the conditions used for the fractionation, it appears likely that the major bioactive constituents are proteins. The active proteins were likely denatured by the low pH (~ 2.1) of the HPLC buffers and/or organic solvent (acetonitrile) presence or precipitated prior to loading the sample.

Accordingly, analysis of the ELISA and RT-qPCR results showed the small molecules are not responsible for the strong suppression seen in SvV. The partially purified small molecules did demonstrate significant levels of TNF suppression, but it was weaker compared to fractions 1 and 5. GABA could, however, still play a role in some of the suppression activity observed in fractions 2 and the SPE fraction. The notion that GABA could help suppress cytokine secretion levels is not implausible, as GABA has been previously shown to modulate the immune system (DeFeudis, 1983; Jin et al., 2013), reducing TNF secretion in a murine model for allergic dermatitis (Duthey et al., 2010), decreasing the release of IL-1ß and IL-6 from mice macrophages in an autoimmune encephalomyelitis model (Bhat et al., 2010), suppressing IL-2 in mice lymph node mononuclear cells (Tian et al., 1999), and lowering the frequency of T cells secreting IFN-γ in prediabetic mice (Tian et al., 2004).

6.4.2.2 S. horrida

Fractions 1, 4 and pooled fractions of this venom had potent immunosuppression. LC-MS analysis revealed that the main constituent of fraction 1 is a molecule of 14,007 Da, and in the case of fraction 4, the main constituent is a molecule of 12,068 Da, although another molecule of 13,776 Da and detectable levels of the 14,007 Da are also found within this fraction. It is possible that the 14,007 Da molecule is one of the main drivers of the significant suppression in both fractions 1 and 4, given that the suppression is seen at both the protein and gene levels for fraction 4. The major proteins observed in fraction 1 for both SvV and ShV (13,690 Da and 14,007 Da, respectively) are likely to be homologues based on the similarity in the molecular weights and the activity. This is also likely for the main proteins in SvV fraction 5 (12,365 Da) and ShV fraction 4 (12,068 Da).

6.4.2.3 Limitations

Some limitations to this study exist. The cytotoxicity assay only used one biological donor. Variability between biological donors is well known (Wilson et al., 1991) and biological replicates are preferred; however, sample limitations prevented use of biological replicates in this cytotoxicity study. Sample limitations also prevented cytotoxicity assay of fraction 2 from SvV, and fraction 4 and pooled fractions from ShV. Based on the cytotoxicity results from crude venom and the other isolated fractions it is unlikely that these untested fractions would be cytotoxic towards hPBMCs. In addition, it was not possible to measure the concentration of fraction 4 from ShV used in the suppression ELISA because it was below the limit of detection of the BCA kit used.

The RT-qPCR results followed the same pattern observed for the ELISA, but the results were below statistical significance. In the assay performed with the SvV fractions, only two donors were used due to sample limitation, which could have played a major role in the lack of statistical significance compared to the ELISA results. In the case of ShV, three donors were used, and it is unknown why the results were not as significant as observed for the ELISA.

6.4.3 Cardio physiological effects of stonefish venoms

The presence of catecholamines such as NE and DA and neurotransmitters such as GABA and ACh in stonefish venom provides an explanation for some of the cardio pathologies and symptoms experienced by sting victims. NE and DA modulate cardiorespiratory effects, which may play a major role upon stonefish envenomation. NE has important roles in the sympathetic regulation of respiratory and cardiovascular systems (Pertovaara, 2006), where it can cause tachycardia (Horwitz et al., 1962) and produce positive chronotropic and inotropic effects (Schroeder and Jordan, 2012). DA has been correlated with increases in cardiac output and stroke volume in healthy volunteers, although the cardiovascular effects produced by DA do not seem to be as significant as NE's (Horwitz et al., 1962).

GABA, a potent inhibitory neurotransmitter, is also capable of modulating cardiovascular function (Jin et al., 2013; Worrall and Williams, 1994). Previous reports have shown a range of effects resulting from intravenous injections of GABA, including transient bradycardia, hypotension and respiratory discomfort (Elliott and Hobbiger, 1959; Kimura et al., 2002).

This study also revealed the presence of choline and ACh in ShV. Choline plays several biological roles in the human body, and it is the precursor of the neurotransmitter ACh (Blusztajn, 1998). The presence of ACh was also found in relative high concentrations in *P. volitans* venom (Cohen and Olek, 1989). It was reported that a *P. volitans* venom extract caused negative inotropic and chronotropic effects in isolated frog hearts, where a synergistic effect of ACh and venom toxicity could have enhanced toxicity by producing local vasodilation or pain and might have also accounted for cardiac physio pathologies seen in previous reports (Cohen and Olek, 1989). In the case of stonefish envenomations, there is a dose-dependent cardiovascular effect, which caused much controversy in the past due to varied results indicating negative to positive and biphasic cardiac effects (Church and Hodgson, 2002). It has been suggested that cardiovascular collapse occurs primarily due to venom acting directly on adrenoceptors and to also have an effect on muscarinic receptors (Church and Hodgson, 2000). Therefore, depending on the individual concentrations of NE, DA, GABA, ACh and choline in stonefish venoms, and their net effect according to their ability to penetrate surrounding tissues (Haavaldsen and Fonnum, 1963), they could play an important role in the cardiorespiratory effects seen in clinical and laboratory stonefish envenomation.

6.4.4 Neuromuscular effects

The presence of ACh in *S. horrida* venom could also explain some of the neuromuscular effects produced by this venom. The effects of ShV and a toxic fraction (trachynilysin) were tested on murine and frog neuromuscular junctions. The neuromuscular toxicity observed in the study was believed to be due to massive increase in quantal release of ACh from nerve terminals at low concentrations, and by neuromuscular damage caused by higher venom concentrations (Colasante et al., 1996; Kreger et al., 1993). Another study using ShV on rat brain synaptosomes reported venom-driven release of ACh endogenous stores (Khoo et al., 1992). Furthermore, when ShV was applied to longitudinal smooth muscle of guinea pigs, venom responses were reduced by atropine, suggesting either a direct or indirect activity at muscarinic receptors (Hopkins et al., 1994). This effect was similar to the effects reported for a *P. volitans* venom extract on frog nerve-muscle preparations, which induced muscle fibrillation and blockage of neuromuscular signal transmission through substantial release with consequent depletion of ACh from nerve terminals (Cohen and Olek, 1989). Some of the results from the aforementioned studies supported indirect effects of the venom as the cause of activity in muscarinic receptors. However, direct effects could be explained by the presence of ACh (a muscarinic

agonist) in the venom, which until now had not been confirmed. The report of ACh presence in *S. horrida* venom in this dissertation supports a direct neuromuscular effect of the venom.

