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Stonefish (*Synanceia* spp.) Toxin Ecology and Therapeutic Potential

Thesis submitted by

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on the 21st of December 2024

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Statement of the Contribution of Others

Danica Lennox-Bulow is the primary author of this thesis and undertook the majority of the work under the supervision of Professor Jamie Seymour, Dr Michael Smout, Dr Robert Courtney, and Distinguished Professor Alex Loukas. Jayden Zieth volunteered his time to assist with data collection and editing the written work. Sally Turner and the Cairns Turtle Rehabilitation Facility assisted with animal husbandry. The Higher Degree by Research Enhancement Scheme under the College of Public Health, Medical, and Veterinary Sciences at James Cook University provided financial aid for publishing some of the written work in this thesis. The Australian Government's Research Training Program scholarship provided financial support for Danica Lennox-Bulow throughout her candidature.

Statement of the Use of Generative AI

Generative AI technology was not used in the preparation of any part of this thesis.

Abstract

A thorough understanding of the toxin ecology of any organism is scant in the zoology world. Often an understanding of the toxins effects on predators is known (e.g., blue ringed octopus), or its effects on prey (e.g., snakes), but seldom is a complete picture of these toxins compiled. Stonefish (*Synanceia* spp.) are one of only a few known organisms that harness two distinct types of toxins; a defensive venom associated with each of their dorsal spines, and a poison, known as ichthyocrinotoxin, housed within specialised skin glands (i.e., tubercles). As such, developing a high-resolution image of their toxin ecology is twice as challenging as that of a venomous-only or poisonous-only organism. Furthermore, toxins represent a vast and largely untapped reservoir of novel compounds, some of which may have practical applications as therapeutics or industrial products. Due to the deleterious nature of stonefish venom to people, the biodiscovery potential of stonefish toxins had not been previously considered. This thesis aimed to improve the current understanding of the ecology of stonefish and their toxins and leverage this knowledge to highlight and explore the potential practical applications of these toxins.

An initial comprehensive review of ichthyocrinotoxins produced by fish indicated that these were used predominantly for protection against larger predators, mainly as antifeedants. In investigating the behavioural ecology of stonefish, which are highly cryptic, my work showed they are crepuscular in movement, transitioning from areas of high structural diversity at dusk, to areas of low structural diversity, and then back to high structural diversity at sunrise. This suggested that their camouflage may not be entirely effective against predators. Given that the two species studied in this thesis occupy different ecological niches, it was hypothesised that the venom they produce may be different between species or locations. Variations in venom composition were found; however, they were minimal. This suggested that either the predators of the different species of stonefish were similar, or that the venom components are generalist in their defensive activity. Based on these results, I determined that a more thorough investigation into the ichthyocrinotoxins may provide a better understanding of the toxin ecology of these animals.

Stonefish ichthyocrinotoxins exhibited considerable variation in composition both between species, as well as within species across geographically isolated populations. Like the venom, this suggested that differences in the ecology of these animals may be driving the differences in their ichthyocrinotoxins. However, as it is believed that ichthyocrinotoxins are

predominantly used to defend against predators larger than the prey, I conducted a series of experiments to assess this hypothesis for stonefish. These demonstrated that, unlike ichthyocrinotoxins belonging to other fish taxa, stonefish ichthyocrinotoxins do not appear to play a major role in antifeedant defence. Instead, given that stonefish lack scales and exhibit a benthic and predominantly sedentary lifestyle, I speculated that these animals would likely be highly susceptible to parasites. This led to the working hypothesis that stonefish ichthyocrinotoxins may be used to prevent parasites.

Experiments conducted on rodent parasites supported this hypothesis, suggesting that compositional differences in the ichthyocrinotoxins between these species may reflect differences in the parasite dynamics to which they are exposed to. Given the observed toxicity of stonefish ichthyocrinotoxins to a parasite species that these fish are unlikely to encounter naturally, further experiments were conducted to evaluate their potential as novel therapeutic agents. The results showed that small (<3 kDa) components isolated from *S. horrida* ichthyocrinotoxin possess potent anthelmintic activity while exhibiting minimal cytotoxicity to various mammalian cell lines *in vitro*. While the activity of the larger components within this toxin remains unknown, these findings demonstrate that further research into the ecology of stonefish ichthyocrinotoxins could uncover additional opportunities for biodiscovery.

In conclusion, this thesis explored various aspects of the ecology of stonefish and their toxins. In doing so, it highlighted that the complex and dynamic nature of stonefish toxins, which appear to be dictated by the ecology of the animal, presents a promising source of novel compounds that could be targeted for biodiscovery. Ultimately, this work demonstrates how an improved understanding of the ecology of an organism and its toxins can be used to guide biodiscovery research efforts.

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List of Publications Included as Part of the Thesis

Chapter 2:

Lennox-Bulow, D., Smout, M., Loukas, A., & Seymour, J. (2023). Stonefish (*Synanceia* spp.) Ichthyocrinotoxins: An Ecological Review and Prospectus for Future Research and Biodiscovery. *Toxicon*, **236**, 107329. doi: 10.1016/j.toxicon.2023.107329

Chapter 3:

Lennox-Bulow, D., Courtney, R., Ebner, B.C., & Seymour, J. (2023). Diel Movement Patterns and Habitat Preferences of Stonefish (*Synanceia* spp.) in Captivity. *Memoirs of the Queensland Museum – Nature*, 65: 1-7. doi: 10.17082/j.2204-1478.65.2024.2023-04

Chapter 4:

Lennox-Bulow, D., Courtney, R., & Seymour, J. (2025). Geographic Variation in Stonefish (*Synanceia* spp.) Venom. *Toxicon*, **254**, 108222. doi: 10.1016/j.toxicon.2024.108222

Chapter 5:

Lennox-Bulow, D., Smout, M., Wilson, D., & Seymour, J. (2023). Interspecies Variation in Stonefish (*Synanceia* spp.) Ichthyocrinotoxins; An Ecological Perspective. *Toxicon*, **221**, 106977. doi: 10.1016/j.toxicon.2022.106977

Chapter 6:

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Chapter 7:

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Chapter 8:

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Chapter 1

Introduction

1.1 Using Ecology to Guide Biodiscovery

The ecology of an animal drives all its other physiological processes, one of those being the toxins it uses. Toxins are substances that disrupt the physiological processes of other living organisms (Nelsen *et al.*, 2014). Organisms that possess toxins, including both plants and animals, utilise these specialised bioweapons to capture prey, defend against a threat, or a combination of both (Albuquerque-Pinna *et al.*, 2024; Schendel *et al.*, 2019). There are two types of toxins, venoms and poisons, which are broadly grouped based on their mechanism of delivery. Venoms are toxins produced within glands that are associated with a specialised weapon (i.e., spine, stinger, fang) that is used to inject the toxin directly into the victim (Nelsen *et al.*, 2014). In contrast, poisons are secreted toxins acquired from consumed metabolites (i.e., tetrodotoxin), or produced within glands (i.e., ichthyocrinotoxin), that can be detrimental when ingested or absorbed (Nelsen *et al.*, 2014).

Australia is home to a wide variety of venomous and poisonous plants and animals, among them are stonefish. Stonefish (*Synanceia* spp.) are a group of benthic, highly camouflaged, and predominantly sedentary fish that are widely distributed throughout the tropical and subtropical waters of the Indo Pacific (Saggiomo *et al.*, 2021; Endean, 1961). Remarkably, they are one of only a few known animals that possess two distinct types of toxins in the form of a venom and a poison. Stonefish venoms are housed within twin glands that are equipped to each of their 13 dorsal spines (Saggiomo *et al.*, 2021; Endean, 1961). In contrast, poisons produced by stonefish, called ichthyocrinotoxins, are secreted from specialised glandular cells, called tubercles, that are scattered across the surface of their skin (Cameron *et al.*, 1981). The deleterious nature of stonefish venom to people has earned these animals the title of ‘the world’s most venomous fish’ (Saggiomo *et al.*, 2021). This reputation has, unfortunately, limited much of the research on these animals to their medical significance. Consequently, very little is known about the ecology of stonefish, or the toxins they possess.

Stonefish are an ideal model organism for toxin ecology studies as their dual-toxin nature allows investigations into how an animal’s ecology can shape two different types of toxins. Toxins, especially venoms, are often comprised of a multifarious mixture of proteins,

peptides, and small molecules (Schendel *et al.*, 2019; Chen *et al.*, 2018). These concoctions exhibit considerable diversity in their composition and bioactivities between taxa, as well as within (Tasoulis *et al.*, 2020; Schaffrath *et al.*, 2018) and between species (van Thiel *et al.*, 2023; Duran *et al.*, 2020), and individuals (Dutertre *et al.*, 2014). A multitude of environmental and physiological factors have been identified to drive this, including, predator-prey dynamics (Smith *et al.*, 2023; Chang & Duda, 2016; Daltry *et al.*, 1996), sex (Amorim *et al.*, 2018; Zelanis *et al.*, 2016; Herzig & Hodgson, 2009), ontogeny (da Silva Aguiar *et al.*, 2020; Chang & Duda, 2016; Underwood & Seymour, 2007), temperature (Smith *et al.*, 2023; O'Hara *et al.*, 2018), and season (Tasima *et al.*, 2024; Junior *et al.*, 2010). Toxins are also highly dynamic substances, capable of changing in composition and/or function within a lifetime (plasticity) in response to specific environmental or physiological conditions (Gangur *et al.*, 2017; Dutertre *et al.*, 2014), or over generations (evolutionary) to suit an ecological niche (Weese & Duda, 2019; Daltry *et al.*, 1996). The compositional and functional diversity and complexity of toxins, coupled with their dynamic nature, makes these substances a rich resource for biodiscovery. While organisms that harness toxins are ubiquitous throughout the natural world, only a handful of toxin-derived molecules (< 15) have presently been approved for pharmaceutical use (Bordon *et al.*, 2020). This suggests that the current methods for biodiscovery could be improved.

Throughout the late 20th century, researchers, backed by the funding of pharmaceutical companies, indiscriminately screened vast numbers of plant and animal extracts against various biological targets (Appendino *et al.*, 2010). These traditional approaches often lacked ecological context, were time-consuming, resource-intensive, and yielded low success rates, and hence, poor financial returns. As a result, many pharmaceutical companies have substantially reduced their investment into natural product research, shifting the burden to underfunded research organisations (Appendino *et al.*, 2010). These traditional approaches to biodiscovery are therefore no longer a viable option. The advent of high-throughput screening systems and omics-based technologies (i.e., genomics, transcriptomics, proteomics, and metabolomics) have since revolutionised the field of biodiscovery (Mohana *et al.*, 2018; Thomford *et al.*, 2018; Rocha-Martin *et al.*, 2014). These modern analytical techniques generate large databases, often at considerably lower costs (both financial and resource), and with greater efficiency compared to traditional methods (Mohana *et al.*, 2018; Thomford *et al.*, 2018; Rocha-Martin *et al.*, 2014). However, without a solid understanding of the ecology of the organism, mining these vast libraries of data for promising drug leads becomes akin to finding a needle in a haystack.

Toxins represent a vast and largely untapped reservoir of novel compounds, some of which may have practical applications as therapeutics or industrial products. Efficiently bioprospecting toxins for novel compounds of interest requires an integrated approach, combining ecological insights with modern analytical techniques to guide biodiscovery research efforts. Unfortunately, this is seldom the approach to biodiscovery research, and there appears to be a disconnect between disciplines, particularly between ecology and medicine. As such, using stonefish as a model organism, the focus of this thesis was to demonstrate the advantages of cross-disciplinary research by improving the current understanding of the ecology of stonefish and their toxins, and leveraging this knowledge to explore potential biodiscovery pathways.

First I reviewed the literature on ichthyocrinotoxins produced by fish. This revealed a significant gap in our understanding of the ecology of stonefish and their toxins, specifically their ichthyocrinotoxins. To begin to address this gap, I conducted a series of biological studies to gain a better understanding of the ecology, particularly the toxin ecology, of these animals. These included examining the diel behaviours and habitat preferences of stonefish, as well as the effect of species and geographic location on the composition of their toxins (both venom and ichthyocrinotoxin). This research led to the hypothesis that one potential role of stonefish ichthyocrinotoxins may be parasite defence. To test this hypothesis, I sought a reliable method to assess the antiparasitic potential of stonefish ichthyocrinotoxins. While these exist (i.e., the xWORM assay), they lacked robustness. Therefore, I refined the xWORM assay by determining the optimal parasite density, media type, and media concentration to support the vitality of my target parasite species and life stage. This improved assay enabled me to demonstrate that stonefish ichthyocrinotoxins indeed possess antiparasitic properties. This further prompted me to investigate the therapeutic potential of stonefish ichthyocrinotoxins by identifying the bioactive compounds responsible for the anthelmintic activity and assessing their toxicity to various mammalian cells *in vitro*. Finally, I synthesise the findings of this research and highlight where future research should focus in relation to stonefish ichthyocrinotoxins and novel therapeutics.

1.2 Thesis Description and Aims

Chapter Two consolidates existing knowledge on the ecological roles and biological activities of ichthyocrinotoxins produced by fish, and, in doing so, identifies a significant gap in the current understanding of stonefish ichthyocrinotoxins. By juxtaposing the known characteristics of ichthyocrinotoxins from other fish taxa with the current state of knowledge of stonefish ecology, this chapter highlights promising avenues for future research on stonefish ichthyocrinotoxins and their potential for biodiscovery.

Chapter Three delves into the diurnal movement patterns and habitat preferences of *Synanceia horrida* (Estuarine Stonefish) and *Synanceia verrucosa* (Reef Stonefish) in captivity. Although stonefish are commonly perceived as a benthic and predominantly sedentary species, this chapter reveals a more complex picture of their environmental interactions. By examining the intricate relationship between stonefish and their environment, this chapter contributes to a deeper understanding of stonefish ecology, which is likely to influence their toxin ecology.

Chapter Four compares the venom composition profiles between stonefish species (*S. horrida* and *S. verrucosa*), as well as within species between geographically isolated populations. Utilising the known drivers of venom variation in other taxa as a foundation, this chapter discusses the ecological factors that may be driving the observed compositional differences in stonefish venom. Finally, this chapter highlights the potential clinical significance of geographic variation in stonefish venom composition, emphasising the need for further research into stonefish toxin ecology.