6.4.5 Nociception mechanisms in stonefish envenomation

It is unclear whether the severe effects associated with stonefish envenomations are a consequence of venom-modulated cardio physiological toxicity, venom-driven neuromuscular collapse, or the excruciating pain that causes stress-induced pathologies on the cardiorespiratory system (Ramachandran, 2015). The involvement of molecules such as NE could potentially play a role in venom nociception, as noradrenergic mechanisms in the peripheral tissues increase nociceptor excitability upon tissue injury, ultimately leading to pain aggravation (Pertovaara, 2006; Schroeder and Jordan, 2012). In addition, vasoconstriction due to noradrenergic effects may promote ischemic states in peripheral tissues, which may lead to ischemic pain (Pertovaara, 2006).

In this study, SEC fractions from *S. verrucosa* were tested on various Na^+ channels, where $Na_V1.3$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ play a major role in nociceptive signaling (De Lera Ruiz and Kraus, 2015). No activity was observed at these channels for the *S. verrucosa* SEC fractions. Therefore, it is unlikely that the venom-driven pain modulation involves these Na^+ channels in *S. verrucosa* envenomation. Therefore, investigating the molecular make up of these venoms and their respective nociceptive cascade effects could be an important avenue for future research.

6.5 Conclusions and future directions

This study discovered and identified molecules that had never been reported in fish venoms before, such as GABA. Choline and acetylcholine were also reported for the first time in stonefish venoms. In addition to these three molecules, many other venom constituents revealed by both NMR spectrometry and LC-MS data remain unidentified at the time of writing.

In addition to hypothesizing on the mechanisms of action of *Synanceia* spp. venoms regarding cardiac, nociception and neuromuscular effects upon envenomation, this study also shed light on the immunological potential found within these venoms. The five small molecules identified in this study most likely do not contribute to the significant immunosuppression observed in the venoms. However, four main small proteins ranging in size from 12 to 17 kDa were revealed by LC-MS in the fractions that caused significant suppression of TNF secretion levels. It was theorized that these proteins could be driving the immunosuppression observed. Further testing on these molecules is warranted to understand what roles they might play in the venoms, if any. In addition, the presence of GABA in earlier eluting fractions together with small proteins was both unexpected and interesting. One explanation for this result is that GABA has some form of interaction with the co-eluting protein.

Further study to establish if an interaction is present, characterisation of the protein, and the role of the protein and the interaction is warranted.

Furthermore, venoms of the different stonefish species differ in composition, so a comparison and contrast of the species, with focus on the pathologies and symptoms, might show that the different species cause different pathophysiological effects. This could translate to improved and more effective treatment of sting victims, or even provide opportunities for novel drug discovery. The pulsed-wave Doppler technology could aid in this endeavour with animal models. Lastly, as technologies and methods improve, so does biodiscovery, so it is essential to continue investigating stonefish venoms to improve treatment modalities and to explore their potential as drug leads or as physiological tools.

6.6 References

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Chapter 7

7 Conclusions and future directions

7.1 Overview and summary of results

Chapter 1 of this dissertation introduced the importance of studying venomous animals. Envenomation caused by venomous marine animals is a global public health problem, and stings caused by jellyfish and stonefish, in particular, have the potential to increase due to rising ocean temperatures and consequent broader distribution of these animals (Carrette et al., 2012; Hifumi et al., 2020). Current treatments focus on alleviating the venom-induced symptoms as they are observed by the attending clinicians. Outcomes from current treatments, therefore, vary with some patients experiencing long-term side-effects or requiring surgical procedures post-envenomation, while others require little to no medical intervention (Carrette et al., 2012; Saggiomo et al., 2021). This range of symptoms experienced by sting victims might be explained by the diverse mechanisms of action of these venoms and respective venom components and presents an area of research importance. The studies within this dissertation were designed to address important questions related to jellyfish envenomation and stonefish venom-derived immunological effects.

Chapter 2 of this dissertation investigated jellyfish envenomation in a live mouse model using non-invasive methods. The project focused on two distinct species of jellyfish (*Chironex fleckeri* and *Carukia barnesi*) that are known to cause severe and lethal human envenomation. The study addressed the systolic and diastolic functions of the left ventricle in mice, since the venoms are known to cause severe cardiac pathologies, which result in significant morbidity and mortality in victims. Data presented in this dissertation showed that venom from *C. fleckeri* had rapid and significant positive inotropic effects without chronotropic effects. The rapid onset of cardiac effects strongly supports the hypothesis that the venom effects occur through a myocardial toxin acting directly on cardiomyocytes. In contrast, the Irukandji Syndrome-causing jellyfish, *C. barnesi*, had moderate but immediate inotropic and chronotropic effects, which were maintained until precipitous cardiac deterioration, indicating that the venom most likely causes cardiomyopathy through catecholaminergic effects. The technique shown in this chapter has the potential to be further used to aid investigations into the current and future effectiveness of treatment modalities of envenomed victims, ultimately improving patient outcome.

Due to the unforeseen consequences of the COVID-19 pandemic, the focus of this dissertation was altered to stonefish venom immunological properties. Therefore, the second stage of this thesis investigated stonefish venom from two distinct species (*Synanceia verrucosa* and *Synanceia horrida*), shedding light on ecological parameters driving venom yield and immunological effects on human

cells. Chapter 3 reviewed the literature of stonefish envenomation and venom components. This review study created, for the first time, a global distribution map of all *Synanceia* spp. known at the time, collated all available case and clinical reports worldwide that implicated specimens within this genus, discussed updated information on stonefish antivenom use and summarised the current knowledge while opening pathways for future studies.

Chapter 4 focused on ecological parameters that might influence venom production and composition in *S. horrida*. The results indicated that although venom quantities were significantly affected by starvation, FPLC and SDS-PAGE gel analyses indicated that venom composition was similar after a period of suspended feeding compared to the fed individuals. These results were important to identify the minimum time required between repetitive milking events of the same individual. The implications of this study were twofold: this ensured venom profiles were adequately restored, and therefore were consistent for the immunological studies presented in the following chapters; and stonefish venom production did not decrease while withstanding long periods without nourishment, which indicated that animals could be milked even if they stopped eating, mitigating the risk of insufficient venom availability.

The overall objective of Chapters 5 and 6 was to investigate the immunological modulation potential of venoms extracted from two species of stonefish, *S. verrucosa* and *S. horrida*. Chapter 5 specifically focused on exploring crude venom-driven effects on cytokine secretion from hPBMCs at concentrations that did not cause cytotoxicity. Venom treatment following cellular stimulation demonstrated that both venoms have potent suppression activity towards TNF and IL-10 secretion levels. SvV also significantly downregulated the levels of IL-12, IFN-γ, MCP-1, and IL-6. Both venoms demonstrated greater suppression activity in LPS-stimulated cells and Th1 cytokines, which provided evidence for a mode of action related to LPS-mediated regulatory pathways. Interestingly, there was no evidence of a pro-inflammatory response to the venom at the concentrations tested, in contrast to the clinical symptoms observed.

Chapter 6 expanded on the findings in Chapter 5, investigating the influence of fractionated venoms and small molecules on venom immunomodulation. Analytical assays demonstrated that small molecules that had been previously described in stonefish venoms were not found in our studies (tryptophan and epinephrine in either venom, and dopamine in SvV). More importantly, GABA, a potent inhibitory neurotransmitter, was found in both SvV and ShV and provided the first report of this molecule in marine venoms. In addition, choline and acetylcholine were also identified in ShV for the first time in a stonefish venom. To further comprehend the immunosuppressive activity of these venoms, immunoassays demonstrated that some SEC fractions caused potent suppression of TNF

secretion levels from hPBMCs. Various small molecules, small proteins, or peptides were identified in the immunosuppressive fractions, which might be involved in the immunosuppression activity of these venoms. These findings highlighted the pharmaceutical potential hidden within stonefish venoms.

7.2 Future directions

Venoms are complex mixtures of different compounds, ranging from large proteins to small molecules and salts (Fry et al., 2009; King, 2011). This diversity in molecular composition leads to modulation of a range of physiological responses (King, 2011), which could be either harmful or beneficial to humans. When looking at ameliorating the harmful effects of venoms, treatment modalities and protocols can be improved by understanding their mode of action. When diving into the potential benefits of venoms, characterizing venom components is of the upmost importance.

Although much has been discovered in the realm of jellyfish venoms, a gap still exists between understanding the physiological and pharmacological pathways activated by venoms and treating victims effectively, especially in relation to Irukandji Syndrome victims. Furthermore, the use of antivenom for *C. fleckeri* stings is reported to have varied effectiveness and is a point of contention in the literature (Currie, 2003). Consequently, practitioners treat the symptoms and pathologies observed, instead of directly annulling the venom activity, which would lead to improved and effective recovery. The murine model developed during this doctorate could be used to verify treatment hypotheses and test readily available drugs prior to clinical trials. One example is the use of magnesium for Irukandji Syndrome victims, which is reported to have varied success in the clinic (Corkeron, 2003; McCullagh et al., 2012). Murine models using the pulsed-wave Doppler technology might offer an opportunity to better investigate the effectiveness of magnesium and test its administration as an anti-catecholaminergic agent. Other drugs, both existing and novel, could be tested on mice or rats prior to initiating clinical trials, potentially reducing trial costs, and improving patient outcome. In addition, this model could be used with stonefish venom, which also causes electrocardiographic changes in experimental models and severe envenoming is associated with cardiorespiratory complications.

Venoms can have beneficial applications in the development of pharmacological tools, insecticides, and therapeutics (Smith et al., 2013; Vetter et al., 2011). This dissertation specifically investigated stonefish venoms and their immunomodulatory activities. My results show that stonefish venoms possess potential pharmacological riches, as venoms from two distinct species were capable of potent reduction secretion levels of cytokines and chemokines from hPBMCs. This study revealed fractions with potent suppression activity of TNF secretion levels. The venoms were discovered to possess an

array of small molecules, small proteins, or peptides that might be responsible for this immunosuppression activity. The identification of GABA, choline and acetylcholine in these stonefish venoms was reported for the first time, and the novel components present, when characterised, could pave the way to a drug lead or a drug scaffold.

To date, *S. verrucosa* and *S. horrida* have been the most, if not the only, species studied within the genus *Synanceia*. It is important to highlight that other species exist, and a new species (*S. quinque*) was described during this PhD candidature, bringing the total number of *Synanceia* species to six. Further characterisation of these venoms is warranted, and venomics analyses could aid in the exploration, discovery, and identification of novel molecules in these venoms as well.

It is undeniable that the study of marine venoms is an important field. The discovery, characterization, isolation, and synthesis of novel venom compounds have the potential to improve treatment modalities of sting victims and also the discovery of novel pharmaceutical compounds. I encourage further research to be performed into stonefish venom cardio physiology using the pulsed-wave Doppler technology as much as I encourage immunological investigations into cubozoan jellyfish venom. This doctorate dissertation has closed some of the gaps of one of the most important aspects of these venoms, and yet more opportunities to understanding them seem to follow these discoveries. This is one of the many reasons why marine venom research is important, stimulating, and necessary.

7.3 References

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Appendices

Appendix A: Name and explanation of the relevant cardiac indices measured in Chapter 2.