Chapters Five and Six proceed to investigate the effect of species and geographic location on the composition of stonefish ichthyocrinotoxins. Resultingly, these chapters provided the first compositional profiles of ichthyocrinotoxins secreted by *S. horrida* and *S. verrucosa*. By drawing on the existing knowledge of stonefish ecology, these chapters discuss the potential ecological drivers of the observed compositional variation in their ichthyocrinotoxins. This research further highlights that there is a significant gap in our understanding of how ecological factors influence the composition of secreted toxins produced *de novo*, a category that likely includes stonefish ichthyocrinotoxins.

Chapter Seven evaluated the palatability of stonefish ichthyocrinotoxins to two potential predator species. Given that ichthyocrinotoxins secreted by most of the well-studied fish taxa serve in predator defence, either as defensive toxins or antifeedants, the primary aim

of Chapter Six was to determine whether stonefish ichthyocrinotoxins serve a similar purpose. Ultimately, this chapter challenges the conventional view of stonefish ichthyocrinotoxins playing a major role in antifeedant defence and further proposes that the toxin may serve an alternate defensive role.

Chapter Eight explored the effect of key xWORM assay parameters, including media type, media concentration, in-well parasite density, and assay duration, on the vitality of the infective third-stage larvae (L3) of two hookworm species: *Nippostrongylus brasiliensis* (Rodent Hookworm) and *Necator americanus* (Human Hookworm). The goal of Chapter Eight was to determine the optimal xWORM assay conditions for novel drug trials involving these parasites. Additionally, this chapter provides a framework for optimising xWORM assay conditions for other parasite species and life stages. The findings of this research underscore the importance of not only considering the ecology of the organism being bioprospected, but also that of the organism being targeted for disruption.

Chapter Nine quantifies the anthelmintic properties and therapeutic potential of stonefish ichthyocrinotoxins. Employing the optimised xWORM assay developed in Chapter Eight, the anthelmintic activity of ichthyocrinotoxins from *S. horrida* and *S. verrucosa* were evaluated against *N. brasiliensis* L3. To assess the therapeutic potential of stonefish ichthyocrinotoxins, cytotoxicity assays were conducted on various mammalian cell lines *in vitro*, including human skin cells (3GN), human bile duct cells (H69), and human and rat red blood cells. This culminated in the discovery of a promising novel anthelmintic drug lead from *S. horrida* ichthyocrinotoxin. While additional research is required to fully characterise the anthelmintic components and evaluate their clinical suitability, this study demonstrates the potential of stonefish ichthyocrinotoxins as a source of novel anthelmintic therapeutics.

The final chapter, **Chapter Ten**, synthesises the knowledge gained from the previous chapters to expand on the current understanding of the ecology of stonefish and their toxins. This chapter showcases how this understanding facilitated the exploration of stonefish ichthyocrinotoxins as a potential source of novel anthelmintic therapeutics. The chapter further outlines avenues for future research on stonefish toxin ecology and biodiscovery. Finally, it proposes that my applied approach, which integrates ecological understanding with biodiscovery, could be applied to bioprospecting other species.

The primary aims of this research were to:

- i) Investigate the diel activity patterns and habitat preferences of stonefish to gain a better understanding of how the animal interacts with its environment, and how its toxin ecology is integrated into this (Chapter 3).
- ii) Assess the effect of species and geographic location on the composition of venom from *Synanceia horrida* (Estuarine Stonefish) and *Synanceia verrucosa* (Reef Stonefish, Chapter 4).
- iii) Determine whether the composition of stonefish ichthyocrinotoxins vary between typically reef-dwelling (*S. verrucosa*) and estuary-dwelling (*S. horrida*) species (Chapter 5).
- iv) Determine whether the composition of *S. horrida* ichthyocrinotoxin varies between geographically isolated populations (Chapter 6).
- v) Quantify the palatability of *S. horrida* and *S. verrucosa* ichthyocrinotoxins to potential predators (e.g., *Dendrochirus zebra*, *Gymnothorax pseudothyrsoides*) to evaluate their role in predator defence (Chapter 7).
- vi) Identify the optimum xWORM assay conditions (i.e., media type, media concentration, and parasite density) for measuring the motility of *Nippostrongylus brasiliensis* L3 (infective rodent hookworm larvae) and *Necator americanus* L3 (infective human hookworm larvae, Chapter 8).
- vii) Investigate the potential role of *S. horrida* and *S. verrucosa* ichthyocrinotoxins in parasite defence by measuring their toxicity to *N. brasiliensis* L3 (Chapter 9).
- viii) Isolate and identify the anthelmintic components within the ichthyocrinotoxins of *S. horrida* and *S. verrucosa*, and evaluate their cytotoxicity against human skin and bile duct cells, as well as human and rat red blood cells *in vitro*, to assess their therapeutic potential (Chapter 9).

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Chapter 2

Stonefish (*Synanceia* spp.) Ichthyocrinotoxins: An Ecological Review and Prospectus for Future Research and Biodiscovery

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D Lennox-Bulow is the principal author of this manuscript and was extensively involved in all aspects of this work under the supervision of J Seymour. M Smout and A Loukas assisted with reviewing earlier versions of this manuscript.

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2.1 Abstract

Marine organisms possess a diverse array of unique substances, many with wide ranging potential for applications in medicine, industry, and other sectors. Stonefish (*Synanceia* spp.), a bottom-dwelling fish that inhabit shallow and intertidal waters throughout the Indo-Pacific, harbour two distinct substances, a venom, and an ichthyocrinotoxin. Stonefish are well-known for the potent venom that is associated with each of their dorsal spines as it poses a significant risk to public health. Consequently, much of the research on stonefish focusses on the venom, with the aim of improving outcomes in cases of envenomation. However, there has been a notable lack of research on stonefish ichthyocrinotoxins, a class of toxin that is synthesised within specialised epithelial cells (i.e., tubercles) and exuded onto the skin. This has resulted in a substantial gap in our understanding of these animals. This review aims to bridge this gap by consolidating the literature on the ecological functions and biochemical attributes of ichthyocrinotoxins present in various fish species and juxtaposing it with the current state of knowledge of stonefish ecology. We highlight the roles of ichthyocrinotoxins in predator defence, bolstering innate immunity, and regulating integumentary interactions with parasites and detrimental fouling organisms. The objective of this review is to identify promising research avenues that could shed light on the ecological functions of stonefish ichthyocrinotoxins and their potential practical applications as therapeutics and/or industrial products.

2.2 Introduction

The marine realm, encompassing over 242,000 documented species (Ahyong *et al.*, 2023), offers a trove of unexplored substances potentially useful to humans (Blunt *et al.*, 2018). Among these are ichthyocrinotoxins, a substance produced within the skin of certain fish. The term is derived from Greek to literally mean ‘*ikhthus*’ – fish (Cresswell, 2021), and ‘*crin*’ – to secrete (Klionsky *et al.*, 2007), in combination with the English word ‘toxin’ – substances causing harm to living organisms (Nelsen *et al.*, 2014). Ichthyocrinotoxic fishes are defined as those that produce a toxin within specialised epithelial glandular structures, such as tubercles, that are independent of traumagenic devices like spines (Halstead, 1988). While ichthyocrinotoxins have been revealed as a complex pharmacy of components, only some of which exhibit toxic activity, this review uses the term to refer to the complete substance originating from these specialised glandular structures akin to other multi-component toxin descriptors such as “venom” and “poison”.

Approximately 50 species of fish across at least 13 families are known to harbour an ichthyocrinotoxin, one of these being stonefish (genus *Synanceia*) (Halstead, 1988; Cameron & Endean, 1973). Two prominent species, *Synanceia horrida* (Estuarine Stonefish) and *Synanceia verrucosa* (Reef Stonefish), are found in the Indo-Pacific region, inhabiting shallow intertidal waters, coral reefs, and rocky environments (The Reef Stonefish, *Synanceia verrucosa* (Left), and the Estuarine Stonefish, *Synanceia horrida* (Right), are considered the world’s most venomous fish.) (Saggiomo *et al.*, 2021; Endean, 1961). These species are notorious for possessing the most potent venom among fish, which is an injectable toxin housed within twin glands associated with each of the 13 hypodermic needle-like spines on the animal’s back (Harris *et al.*, 2021; Mohebbi, 2021; Saggiomo *et al.*, 2021). Envenomation can cause a diverse range of symptoms, including excruciating pain, paralysis, cardiac arrest, and in certain cases, death (Harris *et al.*, 2021; Mohebbi, 2021; Saggiomo *et al.*, 2021; Endean, 1961). Due to their cryptic nature, envenomation cases involving stonefish are frequent and accounted for 9.3% of marine animal related incidents resulting in hospitalisation in Australia between 2017 and 2018 (Pointer & Harrison, 2021). Consequently, much of the research on stonefish focusses on the venom, particularly around improving outcomes in cases of envenomation. However, investigations into the nature and biochemical properties of the ichthyocrinotoxins that stonefish secrete onto their epidermis is limited. Therefore, this review aims to consolidate the literature on ichthyocrinotoxins present in various fish species, including the toxins’ function

in predator defence, bolstering innate immunity, and regulating integumentary interactions. By juxtaposing this with the current knowledge of stonefish ecology, we aim to identify promising research avenues that could shed light on the function of stonefish ichthyocrinotoxins and their potential practical applications as therapeutics and/or industrial products.

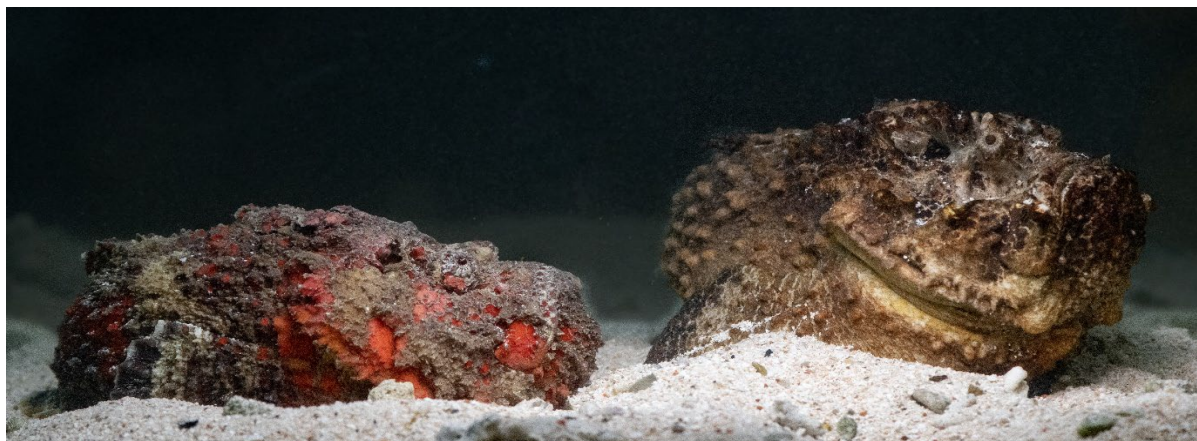


Figure 2.1 Two Prominent Species of Stonefish that occur in Queensland (North-Eastern Australia) Waters. The Reef Stonefish, *Synanceia verrucosa* (Left), and the Estuarine Stonefish, *Synanceia horrida* (Right), are considered the world's most venomous fish.

2.3 The Role of Ichthyocrinotoxins in Predator Defence

Chemical defences, such as venoms and poisons, often play a crucial role in an organism's defence against predators. These defences can function either through sensory aversion (i.e., anti-feedants), or by causing physical harm to cells and disrupting cellular processes (i.e., defensive toxins) (Harris & Jenner, 2019; Tachibana, 1988). Ichthyocrinotoxins are thought to serve both purposes, defending against direct predators, and, in some cases, indirect predators that target the organisms' resources.

2.3.1 Anti-Feedants

Anti-feedants are substances that reduce the rate of herbivory and/or predation and are a common chemical defence employed by a variety of terrestrial and aquatic organisms, particularly plants (Wöll *et al.*, 2013; Chaieb *et al.*, 2009; Bruno *et al.*, 2002; Harborne, 1991) and invertebrates (Kasumyan *et al.*, 2021; Eisner & Aneshansley, 2000; Roitberg & Isman, 1992). The primary function of anti-feedant substances is to deter predation by rendering the user unpalatable (Kasumyan *et al.*, 2021; Wöll *et al.*, 2013; Chaieb *et al.*, 2009; Roitberg & Isman, 1992). Consequently, predators typically react to organisms that possess anti-feedant

substances by immediately expelling and/or regurgitating them during feeding attempts, or by avoiding these organisms altogether (Wöll *et al.*, 2013; Roitberg & Isman, 1992).

The potential of ichthyocrinotoxins to function as anti-feedants was initially highlighted in a study examining the feeding habits of predatory fishes in the West Indies (Randall, 1967). The study reported that no *Rypticus saponaceus* (Greater Soapfish) were present amongst the gut contents of predatory fishes, which the author suggested could be due to the bitter taste of their ichthyocrinotoxins (Randall, 1967). This hypothesis was later supported by a study on the feeding responses of both wild and captive elasmobranchs to ichthyocrinotoxins secreted by *Pardachirus marmoratus*, the Red Sea Moses Sole (Clark, 1983). The study reported noteworthy variations in ingestion times depending on the toxicity of the fish (Clark, 1983). Under typical circumstances, captive *Charcharhinus longimanus* (Whitetip Sharks) reportedly consumed non-toxic fish within a minute (Clark, 1983). In contrast, ichthyocrinotoxic *P. marmoratus* were largely ignored by the sharks for anywhere between 7 and 28 hours, and in most cases were only consumed after being deceased for several hours (Clark, 1983). Furthermore, 14% of predatory attempts on live *P. marmoratus* by *C. longimanus* induced anti-feeding responses (Clark, 1983). These behavioural responses included the sharks jerking away, swimming erratically, and gaping their mouths, ultimately leaving the fish untouched (Clark, 1983). Moreover, baited lines with dead or live *P. marmoratus*, as well as non-toxic teleosts coated with, or in close proximity (up to 30 cm) to the ichthyocrinotoxin, were observed to repel wild *Charcharhinus amblyhinchus* (Grey Reef Sharks) for up to 16 hours (Clark, 1983). However, it is crucial to highlight recent findings indicating the absence of type II taste receptors (T2R's) in cartilaginous predators, which are responsible for perceiving 'bitterness' (Behrens *et al.*, 2021; Sharma *et al.*, 2019). Consequently, if the aversive response of elasmobranchs to *P. marmoratus* and *R. saponaceus* ichthyocrinotoxins indeed stems from sensory factors, it is highly likely that these reactions are linked to olfactory mechanisms rather than gustatory ones. Nevertheless, it has been observed that ichthyocrinotoxins do not consistently deter predators. Previous studies have reported that predatory fish were both attracted to (Lassig, 1981), and would attempt to consume (Gratzer *et al.*, 2015) ichthyocrinotoxic *Gobiiodons* (Coral-Dwelling Gobies) outside of their coral host. Instead, one study has shown that *Gobiodon* ichthyocrinotoxins, particularly those secreted by *G. histrio*, might serve a more strategic role in resource defence (Dirnwoeber & Herler, 2013). In their study, Dirnwoeber and Herler (2013) tracked the predatory encounters initiated by corallivorous Chaedontidae (Butterfly fish) on both *Gobiodon*-inhabited and uninhabited

colonies of *Acropora*, a small-polyp stony coral. The results revealed a significant decrease in predation on *Acropora* colonies that were inhabited by ichthyocrinotoxic *Gobiodon*s compared to those that were not (Dirnwoeber & Herler, 2013). This gave rise to the hypothesis that *Gobiodon* ichthyocrinotoxins might serve as a more potent line of defence against indirect predators, like the coral-eating Chaedontidae, rather than direct predators (Dirnwoeber & Herler, 2013). Furthermore, the authors conjectured that *Gobiodon* ichthyocrinotoxins might function as a resource defence by inducing strong avoidance, and most likely food refusal behaviours in corallivorous Chaedontidae (Dirnwoeber & Herler, 2013).

2.3.2 Defensive Toxins – Fish

Building upon these insights into the complex interplay between ichthyocrinotoxins and predators, it is also worth noting that in certain species, these toxins not only influence palatability, but have also been documented to induce adverse physiological effects on vertebrates. This highlights their potential function as defensive toxins. Unlike anti-feedants, which primarily target the gustatory and/or olfactory senses of predators (Chaieb *et al.*, 2009), defensive toxins produced by fish are designed to damage cells, and/or disrupt cellular processes (Harris & Jenner, 2019; Ellisdon *et al.*, 2015). For instance, certain components present in the venom of *Synanceia* spp. (Stonefish) have been found to form pores in cell membranes, while others bind to nicotinic acetylcholine receptors and interrupt neuronal signalling (Harris *et al.*, 2021; Harris & Jenner, 2019; Ellisdon *et al.*, 2015). It is believed that these combined activities induce significant pain to deter predators and cause localised paralysis – most likely in the predators’ mouths – to improve the fish’s chances of a successful escape (Harris *et al.*, 2021; Ellisdon *et al.*, 2015).

The toxic effects of ichthyocrinotoxins produced by certain teleost fishes are well-documented, with early reports stemming from fishermen’s observations of lethal effects when particular species were kept together (Kalmanzon & Zlotkin, 2009; Randall *et al.*, 1971; Marezki & Del Castillo, 1967). Early ichthyologists discovered that members of several fish families, including Serranidae (Soapfish), Ostraciidae (Boxfish), and Soleidae (Flatfish), secrete large amounts of slime (i.e., ichthyocrinotoxin) in response to stress or excitement (Brock, 1956; Beebe & Tee-Van, 1928). The field collection of some species, such as *Ostracion meleagris* (previously *Ostracion lentiginosus*, Spotted Boxfish), suggested that ichthyocrinotoxins could be noxious to other fish (Brock, 1956).

Studies conducted in a controlled aquarium environment revealed that, when exposed to ichthyocrinotoxins, numerous non-toxic teleost species exhibited motor excitation or hyperactivity, followed by a resting period at the bottom of the tank (Clark & George, 1979; Thomson, 1969; Marezki & Del Castillo, 1967). Recovery from these symptoms depended on the specific species of ichthyocrinotoxin to which the fish were exposed (Thomson, 1969; Marezki & Del Castillo, 1967). Specifically, exposure to *O. meleagris* ichthyocrinotoxins led to irreparable illness and death within an hour, while exposure to *Rypticus saponaceous* (Greater Soapfish) ichthyocrinotoxins could be reversed within one to two hours of transfer to clean, toxin-free water (Thomson, 1969; Marezki & Del Castillo, 1967).

2.3.3 Defensive Toxins – Mammals

Moreover, ichthyocrinotoxins previously identified as ichthyotoxic were also found to exhibit haemolytic properties. Studies reported that the forced consumption or injection of ichthyocrinotoxins belonging to certain fish species had detrimental effects on mammals (Goldberg *et al.*, 1988; Goldberg *et al.*, 1982; Primor & Zlotkin, 1975; Hashimoto *et al.*, 1974; Randall *et al.*, 1971; Hashimoto & Kamiya, 1969; Thomson, 1964). For instance, cats experienced vomiting, increased salivation, and diarrhoea, leading to loss of motor activity, paralysis, and coma within days of ingesting *Pogonoperca punctata* (Spotted Soapfish) ichthyocrinotoxin (Hashimoto & Kamiya, 1969). Likewise, mice injected with ichthyocrinotoxins from *O. meleagris* (Spotted Boxfish), *P. punctata*, or *R. saponaceous* exhibited ataxia and laboured breathing, leading to a comatose state and death (Hashimoto & Kamiya, 1969; Marezki & Del Castillo, 1967; Thomson, 1964). Tests conducted *in vitro* confirmed that the symptoms observed in mammals were likely due to the toxins' haemolytic and/or hemagglutinating activity on mammalian erythrocytes (Goldberg *et al.*, 1988; Goldberg *et al.*, 1982; Primor & Zlotkin, 1975; Hashimoto *et al.*, 1974; Randall *et al.*, 1971; Hashimoto & Kamiya, 1969; Thomson, 1964).

2.3.4 Defensive Toxins – Surfactant Mechanism

Subsequent research discovered that the ichthyotoxic and hemotoxic activities of numerous species' ichthyocrinotoxins are primarily perpetrated by components with similar chemical properties. For instance, pardaxin, pahutoxin, and grammistin, were isolated from the ichthyocrinotoxins of *P. marmoratus* (Primor & Zlotkin, 1975), *O. meleagris* (Boylan & Scheuer, 1967), and various members of the Serranidae family (Randall *et al.*, 1971),

respectively. While grammistin and pardaxin were shown to be α -helical-containing amphiphilic peptides (Primor & Tu, 1980; Primor *et al.*, 1978), and pahutoxin was identified as an amphipathic fatty acid (Kalmanzon & Zlotkin, 2009), all three components were characterised as surfactants.

Surfactants act to reduce surface tension and/or interfacial tension between two immiscible phases and play diverse roles within biological systems, particularly in the exchange of gas and nutrients across cell membranes (Otzen, 2017). However, some surfactants, especially synthetic ones, can harm aquatic life by damaging gill membranes, thereby reducing the animal's ability to osmoregulate, destroying the external mucous layers that provide protection from bacteria and parasites, and diminishing breeding ability and reproductive output (Johnson *et al.*, 2021; Kalmanzon & Zlotkin, 2009). The surfactant components within some ichthyocrinotoxins are believed to have similar detrimental effects, particularly on the gill membranes of predatory species (Primor & Zlotkin, 1975).

Numerous studies have shown that exposure to ichthyocrinotoxins resulted in increased opercular movements in fish, indicating respiratory distress (Primor *et al.*, 1984; Primor & Lazarovici, 1981; Primor & Zlotkin, 1975; Thomson, 1969). Furthermore, the ichthyotoxicity of pardaxin was found to increase with the salinity of the surrounding media, suggesting that it may influence osmoregulatory processes (Primor *et al.*, 1980). It was also reported that pardaxin inhibits (Na⁺ K⁺)-dependent ATPase (i.e., the sodium-potassium pump), which would result in an influx of sodium ions across gill membranes (Primor & Lazarovici, 1981; Primor *et al.*, 1980). Some grammistins (e.g., lipogrammistin A) were found to produce similar effects (Onuki *et al.*, 1998; Onuki *et al.*, 1993). For instance, lipogrammistin A inserts three to four monomers in the inner leaflet of lipid bilayers, causing a transformation in cell shape (Kobayashi *et al.*, 1999). This cell transformation results in small lesions in the bilayer, allowing the entry of small molecules (i.e., salts and urea) into the cell, creating an osmotic imbalance across the membrane which ultimately leads to colloid-osmotic lysis of erythrocytes (Kobayashi *et al.*, 1999).

The toxic activity of pardaxins (Primor *et al.*, 1984) and grammistins (Shiomi *et al.*, 2000) were initially believed to operate non-selectively, affecting any gill membrane in their immediate vicinity. However, one study revealed that soapfish were immune to their own toxin and exhibited cannibalistic behaviours towards other members within the family (Randall *et al.*, 1971). Similarly, *Ostracion cubicus* (Yellow Boxfish) that released their ichthyocrinotoxin in

a closed aquarium system remained unaffected (Kalmanzon *et al.*, 1999). It was later hypothesised that the toxic factors, once considered non-specific, might be interacting with smaller receptor-mediated proteins (Kalmanzon & Zlotkin, 2000; Kalmanzon *et al.*, 1999). The interaction between this protein and its corresponding surfactant (i.e., pahutoxin) was suggested to enable a high degree of specificity towards certain critical target sites (i.e., the gill membranes of predator fish), that are presumably absent in the boxfish's own cell membranes (Kalmanzon *et al.*, 2003; Kalmanzon *et al.*, 1999). It is essential to note that although extensive compositional and structural data exist for the ichthyocrinotoxins of some species, others, such as those belonging to members of *Synanceia*, remain relatively unexplored.

2.3.5 Harnessing both Venom and Ichthyocrinotoxin

Contrary to common perception, not all ichthyocrinotoxins serve a defensive role against predators. Despite their ichthyotoxic and hemotoxic properties, ichthyocrinotoxins secreted by *Arius bilineatus* (Arabian Gulf Catfish) do not appear to play a direct role in predator defence (Al-Hassan *et al.*, 1987). Similar to observations in *Gobiodon* spp., one study showed that predatory fish were attracted to, and caught on, both baited and bare fishing hooks that had been coated with the toxin from *A. bilineatus* (Al-Hassan *et al.*, 1985; Al-Hassan *et al.*, 1982). Given that *A. bilineatus* are not site-attached, it is unlikely that their ichthyocrinotoxin is designed to protect resources. However, it is important to note that *A. bilineatus* also possess a venom stored in glands located at the base of two serrated pectoral spines (Al-Hassan *et al.*, 1987; Thulesius *et al.*, 1983). People envenomed by *A. bilineatus* reportedly experience long-lasting pain, local muscle cramping, paralysis, oedema, and gangrene (Thulesius *et al.*, 1983). Although not directly tested, *A. bilineatus* venom is hypothesised to function as a predator defence (Al-Hassan *et al.*, 1987; Thulesius *et al.*, 1983; Al-Hassan *et al.*, 1982), which further suggests that their ichthyocrinotoxins likely serve an alternative function.

In contrast to *A. bilineatus*, stonefish (*Synanceia* spp.) ichthyocrinotoxins demonstrated minimal haemolytic and ichthyotoxic activity (Cameron *et al.*, 1981). For instance, *Gambusia* spp. (Mosquito Fish), were unaffected for up to 22 hours when immersed in 1/200 and 1/100 dilutions of *Synanceia horrida* (Estuarine Stonefish) ichthyocrinotoxin but did not survive in 1/50 conditions (Cameron *et al.*, 1981). *Petroscirtes japonicus* (Muzzled Blenny), on the other hand, exhibited some discomfort and a reddening of the cloacal region after immersion (1/200 and 1/100 dilutions) but returned to normal two hours after being transferred to uncontaminated conditions (Cameron *et al.*, 1981). While stonefish ichthyocrinotoxins do not possess the

harmful properties characteristic of defensive toxins, it remains to be determined whether they induce food refusal behaviour directly or indirectly. It is worth noting that, akin to *A. bilineatus*, stonefish also produce a potent venom stored within twin glands associated with each of the 13 dorsal spines (Saggiomo *et al.*, 2021; Endean, 1961). These spines are known to become erect, and, in some cases, are strategically positioned when the animal perceives a threat (Saggiomo *et al.*, 2021; Endean, 1961).

The evolution of fish venoms has been proposed to stem from the ability of the epidermis to generate toxic proteinaceous secretions, such as ichthyocrinotoxins (Harris & Jenner, 2019; Cameron & Endean, 1973). Linking a harmful substance like ichthyocrinotoxin to an injection mechanism is thought to offer a selective advantage, especially when used for defence against predators (Cameron & Endean, 1973). Injecting a defensive substance directly into a predator likely enhances the toxin's efficacy while minimising the amount of toxin needed to deter the predator effectively (Cameron & Endean, 1973). This notion implies that the venom of *A. bilineatus* and *Synanceia* spp. may be better suited for predator defence. It also emphasises that the ichthyocrinotoxins of certain species may serve alternate defensive functions, such as in various aspects of innate (non-specific) immunity (Al-Hassan *et al.*, 1982; Liguori, 1963; Endean, 1961).

2.4 The Role of Ichthyocrinotoxins in Innate Immunity

The innate immune system serves as the primary line of natural defence against illness and injury in vertebrates (Sitjà-Bobadilla *et al.*, 2015; Coelho, 2013). This system consists of two main components. The first component comprises physical barriers, such as scales, skin, and mucosal secretions, which function to obstruct, or ensnare, invading organisms before they can infiltrate the body (Elumalai *et al.*, 2019; Sitjà-Bobadilla *et al.*, 2015). These physical barriers, like scales, not only play a role in thwarting harmful agents, but also serve as a protective armour, shielding the organism from external damage, and lessening the severity of injuries (Coelho, 2013; Cameron & Endean, 1973).

The second line of innate defence consists of chemical barriers, including lectins, lysozymes, and anti-microbial peptides, which are designed to eliminate pathogens both before and after they enter the body (Elumalai *et al.*, 2019; Sitjà-Bobadilla *et al.*, 2015). Although ichthyocrinotoxic species typically lack physical barriers, such as scales (Cameron & Endean,

1973), their skin secretions have demonstrated the ability to influence wound healing and impede pathogen development.