Stroke Distance (SD)	Refers to the area under PW Doppler Sonogram. The stroke volume (SV) of a cardiac contraction can be calculated by multiplying Stroke Distance (SD) with the cross-sectional area of the related cardiac valve (Θ), SV = SD x Θ. In this paper we have assumed that the cross-sectional area of the mouse's valve does not change during the observational period, and consequently Δ%SD is equivalent to Δ%SV. Furthermore, as cardiac output (CO, Figure 2.9) is the product of SV and heart rate (HR), a Δ%SD is directly proportional to a Δ%CO (%CO = 100 x ((HRxSDxΘ)/HRxSDxΘ). SD is provided by the Indus Instruments Signal Analysis software.
Peak Flow Velocity (PFV)	PFV refers to the peak velocity measured through the aortic valve (Figure 2.1). This reflects the strength of left ventricular contraction.
IsoVolumetric Contraction time (IVCT)	IVCT is the time it takes from initiation of ventricular contraction to the time that the aortic valve opens. In the presence of reduced SD an increase in IVCT reflects a reduction in ventricular contractility.
IsoVolumetric Relaxation Time (IVRT)	IVRT is the time between the end of ventricular contraction and the opening of the mitral valve (Figures 2.2 and 2.5). An increase in this time represents dysfunctional ventricular relaxation.
E/A Ratio	The ratio between peak velocities for the passive E-filling (E-PV, ventricular relaxation) and the active A-filling (A-PV, atrial contraction) of the left ventricle (Figure 12). In humans, the E/A Ratio is between 2 and 4. In a normal state, up to 85% of left ventricular filling is passive (E-wave: E-SD, E-PV), while the remaining 15% of ventricular filling occurs through atrial contraction (A-wave: A-SD, A-PV). As HR increases to greater than 500/min morphological changes occur to the mitral wave sonogram with progressive equivalisation of the waves, and eventual reversal of the E-PV and A-PV. Additionally, the E and A waves progressively approach each other and fuse. In diastolic dysfunction (dysfunctional relaxation), the E-SD and E-PV reduce while there is compensatory increase in A-SD and A-PV. Consequently, there is equivalisation in the E/A Ratio.

Appendix B: List of case reports where stonefish from the genus* Synanceia *have been reported as the culprit for human envenomations*. As identification of the animal is difficult, caution should be used when stating the causative agent of hospitalizations, as envenomation may have been caused by another venomous fish found in the region.*

Location	Number of Cases	Year	Reference
Australia			
Cairns	1	-	(Flecker, 1956)
Cooktown	1	-	(Flecker, 1956)
Western Australia	1	-	(Jurat et al., 2019)
Tropical northern Australia	1	-	(Isbister, 2001)
Cook Islands	1	-	(Dall et al., 2006)
Egypt			
Taba	1	-	(Rishpon et al., 2008)
French Polynesia			
Bora Bora	1	2018	(Maillaud et al., 2020)
Tubuai Island	1	2014	(Maillaud et al., 2020)
Guam	2	-	(Lopez et al., 2019; Prentice et al., 2008)
Indonesia			
Lembeh, North Sulawesi	1	-	(Brenneke and Hatz, 2006)
Indo-Pacific region	1	-	(Nistor et al., 2010)
Japan			
Okinawa	15	2013–2017	(Hifumi et al., 2020)
Kingdom of Bahrain	1		(Ghanem et al., 2019)
Malaysia			
Kota Kinabalu, Sabah	1	-	(Ongkili and Cheah, 2013)
Mozambique			
Pinda	1	1956	(Smith, 1957)
New Caledonia	1	2008	(Maillaud et al., 2020)
Papua New Guinea			
Trobriand Islands	12	-	(Phleps, 1960)
People's Republic of China			
Hong Kong	1	2002	(Tang et al., 2006)
Hong Kong	1	2003	(Tang et al., 2006)
Hong Kong	7	2005–2008	(Chan et al., 2010)
Not clearly stated—possibly Hong Kong	1	2008	(Ling et al., 2009)
Hong Kong	32	2008–2018	(Poon et al., 2020)
Not clearly stated	1	-	(Lehmann and Hardy, 1993)
Seychelles			
Pont Larue, Mahe	1	1956	(Smith, 1957)
Singapore			
Pulau Bukom	81	4 years	(Phoon and Alfred, 1965)
Location not stated	1	2001–2003	(Lee et al., 2004)
Location not stated	7	1.25 years	(Lee et al., 2004)
Location not stated	30	2004–2006	(Ngo et al., 2009)

Appendix C: Geographical locations of* Synanceia *specimens throughout the globe. *Coordinates are shown as they were presented in the literature.*

Location per Continent	Number of Specimens	Coordinates	Reference
		S. verrucosa	
AFRICA			
Kenya–Andromache Reef, S of Port Kilindini of Mombasa Harbor	1	4°05′ 05″ S, 39°40′ 39″ E	(Eschmeyer and Rao, 1973)
Mauritius—location not stated	1		(Eschmeyer and Rao, 1973)
Mozambique—Pinda Peninsula	1	14°14°12″ S	(Smith, 1951)
Seychelles—location not stated	2		(Eschmeyer and Rao, 1973)
ASIA			
Ceylon—Trincomalee, inside base of Royal Navy of Ceylon	3		(Eschmeyer and Rao, 1973)
Cyprus—Kumyali	1		(Akbora et al., 2021)
Japan—Okinawa	1		(Eschmeyer and Rao, 1973)
Mediterranean Sea			
Gaza City, State of Palestine	1	31°31′ 3.32″ N, 34°25′ 18.66″ E	(Bariche et al., 2019)
Israel—Palmakhim	1	31°56.36′ N, 34°42.16′ E	(Edelist et al., 2011)
Lebanon—Tyr	1	33.290657°N, 35.184459°E	(Crocetta et al., 2015)
Syria—Lattakia city	1	35°31.5′ 5.97″ N, 35°42′ 48.57″ E	(Ibrahim et al., 2019)
Turkey—Yumurtalik, Iskenderum Bay	1		(Bilecenoglu, 2012)
Phillipines			
Dumaguette	1		(Eschmeyer and Rao, 1973)
Sitankai, Sulu Province	2		(Eschmeyer and Rao, 1973)
Location not stated	1		(Eschmeyer and Rao, 1973)
Red Sea			
Location not stated	1		(Eschmeyer and Rao, 1973)
Thailand—Ko Tao, Sairee Beach, Gulf of Thailand	1		(Scraps and Scott, 2014
Western Indian Ocean			
Southern Andaman Islands, S of Corbyn's Cove, Port Blair	1		(Eschmeyer and Rao, 1973)
OCEANIA			
Australia			
Capricorn Islands, One Tree Island, W side, QLD	1		(Eschmeyer and Rao, 1973)