2.4.1 Catalysing Wound Healing

Similar to other ichthyocrinotoxic species, *A. bilineatus* secrete copious amounts of their ichthyocrinotoxin when threatened (Al-Hassan *et al.*, 1982). It was initially assumed that the ichthyocrinotoxins of these species served as a defensive mechanism, containing repellent or harmful substances that worked in conjunction with the venom in their pectoral spines to deter predators (Al-Hassan *et al.*, 1982). One study reported that *A. bilineatus* ichthyocrinotoxins are complex mixtures comprising over 60 unique proteins (Al-Hassan *et al.*, 1985). However, these components were found to lack protease and nuclease activity, suggesting that the toxin does not possess any qualities that could aid the fish in deterring predators (Al-Hassan *et al.*, 1985).

Instead, these secretions were found to contain factors that promote accelerated clotting, vasoconstriction, and red blood cell agglutination, which are typical responses to injury and infection (Al-Hassan *et al.*, 1991; Al-Hassan *et al.*, 1987; Al-Hassan *et al.*, 1985; Al-Hassan *et al.*, 1982). Furthermore, the substances catalysing each of these reactions were generally not present, or only found in very low levels in the skin secretions of non-ichthyocrinotoxic fish (Al-Hassan *et al.*, 1985). Consequently, researchers proposed that the ecological function of *A. bilineatus* ichthyocrinotoxins is not to reduce the likelihood or severity of predatory encounters, but rather to facilitate wound healing by secreting a substance that catalyses the process (Al-Hassan *et al.*, 1985; Al-Hassan *et al.*, 1983, 1982).

Further studies confirmed that the epidermal secretions of *A. bilineatus* promote wound closure and accelerate the healing of wounds and diabetic ulcers in both laboratory animals and humans (Al-Hassan *et al.*, 1991; Al-Hassan *et al.*, 1983). Animals treated with *A. bilineatus* ichthyocrinotoxin preparations demonstrated rapid passage through the inflammatory phase of wound repair, and entered the proliferative phase within a week (Al-Hassan *et al.*, 1991). Remarkably, the toxin significantly accelerated clotting and reduced the healing time of various wounds in rodents and humans by up to 60 percent (Al-Hassan *et al.*, 1991; Al-Hassan *et al.*, 1983). To date, *A. bilineatus* remains the only ichthyocrinotoxic species investigated for this activity. It is yet to be determined whether ichthyocrinotoxins from other species, such as those secreted by members of *Synanceia*, also accelerate wound repair. Given that *Synanceia* ichthyocrinotoxins, as well as those from other well-studied fish species, lack

haemagglutinating properties, it remains plausible that this function is unique to ichthyocrinotoxins secreted by *A. bilineatus*.

2.4.2 Microbial Defence

When organisms sustain injuries, they become more vulnerable to bacterial attacks, making it essential for the innate immune system to prevent infection while facilitating wound repair (Rakers *et al.*, 2013). In non-ichthyocrinotoxic teleosts, this is achieved through the presence of anti-microbial compounds in their epidermal mucosal secretions (Rakers *et al.*, 2013). Similarly, some species' ichthyocrinotoxins have also been found to contain anti-microbial agents. For example, pardaxin (*P. marmoratus* ichthyocrinotoxin), which principally serves as a predator defence, also exhibits anti-bacterial activity against Gram-negative *Escherichia coli*, but not against Gram-positive *Staphylococcus* (Thennarasu & Nagaraj, 1996). Likewise, some members of the Serranidae family have demonstrated anti-microbial activity in their ichthyocrinotoxins. Liguori (1963) reported that both aqueous and 95% ethanol extracts of lyophilised material from *G. sexlineatus* inhibited the growth of *E. coli* in a concentration-dependant manner. In contrast, *P. punctata* ichthyocrinotoxins reportedly showed no anti-bacterial activity against six species of bacteria (Oshima, 1974). However, newer studies have cast doubts on these initial findings, as the grammistins belonging to both *G. sexlineatus* and *P. punctata* were confirmed to exhibit broad-spectrum anti-bacterial activity against nine species, including both Gram-negative and Gram-positive strains (Yokota *et al.*, 2001). While our distinction between anti-microbial peptides present within the mucosal secretions of all fish, and those specific to ichthyocrinotoxins, hinges on the source of the secretion – i.e., general mucosal cells and specialised ichthyocrinotoxin-producing glandular structures, respectively – it is important to consider the potential coexistence of both types within the epidermal environment of ichthyocrinotoxic species. This coexistence may have the potential to bolster the microbial defences of these species, however, further research is required to substantiate these claims.

Interestingly, anti-microbial pardaxins and grammistins share a similar structural motif (i.e., as amphipathic α -helicies) with those identified from various other biological sources, such as melittin from honeybee venom, and cecropins from the hemolymph of *Cecropia* moths (Thennarasu & Nagaraj, 1996). While grammistins possess anti-biotic properties without haemolytic action (Sugiyama *et al.*, 2006; Shiomi *et al.*, 2001; Shiomi *et al.*, 2000), the structure of pardaxins is strongly linked with its haemolytic activity, presenting a challenge for

researchers seeking to develop them into therapeutics. However, it was discovered that the anti-bacterial activity of pardaxin could be significantly increased while reducing its haemolytic activity by applying a positive charge to the N-terminal and C-terminal domains, suggesting that a synthetic redesign of these compounds could make them viable anti-microbial therapeutics (Thennarasu & Nagaraj, 1996).

It is clear that some species of fish have evolved unique strategies to compensate for the absence of conventional physical barriers, relying on an interplay between their secretions and chemical barriers to maintain their health and protect themselves from harm. However, current knowledge of the wound-healing and anti-microbial activity of ichthyocrinotoxins is limited across numerous species, especially for those like *Synanceia*, whose skin toxins seemingly do not serve a role in predator defence.

2.5 The Role of Ichthyocrinotoxins in Regulating Integumentary Interactions

In addition to exhibiting reduced or absent scales, many ichthyocrinotoxic organisms also demonstrate a sedentary and/or site-attached lifestyle (Cameron & Endean, 1973; Endean, 1961). This behaviour increases the vulnerability of ichthyocrinotoxic species to various benthic organisms including parasites, as well as fouling flora and fauna (Cameron & Endean, 1973; Endean, 1961). As such, it has been proposed that the ichthyocrinotoxins produced by some species may function to regulate these interactions with their integument.

2.5.1 Parasite Defence

A substantial proportion of the animal diversity found in coral reef ecosystems is comprised of parasites, with some estimates indicating that they constitute at least 30 percent of the total population (Adlard *et al.*, 2010). Helminths (particularly flukes), as well as myxozoans are among the most prevalent endoparasites affecting tropical teleosts; flukes are typically found in the gut lumen, while myxozoans inhabit the muscle, brain and gall bladder (Cribb *et al.*, 2014). The majority of the thirteen families of ichthyocrinotoxic teleosts also reside in tropical waters within coral reef ecosystems, thus exposing them to the myriad of parasites that flourish in these environments (Cameron & Endean, 1973). As primarily bottom-dwelling or site-attached organisms with reduced or absent scales (Cameron & Endean, 1973), ichthyocrinotoxic species are likely to be particularly susceptible to parasitic invasion.

Synanceia (stonefish) exemplify this vulnerability, as they possess a scale-free epidermis and primarily adopt a sedentary lifestyle, often burrowing into the substratum (Cameron & Endean, 1973; Endean, 1961). Researchers have hypothesised that utilising ichthyocrinotoxin as an anti-parasitic agent, rather than physical barriers like scales, could offer selective advantages for sedentary, non-pelagic fish such as stonefish, since scale pockets may facilitate parasitic invasions (Cameron & Endean, 1973). Despite this, very little is currently known about the potential anti-parasitic properties of *Synanceia* ichthyocrinotoxin. However, the limited number of parasites recovered from stonefish provides some support for this notion. To date, only six parasite taxa including; two myxosporeans (Miller *et al.*, 2018), two digenetic trematodes (Liu *et al.*, 2010; Wang, 1985), an ergasilid copepod (Amado *et al.*, 2001), and an endo-parasitic turbellarian (Ogawa, 2011), have been formally reported and are unique to the nine genera comprising the Synanceiidae family. However, among all known ichthyocrinotoxic species, only one study by Munday *et al.* (2003) has directly investigated the possible anti-parasitic properties of ichthyocrinotoxins.

Munday *et al.* (2003) examined the interactions between ichthyocrinotoxic *Gobiodons* and non-toxic *Paragobiodons* with external parasites in both field and laboratory settings. Field observations revealed an absence of parasites in the toxic *Gobiodons*, while 41% of non-toxic *Paragobiodons* were infected (Munday *et al.*, 2003). However, contrary to these findings, a laboratory-based experiment suggested that the rates of parasite attachment did not differ between the two species (Munday *et al.*, 2003). Instead, the site of attachment was restricted to toxin gland-sparse areas, such as the gills or fins in toxic species (Munday *et al.*, 2003). The lack of studies in this area highlights the need for further exploration, particularly for more sedentary species such as members of *Synanceia*.

2.5.2 Selective Anti-Foulant

Epibiosis, which is the non-parasitic growth of one organism (the epibiont) on another (the basibiont), can present both advantages and disadvantages for the basibiont (Wahl, 1989). In aquatic organisms, the benefits of epibiosis, may encompass enhanced nutrient flow (i.e., vitamins, nitrogenous compounds), physical or chemical protection (i.e., camouflage), and drag reduction in certain cases (Fernandez-Leborans, 2010; Wahl, 1989). However, the detrimental effects of epibiosis tend to be more numerous and, for fish, can result in increased weight and reduced buoyancy, limited mobility, greater surface friction, and damage to soft tissues caused by mechanical attachment or changes in pH levels around the settlement surface (Fernandez-

Leborans, 2010; Wahl, 2008, 1989). Epibiosis can also adversely impact aquatic plants (i.e., seagrasses) through increased shading, competition for nutrients, and herbivory (Hay *et al.*, 2004; Wahl, 1989). Given the potential harmful consequences of epibiosis, marine organisms have evolved three primary strategies against it: tolerance, avoidance, and chemical defences (Wahl, 1989). Some species, such as stonefish (*Synanceia* spp.), are thought to employ ichthyocrinotoxins to regulate interactions with their integument, allowing the settlement of beneficial epibionts while inhibiting the attachment of less-favourable species (Cameron & Endean, 1973; Fishelson, 1973; Endean, 1961).

Synanceia members host a diverse assortment of epibionts, including algae, diatoms, and hydrozoans, which reportedly match those covering their surrounding substrata (Fishelson, 1973; Endean, 1961). The combination of these epibionts, with the intricate rock-like texture of the animals' skin, and their predominantly sedentary lifestyle enables stonefish to blend seamlessly into their environment (Fishelson, 1973; Endean, 1961). It has been reported that stonefish undergo a sloughing process two to three times a year to remove accumulated epibionts from their skin (Fishelson, 1973). Sloughing, similar to the process in reptiles, involves keratinisation of the top three to four epidermal layers, followed by detachment from the younger, cell-producing layers over a period of three to four hours (Fishelson, 1973). While sloughing is suggested to be a response to the build-up of algae and hydrozoans on stonefish skin (Fishelson, 1973), the reason for shedding beneficial epibionts remains unclear.

One possibility is that sloughing serves as a maintenance system to prevent overgrowth of these organisms which could hinder the animal's sensory (i.e., vision) and/or physical (i.e., speed) ability to effectively ambush prey. Furthermore, since these organisms play a critical role in the animal's camouflage, stonefish might also use a chemical simulant, like ichthyocrinotoxin, to expedite the reattachment of these organisms. Whether stonefish ichthyocrinotoxins aid plant attachment is currently untested and should be a focus of future research exploring the ecology of these toxins. Additionally, the absence and potential control of fouling organisms, such as barnacles that could damage stonefish skin, remain poorly understood and unreported. Considering that stonefish undergo sloughing only a few times per year (Fishelson, 1973), while organisms like barnacles are likely to attach much more frequently, it is probable that stonefish utilise other mechanisms, such as ichthyocrinotoxin, to safeguard their integument from detrimental interactions.

Although there is no direct evidence supporting these hypotheses, a study conducted by Cameron *et al.* (1981) offers valuable insight into the biological activity of *Synanceia* ichthyocrinotoxins. The researchers discovered that a component within *S. horrida* ichthyocrinotoxin, designated as FI, immobilised two species of ciliated protozoans, *Blepharisma* and *Paramecium*, within 30 seconds of immersion in 1/500 solutions (Cameron *et al.*, 1981). Furthermore, 60% of prawns intra-muscularly injected with FI displayed violent tail flick twitching, while the remaining prawns slowly rolled over onto their sides and were unable to right themselves within three minutes following the injection (Cameron *et al.*, 1981). In contrast, another component, referred to as FII, had no effect on either ciliated protozoans or prawns (Cameron *et al.*, 1981). Instead, FII increased the tone of barnacle depressor scutorum rostralis muscles (i.e., the muscle that controls the scuta lips) for up to one hour after immersion in *S. horrida* ichthyocrinotoxin, and did so to a slightly greater extent (20%) than FI (Cameron *et al.*, 1981).

Both components were also found to halt the ciliary beating of mussel gills within minutes of exposure to crude toxin extracts, but within 40 to 160 minutes of exposure to ten to 50% preparations (Cameron *et al.*, 1981). As a result, the authors suggested that one potential function of *Synanceia* ichthyocrinotoxins could be to deter ciliated and/or flagellated pathogens (i.e., helminths), as well as metazoan larvae such as barnacle cyprids (Cameron *et al.*, 1981). Nonetheless, additional research is required to substantiate these claims.

2.6 Future Research Directions for Stonefish Ichthyocrinotoxins

Ichthyocrinotoxins encompass a broad array of ecological functions and biochemical activities which are primarily centred around defence (**Table 2.1**). While some of these functions are well-documented across multiple species, there is a paucity of data specific to stonefish ichthyocrinotoxins. Therefore, further research is required to determine the natural function of stonefish ichthyocrinotoxins, as well as their potential therapeutic or industrial applications.

Table 2.1 A Summary of the Reported Ecological Functions of Ichthyocrinotoxins Secreted by Fish.

Function	Biochemical Activity	Species	References
Anti-feedant	Deter predation by making the user/resource unpalatable	<i>Rypticus saponaceus</i> <i>Pardachirus marmoratus</i> <i>Gobiodon histrio</i>	Randall, 1967; Clark, 1983; Dirnwoeber & Herler, 2013
Defensive Poison	Damages gill membranes, inhibits (Na ⁺ K ⁺)-dependent ATPase, disrupts osmoregulatory processes	<i>Pardachirus marmoratus</i> <i>Ostracion cubicus</i> <i>Ostracion meleagris</i> various Serranidae species	Primor & Zlotkin 1975; Primor et al., 1980; Onuki et al., 1988; Shiomi et al., 2000
Wound Healing	Accelerates blood coagulation, causes vasoconstriction and haemagglutination of red blood cells	<i>Arius bilineatus</i>	Al-Hassan et al., 1991; Al-Hassan et al., 1983
Microbial Defence	Inhibits bacterial growth	<i>Pardachirus marmoratus</i> <i>Grammistes sexlineatus</i> <i>Pogonoperca punctata</i>	Thennarasu & Nagaraj, 1996; Yokota et al., 2001
Parasite Defence	Inhibits/reduces parasitic invasion, influences the attachment site of parasites	<i>Gobiodon spp.</i> <i>Synanceia horrida</i>	Munday et al., 2003; Miller et al., 2018; Cameron et al., 1981
Mitigating Epibiosis	Increases tone of barnacle scutorum rostralis muscle, immobilises ciliated protozoans, Algae present on skin	<i>Synanceia horrida</i>	Cameron et al., 1981; Fishelson 1973 Berger

One area of research that warrants further exploration is the potential role of stonefish ichthyocrinotoxins in predator defence. Although stonefish ichthyocrinotoxins, unlike their venom, were shown to lack the noxious attributes of a defensive toxin (Saggiomo *et al.*, 2021; Cameron *et al.*, 1981), future studies should aim to assess their ability to function as an anti-feedant substance. It may be that stonefish toxins serve a dual anti-predatory approach, whereby the ichthyocrinotoxins act as a first line of defence aimed at sensory and/or gustatory repulsion, which would thus allow the venom to be conserved as a ‘more extreme’ second line of defence against persistent pursuers. As such, it is imperative to design a feeding study that incorporates several known or potential predators of stonefish to accurately gauge the toxins effect on their olfactory and/or gustatory senses. Although the possible anti-predatory properties of stonefish ichthyocrinotoxins are unlikely to have medical or industrial significance, it could reveal crucial information regarding the ecology of these animals.

Another promising research avenue is determining the role of stonefish ichthyocrinotoxins in parasite defence. Stonefish are both ecologically and physiologically vulnerable to parasitic invasion, yet the number of parasite taxa identified in *Synanceia* is limited (Miller *et al.*, 2018; Cameron & Endean, 1973; Endean, 1961). To further explore these phenomena, a well-designed study assessing the activity of these toxins on parasites *in vitro*, with a focus on species such as helminths and myxozoans that are likely to interact with stonefish, is essential. Biomolecular separation and purification techniques would then be necessary to identify the responsible constituents. Investigations into the anti-parasitic properties of stonefish ichthyocrinotoxins are likely to uncover novel defence mechanisms employed by these animals, which would contribute significantly to our understanding of stonefish ecology. Additionally, such research has the potential to provide valuable insights for the development of novel treatments in both human and veterinary medicine, addressing the severe and devastating impacts that parasitic diseases have on almost all taxa across the animal kingdom (Adade & Souto-Padrón, 2015; Brooker *et al.*, 2009; Ouellette & Ward, 2003). Furthermore, given the increasing problem of parasite resistance to current anthelmintic treatment options, particularly within agricultural sectors (Charlier *et al.*, 2022; Nielsen, 2022; Kotze & Prichard, 2016; Besier & Love, 2003), this research becomes even more imperative.

In addition to parasite defence, exploring the potential influence of stonefish ichthyocrinotoxins on epibiosis represents another important area in need of further study. Stonefish are known to host a diverse range of epibionts on their integument and periodically

slough their epidermal layer to remove accumulated organisms (Fishelson, 1973; Endean, 1961). However, the specific role of stonefish ichthyocrinotoxins in either facilitating or inhibiting the attachment of epibiotic species is currently unknown. What is needed is a study comparing the species assemblages and relative abundances of fouling organisms between artificial surfaces both uncoated and coated in stonefish ichthyocrinotoxins. It is also important to note that *S. verrucosa* and *S. horrida* primarily occupy two different marine environments, those being coral reefs and estuaries, respectively (Endean, 1961). Furthermore, reports suggest that the epibionts associated with the epidermis of these species mirror those in their immediate surroundings (Cameron & Endean, 1973; Fishelson, 1973; Endean, 1961). Therefore, further investigation into whether the ichthyocrinotoxins produced by *S. verrucosa* and *S. horrida* confer a selective advantage on epibiosis within their respective primary habitats would be highly beneficial. These pursuits are likely to not only enhance our current understanding of the ecology of these fishes and the function of their skin toxins, but also potentially reveal fascinating evolutionary adaptations between stonefish species. Additionally, the findings of such studies could have practical implications in industrial settings.

One potential industrial application of stonefish ichthyocrinotoxins would be in the discovery of novel, environmentally friendly anti-fouling agents. As biofouling poses significant challenges to marine structures, vessels, and equipment (Davidson *et al.*, 2023; Fonseca, 2022; Schultz *et al.*, 2011), inhibitory compounds derived from stonefish ichthyocrinotoxins could hold significant value from both an operational and biosecurity perspective. Alternatively, stimulatory components within stonefish ichthyocrinotoxins, particularly those that promote the settlement or enhance the growth of algae, could be harnessed to boost commercial aquaculture operations. One notable example is the current interest in establishing a land-based commercial facility for cultivating red seaweed (*Asparagopsis taxiformis*) intended for use as a dietary supplement to reduce the carbon footprint of commercial livestock (Roque *et al.*, 2021). In this context, the development of an effective fertiliser could play a pivotal role in optimising the growth and productivity of algae crops by improving yields and reducing the need for the spatial expansion of these facilities to meet growing demand.

2.7 Conclusion

In conclusion, there is a clear need for further research to unravel the ecological function, therapeutic applications, and industrial potential of stonefish ichthyocrinotoxins. Future investigations should focus on the toxin's role in predator defence, parasite prevention, and regulating epibiosis. Studying the natural function of stonefish ichthyocrinotoxins and their impact on predators, parasites, and fouling organisms would both expand upon our currently limited understanding of stonefish ecology, as well as potentially inspire the development of novel treatments in human and veterinary medicine and offer practical solutions in industrial settings.

2.8 References

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2.9 APPENDIX A

Stonefish (*Synanceia* spp.) Ichthyocrinotoxins: An Ecological Review and Prospectus for Future Research and Biodiscovery.

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Stonefish (*Synanceia* spp.) Ichthyocrinotoxins: An ecological review and prospectus for future research and biodiscovery

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ABSTRACT

Marine organisms possess a diverse array of unique substances, many with wide ranging potential for applications in medicine, industry, and other sectors. Stonefish (*Synanceia* spp.), a bottom-dwelling fish that inhabit shallow and intertidal waters throughout the Indo-Pacific, harbour two distinct substances, a venom, and an ichthyocrinotoxin. Stonefish are well-known for the potent venom associated with their dorsal spines as it poses a significant risk to public health. Consequently, much of the research on stonefish focusses on the venom, with the aim of improving outcomes in cases of envenomation. However, there has been a notable lack of research on stonefish ichthyocrinotoxins, a class of toxin that is synthesised within specialised epithelial cells (i.e., tubercles) and exuded onto the skin. This has resulted in a substantial knowledge gap in our understanding of these animals. This review aims to bridge this gap by consolidating literature on the ecological functions and biochemical attributes of ichthyocrinotoxins present in various fish species and juxtaposing it with the current state of knowledge of stonefish ecology. We highlight the roles of ichthyocrinotoxins in predator defence, bolstering innate immunity, and mitigating integumentary interactions with parasites and detrimental fouling organisms. The objective of this review is to identify promising research avenues that could shed light on the ecological functions of stonefish ichthyocrinotoxins and their potential practical applications as therapeutics and/or industrial products.

1. Introduction

The marine realm, encompassing over 242,000 documented species (Ahyong et al., 2023), is a trove of unexplored substances potentially useful to humans (Blunt et al., 2018). Among these are ichthyocrinotoxins, a substance produced within the skin of certain fish. The term is derived from Greek to literally mean ‘*ikhthus*’ – fish (Cresswell, 2021), and ‘*crin*’ – to secrete (Klionsky et al., 2007), in combination with the English word ‘toxin’ – substances causing harm to living organisms (Nelsen et al., 2014). Ichthyocrinotoxins are defined as those that produce a toxin within specialised epithelial glandular structures, such as tubercles, that are independent of traumagenic devices like spines (Halstead, 1988). While ichthyocrinotoxins have been revealed as a complex pharmacy of components, only some of which exhibit toxic activity, this review uses the term to refer to the complete substance originating from these specialised glandular structures akin to other multi-component toxin descriptors such as “venom” and “poison”.

Approximately 50 species of fish across at least 13 families are

known to harbour an ichthyocrinotoxin, one of these being stonefish, i. e., *Synanceia* spp. (Cameron and Endean, 1973; Halstead, 1988). Two prominent species, *S. horrida* (estuarine stonefish) and *S. verrucosa* (reef stonefish), are found in the Indo-Pacific region, inhabiting shallow intertidal waters, coral reefs, and rocky environments (Fig. 1) (Endean, 1961; Saggiomo et al., 2021).

These species are notorious for possessing the most potent venom among fish, which is an injectable toxin housed within twin glands associated with the 13 hypodermic needle-like spines on the animal's back (Harris et al., 2021; Mohebbi, 2021; Saggiomo et al., 2021). Envenomation can cause a diverse range of symptoms, including excruciating pain, paralysis, cardiac arrest, and in certain cases, death (Endean, 1961; Harris et al., 2021; Mohebbi, 2021; Saggiomo et al., 2021). Due to their cryptic nature, envenomation cases involving stonefish are frequent and accounted for 9.3 percent of marine animal related incidents resulting in hospitalisation in Australia between 2017 and 2018 (Pointer and Harrison, 2021). Consequently, much of the research on stonefish focusses on the venom, particularly around improving

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outcomes in cases of envenomation. However, investigations into the nature and biochemical properties of the ichthyocrinotoxins that stonefish secrete onto their epidermis is limited. Therefore, this review aims to consolidate literature on ichthyocrinotoxins present in various fish species including the toxins' function in predator defence, bolstering innate immunity, and mitigating integumentary interactions. By juxtaposing this with the current knowledge of stonefish ecology, we aim to identify promising research avenues that could shed light on the function of stonefish ichthyocrinotoxins and their potential practical applications as therapeutics and/or industrial products.

2. The role of ichthyocrinotoxins in predator defence

Chemical defences, such as venoms and poisons, often play a crucial role in an organisms' defence against predators. These defences can function either through sensory aversion (i.e., anti-feedants) or by causing physical harm to cells and disrupting cellular processes (i.e., defensive toxins) (Harris and Jenner, 2019; Tachibana, 1988). Ichthyocrinotoxins are thought to serve both purposes, defending against direct predators and, in some cases, indirect predators that target the organisms' resources.

2.1. Anti-feedants

Anti-feedants are substances that reduce the rate of herbivory and/or predation, and are a common chemical defence employed by a variety of terrestrial and aquatic organisms, particularly plants (Bruno et al., 2002; Chaieb et al., 2009; Harborne, 1991; Wöll et al., 2013) and invertebrates (Eisner and Aneshansley, 2000; Kasumyan et al., 2021; Roitberg and Isman, 1992). The primary function of anti-feedant substances is to deter predation by making the user unpalatable (Chaieb et al., 2009; Kasumyan et al., 2021; Roitberg and Isman, 1992; Wöll et al., 2013). Consequently, predators typically react to organisms that harness anti-feedant substances by immediately expelling and/or regurgitating them during feeding attempts, or by avoiding these organisms altogether (Roitberg and Isman, 1992; Wöll et al., 2013).

The potential of ichthyocrinotoxins to function as anti-feedants was initially highlighted in a study examining the feeding habits of predatory fishes in the West Indies (Randall, 1967). The study reported that no *Rypticus saponaceus* (greater soapfish) were present amongst the gut contents of predatory fishes, which the author suggested could be due to the bitter taste of their ichthyocrinotoxins (Randall, 1967). This hypothesis was later supported by a study on the feeding responses of both wild and captive elasmobranchs to ichthyocrinotoxins secreted by *Pardachirus marmoratus*, also known as the Red Sea Moses sole (Clark,

1983). The study reported noteworthy variations in ingestion times depending on the toxicity of the fish (Clark, 1983). Under typical circumstances, captive whitetip sharks (*Charcharhinus longimanus*) reportedly consumed non-toxic fish within a minute (Clark, 1983). In contrast, ichthyocrinotoxic *P. marmoratus* were largely ignored by the sharks for anywhere between seven and 28 hours, and in most cases were only consumed after being deceased for several hours (Clark, 1983). Furthermore, 14 percent of predatory attempts on live *P. marmoratus* by *C. longimanus* induced anti-feeding responses (Clark, 1983). These behavioural responses included the sharks jerking away, swimming erratically, and gaping their mouths, ultimately leaving the fish untouched (Clark, 1983). Moreover, baited lines with dead or live *P. marmoratus*, as well as non-toxic teleosts coated with or in close proximity (up to 30 cm) to the ichthyocrinotoxin, were observed to repel wild grey reef sharks (*Charcharhinus amblyrhynchus*) for up to 16 hours (Clark, 1983). However, it is crucial to highlight recent findings indicating the absence of type II taste receptors ('T2R's) in cartilaginous predators, which are responsible for perceiving 'bitterness' (Behrens et al., 2021; Sharma et al., 2019). Consequently, if the aversive response of elasmobranchs to *P. marmoratus* and *R. saponaceus* ichthyocrinotoxins indeed stems from sensory factors, it is highly likely that these reactions are linked to olfactory mechanisms rather than gustatory ones. Nevertheless, it has been observed that ichthyocrinotoxins do not consistently deter predators. Specifically, previous studies have reported that predatory fish were both attracted to (Lassig, 1981), and would attempt to consume (Gratzer et al., 2015) ichthyocrinotoxic *Gobiiodons* (coral-dwelling gobies) outside of their coral host. Instead, one study has shown that *Gobiiodon* ichthyocrinotoxins, particularly those secreted by *G. histrio*, might serve a more strategic role in resource defence (Dirnwoeber and Herler, 2013). In their study, Dirnwoeber and Herler (2013) tracked the predatory encounters initiated by corallivorous Chaedontidae (butterfly fish) on both *Gobiiodon*-inhabited and uninhabited colonies of *Acropora*, a small-polyp stony coral. The results revealed a significant decrease in predation on *Acropora* colonies that were inhabited by ichthyocrinotoxic *Gobiiodons* compared to those that were not (Dirnwoeber and Herler, 2013). This gave rise to the hypothesis that *Gobiiodon* ichthyocrinotoxins might serve as a more potent line of defence against indirect predators, like the coral-eating Chaedontidae, rather than direct predators (Dirnwoeber and Herler, 2013). Furthermore, the authors proposed that *Gobiiodon* ichthyocrinotoxins might function as a resource defence by inducing strong avoidance and most likely food refusal behaviours in corallivorous Chaedontidae (Dirnwoeber and Herler, 2013).