Exmouth, WA	1	−21.958648, 114.141082	(“Atlas of Living Australia 1,” n.d.)
Fairfax Island, QLD	1		(Eschmeyer and Rao, 1973)
Gulf of Carpentaria, QLD	1	15°1′ 30″ S, 138°41′ 30″ E	(“Atlas of Living Australia 2,” n.d.)
Heron Island, QLD	1		(Endean, 1961)
Lancelin, WA	1	31°0′ S, 115°19′ E	(“Atlas of Living Australia 3,” n.d.)
Magnetic Island, Townsville, QLD	1	19°08′ 00″ S, 146°50′00″ E	(“Atlas of Living Australia 4,” n.d.)
Mermaid Reef, Rowley Shoals, WA	1	−17.083, 119.583	(“Atlas of Living Australia 5,” n.d.)
Shark Bay, New Beach, 45 km S of Carnarvon	1	25°20′ S, 113°56′ E	(“Atlas of Living Australia 6,” n.d.)
Tallow Beach, 300 m S of the southern boundary of Arakwal NP, NSW	1	28°39′ 54″ S, 153°37′ 32″ E	(“Atlas of Living Australia 7,” n.d.)
Melanesia			
Solomon Islands			
Bougainville, E side of Puk Island, outside Poison Lagoon	1		(Eschmeyer and Rao, 1973)
Sikaiana Island	1		(Eschmeyer and Rao, 1973)
Micronesia			
Caroline Islands			
Yap Island, inlet E side of Yap Island	1	9°29′ 48″ N, 138°26′ 57″ E	(Eschmeyer and Rao, 1973)
Location not stated	4		(Eschmeyer and Rao, 1973)
Mariana Islands			
Guam, Hagatna	1		(Eschmeyer and Rao, 1973)
Guam, N of Tringhera Beach in Hagatna Bay	3	13°28′ 53″ N, 144°45′ 45″ E	(Eschmeyer and Rao, 1973)
Guam, SW of Agat village, N side of Bangi Point	1	13°22′ 36″ N, 144°38′ 53″ E	(Eschmeyer and Rao, 1973)
Location not stated	3		(Eschmeyer and Rao, 1973)
Palau Islands			
Angaur Island, in Garangaoi Cove, S of Cape Nagaramudel	1	6°53′ 50″ N, 137°7′ 49″ E	(Eschmeyer and Rao, 1973)
Auluptagel Island, Crocodile Cove	1	7°17′ N, 137°29′ E	(Eschmeyer and Rao, 1973)
Ngadarak Reef SW of Auluptagel Island	2	7°17′ 48″ N, 134°28′ 37″ E	(Eschmeyer and Rao, 1973)
Location not stated	5		(Eschmeyer and Rao, 1973)
New Caledonia—Noumea	1		(Eschmeyer and Rao, 1973)
New Guinea—location not stated	1		(Eschmeyer and Rao, 1973)
Polynesia			
Society Islands			

Moorea, Faatoai village at Papetoai Bay	1		(Eschmeyer and Rao, 1973)
Tahiti	1		(Eschmeyer and Rao, 1973)
Location not stated	4		(Eschmeyer and Rao, 1973)
Fiji			
Location not stated	1		(Eschmeyer and Rao, 1973)
Samoa			
Pago Pago	2		(Eschmeyer and Rao, 1973)
Location not stated	1		(Eschmeyer and Rao, 1973)
Location not stated	4		(Eschmeyer and Rao, 1973)
Tonga Islands			
Location not stated	1		(Eschmeyer and Rao, 1973)
Tuamotu Islands			
Location not stated	3		(Eschmeyer and Rao, 1973)
		S. horrida	
ASIA			
Batavia (Jakarta)	1		(Eschmeyer and Rao, 1973)
Malasia—location not stated			(Le Mare, 1952)
Phillipines			
Atimonan, Tayabas	1		(Eschmeyer and Rao, 1973)
Calaogao, Cauayan, Negros Island	1	10°N, 122°30’ E	(Eschmeyer and Rao, 1973)
Manila Bay	1		(Eschmeyer and Rao, 1973)
Ragay Gulf, Luzon	1		(Eschmeyer and Rao, 1973)
Stankai, Sulu Island	1		(Eschmeyer and Rao, 1973)
Location not stated	2		(Eschmeyer and Rao, 1973)
Singapore			
Pacific Expedition—location not stated	5		(Eschmeyer and Rao, 1973)
Punggol—location not stated	1		(Phoon and Alfred, 1965)
Singapore Market	1		(Eschmeyer and Rao, 1973)
Location not stated	1		(Eschmeyer and Rao, 1973)
Thailand			
Patong Bay, Patong Phuket	2		(Eschmeyer and Rao, 1973)
Rayong Province, SE of Ban Phe Fisheries Station	1	12°35’ 40” N, 101°25’43” E	(Eschmeyer and Rao, 1973)
Location not stated	1		(Eschmeyer and Rao, 1973)
OCEANIA			
New Guinea—Waigeo Island	1		(Eschmeyer and Rao, 1973)
Australia			
Between Moreton Bay and Cairns, Queensland, Australia	52		(Endean, 1961)
Britomart Reef, QLD	1	18°10’ S, 146°43’ E	(“Atlas of Living Australia 8,” n.d.)
Coffs Harbor, NSW	1	30°15’ S, 153°8’ E	(“Atlas of Living Australia 9,” n.d.)

East coast of Northern Queensland	25-30		[150]
Melville Bay and Cape Arnhem area, NT	1	12°15′ S, 136°43′ E	(“Atlas of Living Australia 10,” n.d.)
Moreton Bay			[13]
N side of Main Wharf, Broome, WA	1	−17.967, 122.233	(“Atlas of Living Australia 11,” n.d.)
Northern Territory, Groote Eylandt	5		(Eschmeyer and Rao, 1973)
Port Darwin, NT	1	12°27′ S, 130°48′ E	(“Atlas of Living Australia 12,” n.d.)
Port Hedland, WA	1	20°18′ S, 118°35′ E	(“Atlas of Living Australia 13,” n.d.)
Princess Charlotte Bay, QLD	1	−14.333333, 144.116667	(“Atlas of Living Australia 14,” n.d.)
Shark Bay, blow holes, N of Carnarvon	1	−24.483333, 113.416667	(“Atlas of Living Australia 15,” n.d.)
Sweers Island, Gulf of Carpentaria, QLD	1	17°6′ S, 139°37′ E	(“Atlas of Living Australia 16,” n.d.)
Tryon Island, Capricorn Group, QLD	1	23°15′ S, 151°47′ E	(“Atlas of Living Australia 17,” n.d.)
S. nana			
ASIA			
Pakistan—Karachi Fish Harbor	2		(Osmany and Moazzam, 2018)
Red Sea			
Gulf of Suez—Et-Tur, Sinai Peninsula	1		(Eschmeyer and Rao, 1973)
Gulf of Suez—off Port Safaga	1	27°16′ 15″ N, 33°47′ 30″ E	(Eschmeyer and Rao, 1973)
Israel—Gulf of Aqaba, between Marset Mahash el Ala and Marset Abu Samra	1		(Eschmeyer and Rao, 1973)
Israel—NW coast of the Gulf of Aqaba, bay at Al Himeira	5		(Eschmeyer and Rao, 1973)
Saudi Arabia—Persian Gulf, Tarut Bay, Near Ras Tanura spit	1		(Eschmeyer and Rao, 1973)
S. alula			
OCEANIA			
Nicobar Islands—Nancowry Island	2	8°N, 93°40′ E	(Eschmeyer and Rao, 1973)
Solomon Islands—New Georgia, Munda Lagoon	1		(Eschmeyer and Rao, 1973)
Solomon Islands—New Georgia, Munda Pier	2		(Eschmeyer and Rao, 1973)
S. platyrhyncha			
ASIA			
Ambon Island	1		(Eschmeyer and Rao, 1973)

Appendix D: Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE Guidelines). *This table includes all essential information related to the materials and methods of all RT-qPCR experiments done in Chapters 5 and 6.*

MIQE checklist for authors	
Experimental design	
Definition of control groups	Negative: untreated cells; Positive: LPS or PMA/Ionomycin (refer to Section 5.2.6.2.1 for more information, where cells were supplemented with 10% human serum instead of FBS, and received additional 10mM HEPES and 1% amino acids)
Definition of experimental groups	Venom at different titrations (20, 5 and 1.25 µg/mL) and venom at these titrations incubated with either LPS (10 ng/mL) or PMA/Ionomycin (0.5 X)
Number within each group	Three biological replicates, where single stimulations, single RNA isolations and single RT with qPCR in technical triplicate were done
Assay carried out by	Investigator's laboratory
Acknowledgment of author's contributions	Silvia Saggiomo designed the experiment, thawed, separated hPBMCs, stimulated and lysed cells; Daniel Browne thawed, separated hPBMCs and lysed cells, as well as performed the RT-qPCR assays. Prof Denise Doolan oversaw the experiments and was Daniel Browne's PhD supervisor, Prof Norelle Daly and Dr David Wilson were Silvia Saggiomo's PhD supervisors.
Sample	
Description	Refer to Section 5.2.5
Volume of sample collected	90 mL of blood collected from each donor (refer to Section 5.2.5 for more information)
Microdissection or macro dissection	N/A; no dissection was done, but whole PBMCs were lysed at once
Processing procedure	As previously done by Browne *et al.*2020
If frozen, how and how quickly?	Buffy coats were sourced from Red Cross Australia and processed as per above
If fixed, with what and how quickly?	N/A
Sample storage conditions and duration	Cells were immediately frozen at -80°C for 24 h, then cryogenically frozen in LN_2 with 10% DMSO/FBS. When needed, they were thawed rapidly at 37°C and washed 2x after DNase treatment (refer to section 5.2.5.2 for more information)
Nucleic acid extraction	
Procedure and/or instrumentation	As previously performed by Browne *et al.* 2020.
Name of kit and details of any modifications	MagMAX™ *mirVana*™ Total RNA Isolation kit (Applied Biosystems); as per manufacturer's instructions, with the modification that all RNA extraction reagents were used at 1/4 of the volume recommended by the manufacturer
Source of additional reagents	N/A; all reagents used came from the kits
Details of DNase or RNase treatment	Cells were treated with DNase as per MagMAX™ *mirVana*™ manufactures instructions
Contamination assessment (DNA or RNA)	We did not assess the samples for DNA contamination. This was because we were following Browne *et al.* 2020 protocol. This work demonstrated that gDNA contamination is negligible following the above DNase stage in the MagMAX™ protocol