Fig. 1. Two prominent species of stonefish that occur in Queensland (North Eastern Australia) waters. The reef stonefish, *Synanceia verrucosa* (left), and the estuarine stonefish, *Synanceia horrida* (right) are considered the world's most venomous fishes.

2.2. Defensive toxins - fish

Building upon these insights into the complex interplay between ichthyocrinotoxins and predators, it is also worth noting that in certain species, these toxins not only influence palatability but have also been documented to induce adverse physiological effects on vertebrates. This suggests their potential function as defensive toxins. Unlike anti-feedants, which primarily target the gustatory and/or olfactory senses of predators (Chaieb et al., 2009), defensive toxins produced by fish are designed to damage cells or disrupt cellular processes (Ellisdon et al., 2015; Harris and Jenner, 2019). For instance, certain components present in the venom of *Synanceia* (stonefish) have been found to form pores in cell membranes, while others bind to nicotinic acetylcholine receptors and interrupt neuronal signalling (Ellisdon et al., 2015; Harris and Jenner, 2019; Harris et al., 2021). It is believed that these combined activities induce significant pain to deter predators and cause localised paralysis – most likely in the predators' mouths – to improve the fishes chances of a successful escape (Ellisdon et al., 2015; Harris et al., 2021).

The toxic effects of ichthyocrinotoxins produced by certain teleost fishes are well-documented, with early reports stemming from fishermen's observations of lethal effects when particular species were kept together (Kalmanson and Zlotkin, 2009; Maretzki and Del Castillo, 1967; Randall et al., 1971). Early ichthyologists discovered that members of several fish families, including Serranidae (soapfish), Ostraciidae (boxfish), and Soleidae (flatfish), secrete large amounts of slime (i.e., ichthyocrinotoxin) in response to stress or excitement (Beebe and Tee-Van, 1928; Brock, 1956). The field collection of some species, such as *Ostracion meleagris* (previously *Ostracion lentiginosus*, spotted boxfish), revealed that ichthyocrinotoxins could be noxious to other fish (Brock, 1956).

Studies conducted in a controlled aquarium environment demonstrated that when exposed to ichthyocrinotoxins numerous non-toxic teleost species exhibited motor excitation or hyperactivity, followed by a resting period at the bottom of the tank (Clark and George, 1979; Maretzki and Del Castillo, 1967; Thomson, 1969). Recovery from these symptoms depended on the species of ichthyocrinotoxin to which the fish were exposed (Maretzki and Del Castillo, 1967; Thomson, 1969). For instance, exposure to *O. meleagris* ichthyocrinotoxins led to irreparable illness and death within an hour, while exposure to *Rypticus saponaceus* (greater soapfish) ichthyocrinotoxins could be reversed within one to two hours of transfer to clean, toxin-free water (Maretzki and Del Castillo, 1967; Thomson, 1969).

2.3. Defensive toxins - mammals

Moreover, ichthyocrinotoxins previously identified to be ichthyotoxic were also found to exhibit hemotoxic properties. Studies reported that the forced consumption or injection of ichthyocrinotoxins belonging to certain fish species had severe detrimental effects on mammals (Goldberg et al., 1982, 1988; Hashimoto and Kamiya, 1969; Hashimoto et al., 1974; Primor and Zlotkin, 1975; Randall et al., 1971; Thomson, 1964). For instance, cats experienced vomiting, increased salivation, and diarrhoea, leading to loss of motor activity, paralysis, and coma within days of ingesting *Pogonoperca punctata* (spotted soapfish) ichthyocrinotoxin (Hashimoto and Kamiya, 1969). Likewise, mice injected with ichthyocrinotoxins from *O. meleagris* (spotted boxfish), *P. punctata* (spotted soapfish), or *R. saponaceus* (greater soapfish) exhibited ataxia and laboured breathing, leading to a comatose state and death (Hashimoto and Kamiya, 1969; Maretzki and Del Castillo, 1967; Thomson, 1964). *In vitro* tests confirmed that the symptoms observed in mammals were likely due to the toxins' haemolytic and/or hemagglutinating activity on mammalian erythrocytes (Goldberg et al., 1982, 1988; Hashimoto and Kamiya, 1969; Hashimoto et al., 1974; Primor and Zlotkin, 1975; Randall et al., 1971; Thomson, 1964).

2.4. Defensive toxins – surfactant mechanism

Subsequent research discovered that the ichthyotoxic and hemotoxic activities of numerous species' ichthyocrinotoxins are primarily perpetrated by components with similar chemical properties.

For instance, pardaxin, pahutoxin, and grammistin, were isolated from the ichthyocrinotoxins of *P. marmoratus* (Red Sea Moses sole; Primor and Zlotkin, 1975), *O. meleagris* (spotted boxfish; Boylan and Scheuer, 1967), and various members of the Serranidae family (soapfish; Randall et al., 1971), respectively. While grammistin and pardaxin were shown to be α -helical-containing amphiphilic peptides (Primor et al., 1978; Primor and Tu, 1980), and pahutoxin was identified as an amphipathic fatty acid (Kalmanson and Zlotkin, 2009), all three components were characterised as surfactants.

Surfactants act to reduce surface tension and/or interfacial tension between two immiscible phases and play diverse roles within biological systems, particularly in the exchange of gas and nutrients across cell membranes (Otzen, 2017). However, some surfactants, especially synthetic ones, can harm aquatic life by damaging gill membranes, thereby reducing the animals' ability to osmoregulate, destroying the external mucous layers that provide protection from bacteria and parasites, and diminishing breeding ability and reproductive output (Johnson et al., 2021; Kalmanson and Zlotkin, 2009). The surfactant components within some ichthyocrinotoxins are believed to have similar detrimental effects, particularly on the gill membranes of predatory species (Primor and Zlotkin, 1975).

Numerous studies have shown that exposure to ichthyocrinotoxins resulted in increased opercular movements in fish, indicating respiratory distress (Primor and Lazarovici, 1981; Primor et al., 1984; Primor and Zlotkin, 1975; Thomson, 1969). Furthermore, the ichthyotoxicity of pardaxin was found to increase with the salinity of the surrounding media, suggesting that it may influence osmoregulatory processes (Primor et al., 1980). It was also reported that pardaxin inhibits (Na + K⁺)-dependent ATPase (i.e., the sodium-potassium pump), which would result in an influx of sodium ions across gill membranes (Primor and Lazarovici, 1981; Primor et al., 1980).

Some grammistins (e.g., lipogrammistin A) were found to produce similar effects (Onuki et al., 1998; Onuki et al., 1993). For instance, lipogrammistin A inserts three to four monomers in the inner leaflet of lipid bilayers, causing a transformation in cell shape (Kobayashi et al., 1999). This cell transformation results in small lesions in the bilayer, allowing the entry of small molecules (i.e., salts and urea) into the cell, creating an osmotic imbalance across the membrane and leading to colloid-osmotic lysis of erythrocytes (Kobayashi et al., 1999).

The toxic activity of pardaxins (Primor et al., 1984) and grammistins (Shiomi et al., 2000) were initially believed to operate non-selectively, affecting any gill membrane in their immediate vicinity. However, one study revealed that soapfish were immune to their own toxin and even exhibited cannibalistic behaviours towards other members within the family (Randall et al., 1971). Similarly, *Ostracion cubicus* that released their ichthyocrinotoxin in a closed aquarium system remained unaffected (Kalmanson et al., 1999). It was later hypothesised that the toxic factors, once considered non-specific, might be interacting with smaller receptor-mediated proteins (Kalmanson and Zlotkin, 2000; Kalmanson et al., 1999). The interaction between this protein and its corresponding surfactant (i.e., pahutoxin) was suggested to enable a high degree of specificity towards certain critical target sites (i.e., the gill membranes of predator fish), that are presumably absent in the boxfishes own cell membranes (Kalmanson et al., 1999, 2003). It is essential to note that although extensive compositional and structural data exist for the ichthyocrinotoxins of some species, others, such as those belonging to members of *Synanceia*, remain relatively unexplored.

2.5. Harnessing both venom and ichthyocrinotoxin

Contrary to common perception, not all ichthyocrinotoxins serve a

defensive role against predators. Despite their ichthyotoxic and hemotoxic properties, ichthyocrotinotoxins secreted by *Arius bilineatus* (Arabian Gulf catfish) did not appear to play a direct role in predator defence (Al-Hassan et al., 1987). Similar to the case in *Gobiiodons*, one study showed that predatory fish were attracted to, and caught on, both baited and bare fishing hooks that had been coated with the toxin from *A. bilineatus* (Al-Hassan et al., 1982, 1985). However, since *A. bilineatus* is not site-attached, it is unlikely that their ichthyocrotinotoxin is designed to protect resources. Notably, *A. bilineatus* also possesses a venom stored in glands located at the base of two serrated pectoral spines (Al-Hassan et al., 1987; Thulesius et al., 1983). People envenomed by *A. bilineatus* reportedly experience long-lasting pain, local muscle cramping, paralysis, oedema, and gangrene (Thulesius et al., 1983). Although not directly tested, *A. bilineatus* venom is believed to function as a predator defence (Al-Hassan et al., 1982, 1987; Thulesius et al., 1983).

In contrast to *A. bilineatus*, stonefish (*Synanceia* spp.) ichthyocrotinotoxins demonstrated minimal haemolytic and ichthyotoxic activity (Cameron et al., 1981). Mosquito fish (*Gambusia* spp.), for example, were unaffected for up to 22 hours when immersed in 1/200 and 1/100 dilutions of *S. horrida* ichthyocrotinotoxin but did not survive in 1/50 conditions (Cameron et al., 1981). Blennies, on the other hand, exhibited some discomfort and a reddening of the cloacal region after immersion (1/200 and 1/100 dilutions) but returned to normal 2 hours after being transferred to uncontaminated conditions (Cameron et al., 1981). While stonefish ichthyocrotinotoxins do not possess the harmful properties characteristic of defensive toxins, it remains to be determined whether they induce food refusal behaviour directly or indirectly. It is worth noting that, akin to *A. bilineatus*, stonefish also produce a potent venom stored within twin glands associated with 13 dorsal spines (Endean, 1961; Saggiomo et al., 2021). These spines are known to become erect and, in some cases, are strategically positioned when the animal perceives a threat (Endean, 1961; Saggiomo et al., 2021).

The evolution of fish venoms has been proposed to stem from the ability of the epidermis to generate toxic proteinaceous secretions, such as ichthyocrotinotoxins (Cameron and Endean, 1973; Harris and Jenner, 2019). Linking a harmful substance like ichthyocrotinotoxin to an injection mechanism is thought to offer a selective advantage, especially when used for self-defence against predators (Cameron and Endean, 1973). Injecting a defensive substance directly into the predators' blood stream likely enhances the toxins' efficacy while minimising the amount needed to deter the predator effectively (Cameron and Endean, 1973). This notion implies that the venom of *A. bilineatus* and *Synanceia* spp. may be better suited for predator defence. It also emphasises that the ichthyocrotinotoxins of certain species may serve alternate defensive functions, such as in various aspects of innate (non-specific) immunity (Al-Hassan et al., 1982; Endean, 1961; Liguori, 1963).

3. The role of ichthyocrotinotoxins in innate immunity

The innate immune system serves as the primary line of natural defence against illness and injury in vertebrates (Coelho, 2013; Sitjà-Bobadilla et al., 2015). This system consists of two main components. The first component comprises physical barriers, such as scales, skin, and mucosal secretions, which function to obstruct, or ensnare, invading organisms before they can infiltrate the body (Elumalai et al., 2019; Sitjà-Bobadilla et al., 2015). These physical barriers, like scales, not only play a role in thwarting harmful agents but also serve as a protective armour, shielding the organism from external damage and lessening the severity of injuries (Cameron and Endean, 1973; Coelho, 2013).

The second line of innate defence consists of chemical barriers, including lectins, lysozymes, and anti-microbial peptides, which are designed to eliminate pathogens both before and after they enter the body (Elumalai et al., 2019; Sitjà-Bobadilla et al., 2015). Although ichthyocrotinotoxic species typically lack physical barriers such as scales (Cameron and Endean, 1973), their skin secretions have demonstrated

the ability to influence wound healing and impede pathogen development.

3.1. Catalysing wound healing

Similar to other ichthyocrotinotoxic species, *A. bilineatus* (Arabian Gulf catfish) secrete copious amounts of their ichthyocrotinotoxin when threatened (Al-Hassan et al., 1982). It was initially assumed that catfish ichthyocrotinotoxins served as a defensive mechanism, containing repellent or harmful substances that worked in conjunction with the venom in their pectoral spines to deter predators (Al-Hassan et al., 1982). One study reported that catfish ichthyocrotinotoxins are complex mixtures comprising over 60 unique proteins (Al-Hassan et al., 1985). However, these components were found to lack protease and nuclease activity, suggesting that the toxin does not possess any qualities that could aid the fish in deterring predators (Al-Hassan et al., 1985).

Instead, these secretions were found to contain factors that promote accelerated clotting, vasoconstriction, and red blood cell agglutination, which are typical responses to injury and infection (Al-Hassan et al., 1982, 1985, 1987, 1991). Furthermore, the substances catalysing each of these reactions were generally not present, or only found in very low levels in the skin secretions of non-ichthyocrotinotoxic fish (Al-Hassan et al., 1985). Consequently, researchers proposed that the ecological function of *A. bilineatus* ichthyocrotinotoxins is not to reduce the likelihood or severity of predatory encounters, rather to facilitate wound healing by secreting a substance that catalyses the process (Al-Hassan et al., 1982, 1983, 1985).