Nucleic acid quantification	As per Browne *et al.* 2020 protocol (samples were normalised to cell number). Quantification of RNA concentration is unavailable following this protocol. This is because when extracting from 100,000 hPBMCs the concentration of RNA is below the limit of detection of the available Nano spectrophotometer (<10 ng/µL).
Instrument and method	N/A as per above
Purity (A_{260}/A_{280})	N/A as per above
Yield	N/A as per above
RNA integrity: method/instrument	N/A as per above
RIN/RQI or C_q of 3' and 5' transcripts	N/A as per above
Electrophoresis traces	N/A as per above
Inhibition testing (C_q dilutions, spike, or other)	This protocol followed the protocol tested by Browne *et al.* 2020, who demonstrated that cDNA dilutions had an efficiency between 90-110%. This protocol followed MIQE guidelines. The Efficiencies of the primers of this study were also tested (see section "PCR efficiency calculated from slope")
Reverse transcription	
Complete reaction conditions	RT was performed in two stages as per Browne *et al*. 2020. Stage 1: 50 µM Random Hexamers, 10 mM dNTPs, Superscript IV™ reagents, and Primer Binding Mix were thawed and/or prepared. 0.75 µL of Primer Binding Mix per 0.2 mL PCR tube was added, then 2.5 µL of eluted RNA was added to each tube and incubated for 5 min at 65°C, then cooled to 4°C for at least 1 min. Stage 2: cDNA Synth Mix was then prepared (as per below) and a volume of 1.75 µL was added to each tube. Tubes were then incubated for 10 min at various temperatures as per below.
Amount of RNA and reaction volume	15 µL total volume buffer and RNA, which was diluted (1:4), generating 2.5 µL of RNA
Priming oligonucleotide (if using GSP) and concentration	50 µM Random Hexamers (ThermoFisher)
Reverse transcriptase and concentration	5 units of enzyme per 1 µL of RNA
Temperature and time	Incubation at 65°C for 5 min, then cool to 4°C for at least 1 min; then primer priming and RT for 10 min at 23°C and 10 min at 50°C; then reaction termination at 85°C for 10 min
Manufacturer or reagents and catalogue numbers	SuperScript™ IV First-Strand Synthesis System (ThermoFisher: Catalogue # 18090050) as per manufacturer's instructions, with the modification that the Superscript enzyme was run at 5 U/µL RNA and all cDNA extraction reagents were used at 1/4 of the volume recommended by the manufacturer, inclusive of RT enzyme.
C_qs with and without reverse transcription	A negative RT control was not tested as these samples were treated with DNase. Previous testing (Browne *et al.* 2020) demonstrated that RT negative controls using this protocol were > 35 Ct

Storage conditions of cDNA	cDNA was immediately used after RNA isolation or otherwise frozen at -20°C
qPCR target information	
Gene symbol	Tumour necrosis factor (TNF), interferon-gamma (IFN-γ) and interleukin-10 (IL-10)
Sequence accession number from GenBank	TNF: NM_000594
	IFN-γ: NM_000619.2
	IL-10: NM_000572.2
Location of amplicon	TNF: 556-574
	IFN-γ: 364-386
	IL-10: 217-239
Amplicon length	TNF: 91
	IFN-γ: 93
	IL-10: 112
In silico specificity screen from PrimerBank	TNF: 25952110c2
	IFN-γ: 56786137c1
	IL-10: 24430216c1
Location of each primer by exon or intron	TNF: Both forward and reverse primers are in Exon 4 IFN-γ: Both forward and reverse primers are in Exon 3 IL-10: Both forward and reverse primers are in Exon 3
What splice targets are targeted?	N/A; as above
qPCR oligonucleotides	
Primer sequences	TNF: **Forward** GAGGCCAAGCCCTGGTATG **Reverse** CGGGCCGATTGATCTCAGC
	IFN-γ: **Forward** TCGGTAACTGACTTGAATGTCCA **Reverse** TCGCTTCCCTGTTTTAGCTGC
	IL-10: **Forward** GACTTTAAGGGTTACCTGGGTTG **Reverse** TCACATGCGCCTTGATGTCTG
RTPrimerDB identification number	TNF: NCBI ID: 7124; GenBank Accession: NM_000594; PrimerBank ID: 25952110c2
	IFN-γ: NCBI ID: 3458; GenBank Accession: NM_000619.2; PrimerBank ID: 56786137c1
	IL-10: NCBI ID: 3586; GenBank Accession: NM_000572.2; PrimerBank ID: 24430216c1
Probe sequences	N/A; SYBR chemistry
Location and identity of oligonucleotides	N/A; no modification

Manufacturer of oligonucleotides	Sigma-Aldrich
Purification method	Desalted
qPCR protocol	
Complete reaction conditions	As per Browne *et al.* 2020. Briefly, cDNA was diluted (1:2) with Ultra-Pure water. A PCR master mix was generated with 1X SsoAdvanced, UPH2O and primers were mixed at a concentration of 5 µM each (sufficient amounts for standards, positive controls, and triplicate replicates for each sample, with 0.5 µL added to a 5 µL reaction resulting in a final concentration of 500 nM), 4 µL of master mixes were transferred into the reaction plate and 1 µL of sample was added. After all sample was added, the plate was centrifuged at 200 *g* for 1 sec prior to data collection as per SsoAdvanced SYBR supermix™ protocol.
Reaction volume and amount of cDNA/DNA	5 µL total volume cDNA synthesis, which was diluted (1:4), generating 1 µL cDNA per qPCR
Primer	500 nM in reaction for each primer
Mg^{2+}, and dNTP concentrations	SsoAdvanced™ master mix of 1X concentration as per manufacturer's recommendation (i.e. Mg^{2+} and dNTP concentrations are proprietary information)
Polymerase identity and concentration	Sso7d Fusion polymerase contained in the SsoAdvanced™ Universal SYBR Green Supermix run at a 1 X concentration, 5 µL x reaction volume
Buffer kit identity and manufacturer	SsoAdvanced™ Universal SYBR R® Green Supermix (BioRad)
Additives	N/A
Manufacturer of plates/tubes, and catalogue number	MicroAmp™ Optical 384-well reaction plate (ThermoFisher), 0.2 mL PCR-grade clean tube (Eppendorf: PCR grade), Micro-Amp™ Optical adhesive film (ThermoFisher)
Complete thermocycling parameters	Fast cycling protocol: 30 sec at 95 or 98°C for polymerase activation and DNA denaturation. Amplification: 5-15 sec for denaturation, 15-30 sec for annealing/extension and plate read at 60°C for 35-40 cycles. Met curve analysis at 65-95°C, with 0.5°C increments at 2-5 sec/step
Reaction setup	Manual
Manufacturer of qPCR instrument	QuantStudio 5 (Applied Biosystems)
qPCR validation	
Evidence of optimization (from gradients)	qPCR was optimised by Browne *et al.* 2020. Briefly, samples of $1x10^6$ PBMCs were stimulated with PMA/Ionomycin, mRNA extraction used RNeasy Mini kit, and cDNA synthesis used Superscript III. Amplicon and cDNA standards were tested using SYBR Master mix testing; reference gene stability was also tested
Specificity	A melt curve test was performed after every reaction, and all melt curves demonstrated high specificity
For SYBR Green I, C_q of the NTC	TNF: 36.795 and 5 x undetermined (across two 384-well plates)
	IFN-γ: 6 x undetermined (across two 384-well plates)
	IL-10: 37.897 and 5 x undetermined (across two 384-well plates)
Calibration curves with slope and y intercept	TNF: y = -3.6125x + 38.358
	IFN-γ: y = -3.5199x + 38.481
	IL-10: y = -3.5193x + 38.617

PCR efficiency calculated from slope	TNF: E = 89.15%
	IFN-γ: E = 92.35%
	IL-10: E = 92.37%
r^2 of calibration curve	TNF: r^2 = 0.9976
	IFN-γ: r^2 = 0.9964
	IL-10: r^2 = 0.9973
Linear dynamic range	0 - 40
C_q variation at LOD	TNF: 0.34655629
	IFN-γ: 0.87019037
	IL-10: 0.79956359
Evidence for LOD	10^2 copies/reaction were detected in 3/3 wells for all cytokines tested
If multiplex, efficiency and LOD of each assay	N/A; all singleplex

Data analysis

qPCR analysis program (source, version)	QuantStudio™ 5 Quantitative Thermocycler and QuantStudio™ 5 Design and Analysis Software (Applied Biosystems)
Method of C_q determination	Threshold was set during the exponential phase of amplification at ΔRn0.3
Outlier identification and disposition	No outliers were recognized by Grubb's test (α = 0.05), therefore, no data were disposed of
Results for NTCs	TNF: Mean = 5 x Undetermined + 36.795
	IFN-γ: Mean = 6 x Undetermined
	IL-10: Mean = 5 x Undetermined + 37.897
Justification of number and choice of reference genes	N/A; copies/reaction were normalized to cell number using '*Relative Quantification'* as per Browne *et al.* 2020
Description of normalization method	Absolute quantification of copies/reaction were determined by standard curve and compared to cell number/stimulation
Number and stage (RT or qPCR) of technical replicates	1x stimulation, 1x RNA isolation, 1x cDNA synthesis measured in technical triplicate qPCR per test performed
Repeatability (intraassay variation)	TNF: Ct SD = 20.95 ± 0.076
	IFN-γ: Ct SD = 21.36 ± 0.050
	IL-10: Ct SD = 25.27 ± 0.101
Statistical methods for results significance	One-way ANOVA followed by Dunnett's multiple comparison tests
Software (source, version)	GraphPad Prism ver 9.3.1

Appendix E: LC-MS analysis of stonefish crude venom components and their respective SEC fractions with the molecular weights (MW, Da) listed. *Yellow highlights GABA and NE (103 and 169 Da, respectively) found in crude SvV; green highlights GABA/choline, NE, ACh and DA (104, 169, 145 and 153 Da, respectively) found in crude ShV; grey highlights the major constituents of each SEC fraction per stonefish species. RT is the retention time (min); ✓ marks the presence of the molecule; * marks m/z values (not possible to distinguish GABA from choline because they have similar m/z values despite having distinct MWs); SEC fractions are given by "F" followed by their respective numbers.*

			***S. verrucosa* - SEC**						***S. horrida* - SEC**				
	RT (min)	MW (Da)	Crude	F1	F2	F3	F4	F5	Crude	F1	F2	F3	F4
GABA/Choline	1.7	*104.24**							✓				
		122.126							✓				
		140.097							✓				
		145.105							✓				
		163.083							✓				
		168.079							✓				
		202.247							✓				
		218.028							✓				
		236.014							✓				
		241.034							✓				
	1.9	122.116	✓										
		140.080	✓										
		145.097	✓										
		163.086	✓										
		168.077	✓										
		218.011	✓										
		236.001	✓										
GABA	2.3	103.187	✓										
		151.140	✓										
NE		169.120	✓										
NE		169.001							✓				
ACh	2.4	*146.189**							✓				
	2.6	154.124	✓										
DA	3.2	153.186							✓				
		171.139							✓				
	3.3	307.106	✓										
	4.0	258.128	✓										
		347.088	✓										
	4.8	181.155											
	5.3	157.181							✓				
		211.137							✓				
	5.4	365.179	✓										
		389.320	✓										
	5.5	236.102							✓				
	6.2	275.108							✓				
	7.0	612.212	✓										
	7.9	208.139	✓										

	9.8	301.136							✓						
	10.3	236.105							✓						
	11.5	283.198							✓						
	12.3	287.150							✓						
	12.7	275.156							✓						
	12.9	323.089	✓												
	13.6	659.499							✓						
	14.1	312.134							✓						
	15.2	244.196							✓						
	15.9	564.373							✓						
	16.3	244.249							✓						
		285.275							✓						
	19.2	872.580							✓						
		1000.661							✓						
	19.8	315.388							✓						
	20.1	414.330							✓						
	20.5	379.232							✓						
		424.262							✓						
	20.9	317.215							✓						
	21.6	2387.436							✓						
	23.0	2810.048	✓												
	24.5	2716.891							✓						
	30.8	940.587							✓						
	33.2	12365.952	✓				✓	✓							
	33.6	13617.200							✓						
		13776.956							✓						✓
		15735.610							✓						
	34.4	13232.618	✓				✓								
		13260.574	✓				✓								
	36.8	1189.679							✓						
		11892.910							✓	✓	✓	✓			
		11920.363							✓	✓	✓	✓			
	37.5	17375.576	✓		✓	✓	✓								
	37.7	12068.322							✓						✓
		11920.789							✓	✓					
	38.2	4205.094	✓												
	41.1	14007.152							✓	✓	✓	✓	✓		
	41.8	13960.457	✓	✓	✓	✓	✓								
	45.7	13287.490	✓				✓								
	50.2	375.310							✓						
		392.314							✓						
		420.350							✓						
	50.4	375.298	✓												
		392.325	✓												
		420.352	✓												