Further studies confirmed that the epidermal secretions of *A. bilineatus* promote wound closure and accelerate the healing of wounds and diabetic ulcers in both laboratory animals and humans (Al-Hassan et al., 1983, 1991). Animals treated with *A. bilineatus* ichthyocrotinotoxin preparations demonstrated rapid passage through the inflammatory phase of wound repair and entered the proliferative phase within a week (Al-Hassan et al., 1991). Remarkably, the toxin significantly accelerated clotting and reduced the healing time of various wounds in rodents and humans by up to 60 percent (Al-Hassan et al., 1983, 1991). To date, *A. bilineatus* remains the only ichthyocrotinotoxic species investigated for this activity. It is yet to be determined whether ichthyocrotinotoxins from other species, such as those secreted by *Synanceia*, also accelerate wound repair. Given that *Synanceia* ichthyocrotinotoxins and those from other well-studied species lack hemagglutinating properties, it remains plausible that this function is unique to catfish.

3.2. Microbial defence

When organisms sustain injuries, they become more vulnerable to bacterial attacks, making it essential for the innate immune system to prevent infection while facilitating wound repair (Rakers et al., 2013). In non-ichthyocrotinotoxic teleosts, this is achieved through the presence of anti-microbial compounds in their epidermal mucosal secretions (Rakers et al., 2013). Similarly, some species ichthyocrotinotoxins have also been found to contain anti-microbial agents. For instance, pardaxin (*P. marmoratus* ichthyocrotinotoxin), which principally serves as a predator defence, also exhibits anti-bacterial activity against Gram-negative *Escherichia coli*, but not against Gram-positive *Staphylococcus* (Thennarasu and Nagaraj, 1996). Likewise, some members of the Serranidae family have demonstrated anti-microbial activity in their ichthyocrotinotoxins. Liguori (1963) reported that both aqueous and 95 percent ethanol extracts of lyophilised epidermal material from *G. sexlineatus* inhibited the growth of *E. coli* in a concentration-dependant manner. In contrast, *P. punctata* ichthyocrotinotoxins reportedly showed no anti-bacterial activity against six species of bacteria (Oshima, 1974). However, newer studies have cast doubts on these initial findings, as the graminins belonging to both *G. sexlineatus* and *P. punctata* were confirmed to exhibit broad-spectrum anti-bacterial activity against nine species,

including both Gram-negative and Gram-positive strains (Yokota et al., 2001). While our distinction between anti-microbial peptides present in the mucosal secretions of all fish and those specific to ichthyocrinotoxins hinges on the source of the secretion – i.e., general mucosal cells and specialised ichthyocrinotoxin-producing glandular structures, respectively – it is important to consider the potential coexistence of both types within the epidermal environment of ichthyocrinotoxic species. This coexistence may have the potential to bolster the microbial defences of these species, however, further research is required to substantiate these claims.

Interestingly, anti-microbial pardaxins and grammistins share a similar structural motif (i.e., as amphipathic α -helices) with those identified from various other biological sources, such as melittin from honeybee venom and cecropins from the hemolymph of *Cecropia* moths (Thennarasu and Nagaraj, 1996). While grammistins possess anti-biotic properties without haemolytic action (Shiomi et al., 2000, 2001; Sugiyama et al., 2006), the structure of pardaxins is strongly linked with its haemolytic activity, presenting a challenge for researchers seeking to develop them into therapeutics. However, it was discovered that the anti-bacterial activity of pardaxin could be significantly increased while reducing its haemolytic activity by applying a positive charge to the N-terminal and C-terminal domains, suggesting that a synthetic redesign of these compounds could make them viable anti-microbial therapeutics (Thennarasu and Nagaraj, 1996).

It is clear that some species of fish have evolved unique strategies to compensate for the absence of conventional physical barriers, relying on the interplay between their secretions and chemical barriers to maintain their health and protect themselves from harm. However, the current knowledge of wound-healing and anti-microbial activity of ichthyocrinotoxins is limited for numerous species, especially for those like *Synanceia*, whose skin toxins seemingly do not serve a role in predator defence.

4. The role of ichthyocrinotoxins in mitigating integumentary interactions

In addition to exhibiting reduced or absent scales, many ichthyocrinotoxic organisms also demonstrate a sedentary and/or site-attachment lifestyle (Cameron and Endean, 1973; Endean, 1961). This behaviour increases the vulnerability of ichthyocrinotoxic species to various benthic organisms including parasites, as well as fouling flora and fauna (Cameron and Endean, 1973; Endean, 1961). Consequently, it has been proposed that the ichthyocrinotoxins produced by some species may function to mitigate these interactions with their integument.

4.1. Parasite defence

A substantial proportion of the animal diversity found in coral reef ecosystems is comprised of parasites, with some estimates indicating that they constitute at least 30 percent of the total population (Adlard et al., 2010). Helminths (particularly flukes), as well as myxozoans are among the most prevalent endoparasites affecting tropical teleosts; flukes are typically found in the gut lumen, while myxozoans inhabit the muscle, brain and gall bladder (Adlard et al., 2010; Cribb et al., 2014). The majority of the thirteen families of ichthyocrinotoxic teleosts also reside in tropical waters within coral reef ecosystems, thus exposing them to the myriad of parasites that flourish in these environments (Cameron and Endean, 1973). As primarily bottom-dwelling or site-attached organisms with reduced or absent scales, ichthyocrinotoxic species are likely to be particularly susceptible to parasitic invasion.

Synanceia (stonefish) exemplify this vulnerability, as they possess a scale-free epidermis and primarily adopt a sedentary lifestyle, often burrowing into the substratum (Cameron and Endean, 1973; Endean, 1961). Researchers have hypothesised that utilising ichthyocrinotoxin as an anti-parasitic agent, rather than physical barriers like scales, could offer selective advantages for sedentary, non-pelagic fish such as

stonefish, since scale pockets may facilitate parasitic invasions (Cameron and Endean, 1973). Despite this, very little is currently known about the potential anti-parasitic properties of *Synanceia* ichthyocrinotoxin. However, the limited number of parasites recovered from stonefish provides some support for this notion. To date, only six parasite taxa including; two myxosporeans (Miller et al., 2018), two digenetic trematodes (Liu et al., 2010; Wang, 1985), an *ergasilid* copepod (Amado et al., 2001), and an endo-parasitic turbellarian (Ogawa, 2011), have been formally reported and are unique to the nine genera comprising the *Synanceiidae* family. However, among all known ichthyocrinotoxic species, only one study by Munday et al. (2003) has directly investigated the possible anti-parasitic properties of ichthyocrinotoxins.

Munday et al. (2003) examined the interactions between ichthyocrinotoxic *Gobiiodons* and non-toxic *Paragobiiodons* with external parasites in both field and laboratory settings. Field observations revealed an absence of parasites in the toxic *Gobiiodons*, while 41 percent of non-toxic *Paragobiiodons* were infected (Munday et al., 2003). However, contrary to these findings, a laboratory-based experiment suggested that the rates of parasite attachment did not differ between the two species (Munday et al., 2003). Instead, the site of attachment was restricted to toxin gland-sparse areas such as the gills or fins in toxic species (Munday et al., 2003). The lack of studies this area highlights the need for further exploration, particularly for more sedentary species such as members of *Synanceia*.

4.2. Selective anti-foulant

Epibiosis, which is the non-parasitic growth of one organism (the epibiont) on another (the basibiont), can present both advantages and disadvantages for the basibiont (Wahl, 1989). In aquatic organisms, the benefits of epibiosis, may encompass enhanced nutrient flow (i.e., vitamins, nitrogenous compounds), physical or chemical protection (i.e., camouflage), and drag reduction in certain cases (Fernandez-Leborans, 2010; Wahl, 1989). However, the detrimental effects of epibiosis tend to be more numerous and, for fish, can result in increased weight and reduced buoyancy, limited mobility, greater surface friction, and damage to soft tissues caused by mechanical attachment or changes in pH levels around the settlement surface (Fernandez-Leborans, 2010; Wahl, 1989; 2008). Epibiosis can also adversely impact aquatic plants (i.e., seagrasses) through increased shading, competition for nutrients, and herbivory (Hay et al., 2004; Wahl, 1989). Given the potential harmful consequences of epibiosis, marine organisms have evolved three primary strategies against it: tolerance, avoidance, and chemical defences (Wahl, 1989). Some species, such as stonefish (*Synanceia* spp.), are thought to employ ichthyocrinotoxins to regulate interactions with their integument, allowing the settlement of beneficial epibionts while inhibiting the attachment of less-favourable species (Cameron and Endean, 1973; Endean, 1961; Fishelson, 1973).

Synanceia members host a diverse assortment of epibionts, including algae, diatoms, and hydrozoans, which reportedly match those covering their surrounding substrata (Endean, 1961; Fishelson, 1973). The combination of these epibionts, with the intricate rock-like texture of the animals' skin, and their predominantly sedentary lifestyle enables stonefish to blend seamlessly into their environment (Endean, 1961; Fishelson, 1973). It has been reported that stonefish undergo a sloughing process two to three times a year to remove accumulated epibionts from their skin (Fishelson, 1973). Sloughing, similar to the process in reptiles, involves keratinisation of the top three to four epidermal layers, followed by detachment from the younger, cell-producing layers over a period of three to four hours (Fishelson, 1973). While sloughing is suggested to be a response to the build-up of algae and hydrozoans on stonefish skin (Fishelson, 1973), the reason for shedding beneficial epibionts remains unclear.

One possibility is that sloughing serves as a maintenance system to prevent overgrowth of these organisms which could hinder the animals' sensory (i.e., vision) and/or physical (i.e., speed) ability to effectively

ambush prey. Furthermore, since these organisms play a critical role in the animals' camouflage, stonefish might also use a chemical simulant, like ichthyocrinotoxin, to expedite the reattachment of these organisms. Whether stonefish ichthyocrinotoxins aid plant attachment is currently untested and should be a focus of future research exploring the ecology of these toxins. Additionally, the absence and potential control of fouling organisms, such as barnacles that could damage stonefish skin, remain poorly understood and unreported. Considering that stonefish undergo sloughing only a few times per year (Fishelson, 1973), while organisms like barnacles are likely to attach much more frequently, it is probable that stonefish utilise other mechanisms, such as ichthyocrinotoxin, to safeguard their integument from detrimental interactions.

Although there is no direct evidence supporting these hypotheses, a study conducted by Cameron et al. (1981) offers valuable insight into the biological activity of *Synanceia* ichthyocrinotoxins. The researchers discovered that a component within *S. horrida* ichthyocrinotoxin, designated as FI, immobilised two species of ciliated protozoans, *Blapharisma* and *Paramecium*, within 30 seconds of immersion in 1/500 solutions (Cameron et al., 1981). Furthermore, 60 percent of prawns intra-muscularly injected with FI displayed violent tail flick twitching, while the remaining prawns slowly rolled over onto their sides and were unable to right themselves within 3 minutes following the injection (Cameron et al., 1981). In contrast, another component, referred to as FII, had no effect on either ciliated protozoans or prawns (Cameron et al., 1981). Instead, FII increased the tone of barnacle depressor scutorum rostralis muscles (i.e., the muscle that controls the scuta lips) for up to 1 hour after immersion in *S. horrida* ichthyocrinotoxin, and did so to a slightly greater extent (20 percent) than FI (Cameron et al., 1981).

Both components were also found to halt the ciliary beating of mussel gills within minutes of exposure to crude toxin extracts, but within 40 to 160 minutes of exposure to ten to 50 percent preparations (Cameron et al., 1981). As a result, the authors suggested that one potential function of *Synanceia* ichthyocrinotoxins could be to deter ciliated and/or flagellated pathogens (i.e., helminths), as well as metazoan larvae such as barnacle cyprids (Cameron et al., 1981). Nonetheless, additional research is required to substantiate these claims.

5. Future research directions for stonefish ichthyocrinotoxins

Ichthyocrinotoxins encompass a broad array of ecological functions and biochemical activities which are primarily centred around defence (Table 1). While some of these functions are well-documented across multiple species, there is a paucity of data specific to stonefish ichthyocrinotoxins. Therefore, further research is required to determine the natural function of stonefish ichthyocrinotoxins, as well as their potential therapeutic or industrial applications.

One area of research that warrants further exploration is the potential role of stonefish ichthyocrinotoxins in predator defence. Although stonefish ichthyocrinotoxins, unlike their venom, were shown to lack the noxious attributes of a defensive toxin (Cameron et al., 1981; Saggiomo et al., 2021), future studies should aim to assess their ability to function as an anti-feedant substance. It may be that stonefish toxins serve a dual anti-predatory approach, whereby the ichthyocrinotoxins act as a first line of defence aimed at sensory and/or gustatory repulsion, which would thus allow the venom to be conserved as a 'more extreme' second line of defence against persistent pursuers. As such, it is imperative to design a feeding study that incorporates several known or potential predators of stonefish to accurately gauge the toxins effect on their olfactory and/or gustatory senses. Although the possible anti-predatory properties of stonefish ichthyocrinotoxins are unlikely to have medical or industrial significance, it could reveal crucial information regarding the ecology of these animals.

Another promising research avenue is determining the role of stonefish ichthyocrinotoxins in parasite defence. Stonefish are both ecologically and physiologically vulnerable to parasitic invasion, yet the number of parasite taxa identified in *Synanceia* is limited (Cameron and

Table 1

A summary of the reported ecological functions of ichthyocrinotoxins across fish species.

Function	Biochemical Activity	Species	References
Anti-feedant	Deter predation by making the user/resource unpalatable	<i>Rypticus saponaceus</i> <i>Pardachirus marmoratus</i> <i>Gobiodon histrio</i>	Randall (1967); Clark (1983); Dirnwoeber and Herler (2013)
Defensive Poison	Damages gill membranes, inhibits (Na + K ⁺)-dependent ATPase, disrupts osmoregulatory processes	<i>Pardachirus marmoratus</i> <i>Ostracion cubicus</i> <i>Ostracion meleagris</i> various species	Primor and Zlotkin (1975); Primor et al. (1980); Onuki et al., 1993; Shiomi et al. (2000)
Wound Healing	Accelerates blood coagulation, causes vasoconstriction and haemagglutination of red blood cells	<i>Arius bilineatus</i>	Al-Hassan et al. (1991); Al-Hassan et al. (1983)
Microbial Defence	Inhibits bacterial growth	<i>Pardachirus marmoratus</i> <i>Grammistes sexlineatus</i> <i>Pogonoperca punctata</i>	(Thennarasu and Nagaraj, 1996; Yokota et al. (2001)
Parasite Defence	Inhibits/reduces parasitic invasion, influences the attachment site of parasites	<i>Gobiodon</i> spp. <i>Synanceia horrida</i>	(Munday et al., 2003; Miller et al., 2018; Cameron et al. (1981)
Mitigating Epibiosis	Increases tone of barnacle scutorum rostralis muscle, immobilises ciliated protozoans, Algae present on skin	<i>Synanceia horrida</i>	(Cameron et al., 1981; Fishelson 1973)

Endean, 1973; Endean, 1961; Miller et al., 2018). To further explore these phenomena, a well-designed study assessing the activity of these toxins on parasites *in vitro*, with a focus on species such as helminths and myxozoans that are likely to interact with stonefish, is essential. Bio-molecular separation and purification techniques would then be necessary to identify the responsible constituents. Investigations into the anti-parasitic properties of stonefish ichthyocrinotoxins are likely to uncover novel defence mechanisms employed by these animals, which would contribute significantly to our understanding of stonefish ecology. Additionally, such research has the potential to provide valuable insights for the development of novel treatments in both human and veterinary medicine, addressing the severe and devastating impacts that parasitic diseases have on almost all taxa across the animal kingdom (Adade and Souto-Pradón, 2015; Brooker et al., 2009; Ouellette and Ward, 2003). Furthermore, given the increasing problem of parasite resistance to current anthelmintic treatment options, particularly within agricultural sectors (Besier and Love, 2003; Charlier et al., 2022; Kotze and Prichard, 2016; Nielsen, 2022), this research becomes even more imperative.

In addition to parasite defence, exploring the potential influence of stonefish ichthyocrinotoxins on epibiosis represents another important area in need of further study. Stonefish are known to host a diverse range of epibionts on their integument and periodically slough their epidermal layer to remove accumulated organisms (Endean, 1961; Fishelson, 1973). However, the specific role of stonefish ichthyocrinotoxins in either facilitating or inhibiting the attachment of epibiotic species is currently unknown. What is needed is a study comparing the species assemblages and relative abundances of fouling organisms between artificial surfaces both uncoated and coated in stonefish ichthyocrinotoxins. It is also important to note that *S. verrucosa* and *S. horrida* primarily occupy two different marine environments, those being coral

reefs and estuaries, respectively (Endean, 1961). Furthermore, reports suggest that the epibionts associated with the epidermis of these species mirror those in their immediate surroundings (Cameron and Endean, 1973; Endean, 1961; Fishelson, 1973). Therefore, further investigation into whether the ichthyocinotoxins produced by *S. verrucosa* and *S. horrida* confer a selective advantage on epibiosis within their respective primary habitats would be highly beneficial. These pursuits are likely to not only enhance our current understanding of the ecology of these fishes and the function of their skin toxins, but also potentially reveal fascinating evolutionary adaptations between stonefish species. Additionally, the findings of such studies could have practical implications in industrial settings.

One potential industrial application of stonefish ichthyocinotoxins would be in the discovery of novel, environmentally friendly anti-fouling agents. As biofouling poses significant challenges to marine structures, vessels, and equipment (Davidson et al., 2023; Fonseca, 2022; Schultz et al., 2011), inhibitory compounds derived from stonefish ichthyocinotoxins could hold significant value from both an operational and biosecurity perspective. Alternatively, stimulatory components within stonefish ichthyocinotoxins, particularly those that promote the settlement or enhance the growth of algae, could be harnessed to boost commercial aquaculture operations. One notable example is the current interest in establishing a land-based commercial facility for cultivating red seaweed (*Asparagopsis taxiformis*) intended for use as a dietary supplement to reduce the carbon footprint of commercial livestock (Roque et al., 2021). In this context, the development of an effective fertiliser could play a pivotal role in optimising the growth and productivity of algae crops by improving yields and reducing the need for the spatial expansion of these facilities to meet growing demand.

6. Conclusion

In conclusion, there is a clear need for further research to unravel the ecological function, therapeutic applications, and industrial potential of stonefish ichthyocinotoxins. Future investigations should focus on the toxins role in predator defence, parasite prevention, and mitigating epibiosis. Studying the natural function of stonefish ichthyocinotoxins and their impact on predators, parasites, and fouling organisms would both expand upon our currently limited understanding of stonefish ecology, as well as potentially inspire the development of novel treatments in human and veterinary medicine and offer practical solutions in industrial settings.

Ethical Statement for Solid State Ionics

Hereby, I Danica Lennox-Bulow consciously assure that for the manuscript “Stonefish (*Synanceia* spp.) Ichthyocinotoxins: an ecological review and prospectus for future research and biodiscovery” the following is fulfilled:

- 1) This material is the authors' own original work, which has not been previously published elsewhere.
- 2) The paper is not currently being considered for publication elsewhere.
- 3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
- 4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
- 5) The results are appropriately placed in the context of prior and existing research.
- 6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.
- 7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

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I agree with the above statements and declare that this submission follows the policies of Solid State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

Date: July 14, 2023.

CRedit authorship contribution statement

Danica Lennox-Bulow: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Michael Smout:** Writing – review & editing. **Alex Loukas:** Writing – review & editing. **Jamie Seymour:** Writing – review & editing.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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Chapter 3

Diel Activity Patterns and Habitat Preferences of Stonefish (*Synanceia* spp.) in Captivity

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D Lennox-Bulow is the principal author of this manuscript and was extensively involved in all aspects of this work under the supervision of J Seymour. R Courtney and B Ebner assisted with conceptualisation, data collection, statistical analyses, editing and reviewing earlier versions of this manuscript, and animal husbandry. B Ebner provided photographs of stonefish.

3.1 Abstract

Synanceia verrucosa (Reef Stonefish) and *Synanceia horrida* (Estuarine Stonefish) are known as the world's most venomous fishes. However, little is known about their habitat preferences and activity patterns. This study aimed to determine the habitat preferences and diel activity patterns of stonefish. The movement of four adults of each species offered complex and simple habitat was recorded by filming in captivity during day and night periods. Generally, both species exhibited similar behaviour, except that *S. verrucosa* travelled further per movement than *S. horrida*. Collectively, stonefishes spent 94% of their time associated with complex habitat and moved significantly less frequently on complex (1.6 movements per hour) relative to simple habitat (17.3 movements per hour). Stonefishes were least active around sunset in the late afternoon and evening, which may be related to an active feeding period during the morning or paradoxically to a higher probability of ambush feeding success near sunset. These findings have enhanced our current understanding of the ecology of these medically important species.

3.2 Introduction

The Reef Stonefish, *Synanceia verrucosa*, and the Estuarine Stonefish, *Synanceia horrida*, are members of the Synanceiidae family (**Figure 3.1**) and are known as the world's most venomous fish (Isbister, 2001; Meyers, 1999). Stonefish are widely distributed in both the tropical and sub-tropical coastal marine waters of the Indian and Pacific oceans, including around northern Australia (Saggiomo *et al.*, 2021). *Synanceia verrucosa* is found on reef flats, shallow lagoons and in small intertidal pools, whereas *S. horrida* inhabits estuaries and near shore areas (Burnett *et al.*, 1996; Grobecker, 1983; Endean, 1961). Stonefish are cryptic, ambush predators and remain motionless for extended periods of time (Burnett *et al.*, 1996; Grobecker, 1983; Endean, 1961) but will engulf nearby prey with explosive speed (Ferry-Graham & Lauder, 2001; Grobecker, 1983; Endean, 1961).

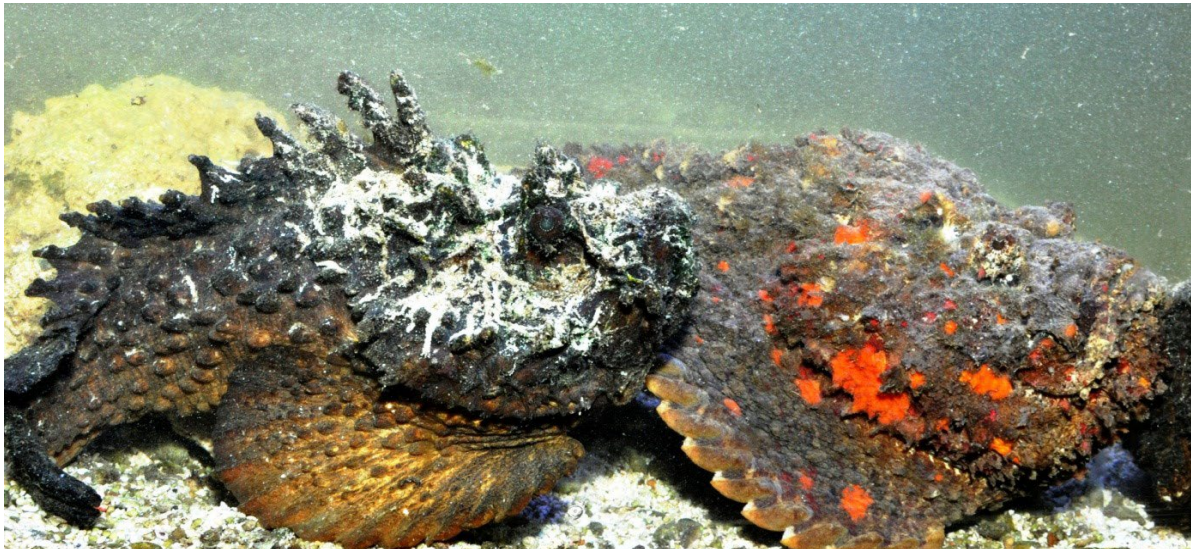


Figure 3.1 Two Stonefish Species found in Queensland (North-Eastern Australia) Waters. Image shows a side view of the Estuarine Stonefish, *Synanceia horrida* (Left), and Reef Stonefish, *Synanceia verrucosa* (Right).

Research conducted on stonefish has primarily focused on their venom and venom delivery system (Isbister, 2001; Burnett *et al.*, 1996; Kizer *et al.*, 1985; Endean, 1961), particularly their associated human medical significance (Ngo *et al.*, 2009; Brenneke & Hatz, 2006). In contrast, scant research attention has been directed toward the ecology of these species including, feeding, habitat use, and activity patterns (but see Grobecker, 1983). Unlike fast-swimming pursuit predators that rely on speed and agility to capture prey (Juanes *et al.*, 2002), stonefish are slow-swimming, ambush predators that mimic benthic microhabitat (Grobecker, 1983; Endean, 1961). Stonefish are highly cryptic, a common morphological adaptation to

avoid detection by prey and predators that are visually oriented (Hairston *et al.*, 1982; Endler, 1981). Furthermore, stonefish can change colour to better match their environment (Loannou & Krause, 2009; Grobecker, 1983; Endean, 1961). Background matching is less effective when animals are in motion; therefore, many cryptic animals tend to stay still (Loannou & Krause, 2009; Zhang & Richardson, 2007). Moreover, evidence suggests that crypsis and lack of motion form a synergistic relationship reducing the probability of detection (Loannou & Krause, 2009). Additionally, stonefish facilitate algal growth on their body surfaces which enhances their ability to blend into their surrounding environment (Ballantine *et al.*, 2001; Burnett *et al.*, 1996) and may also actively attract herbivorous fish and invertebrates into the animals strike range (Grobecker, 1983).

Habitat type has also been shown to be an important factor that contributes not only to background matching, but also to the efficiency of foraging success for many ambush predators (Horinouchi *et al.*, 2009; Schultz *et al.*, 2009; Laurel & Brown, 2006; Savino & Stein, 1989). Structurally complex environments have been shown to reduce the efficiency of pursuit predators through reduced mobility and decreased vision (Laurel & Brown, 2006; Abrahams, 2005). However, the foraging efficiency of ambush predators in structurally complex environments is mediated by the mode of predation, where these predators capitalise on their ability to ambush prey (Schultz *et al.*, 2009; Stephens & Krebs, 1986). Stonefish are commonly associated with complex environments; however, they can also be found buried in the sand amongst simple environments (Burnett *et al.*, 1996; Grobecker, 1983; Endean, 1961). It is unknown which habitat type is preferred by stonefish by day or night. Subsequently, the aims of this study were to determine the habitat preferences and diel activity patterns of *S. verrucosa* and *S. horrida* to gain a better understanding of the ecology of these medically important species.

3.3 Materials and Methods

Two adult *S. verrucosa* and two adult *S. horrida* (330 – 410 mm L_T) were housed in each of two aquaria (dimensions L x W x D: 2.3 x 1.2 x 0.6 m) with a benthos comprising of sand (0.1 m deep) in one half (termed simple habitat) and rock and large coral rubble in the other half (termed complex habitat) (**Figure 3.2**). A ten-day acclimation period was provided before filming commenced. The aquaria were exposed to natural light and shielded from direct sunlight. Filming of each aquarium occurred simultaneously over two 9-hour intervals during the day (08:00 – 17:00 hours) and two 9-hour intervals at night (20:00 – 05:00 hours). These time frames were chosen to compare behaviour patterns between dark and light conditions.



Figure 3.2 View from One of the Experimental Aquariums Used in the Study. Two substrate types were used; simple (Left) comprised of sand only, and complex (Right) containing a mixture of sand, rock, and large coral rubble. Arrows indicate the location of stonefish on complex habitat.

The time each individual spent occupying each habitat type was quantified from film. The position of individuals resting on the boundary between two habitat types was classified according to the habitat containing the majority of the fish's body. A three-way between groups analysis of variance (ANOVA) was conducted using the statistical package SPSS v. 17 to determine the effect of habitat type (complex, simple), condition (light, dark) and species (*S. verrucosa* and *S. horrida*) on the total amount of time spent in each environment. A further

three-way between groups ANOVA was conducted to explore the frequency of movements by each species per hour, in relation to habitat type and day or night.

Distance was calculated from viewed film footage by marking the initial position of each stonefish, tracking each movement on a display monitor and measuring the on-screen distance travelled. The on-screen measure was then converted to the true scale movement distance ± 0.05 m and movements under 0.1 m were excluded. The time each movement occurred was recorded and rounded to the nearest minute. The movement times were then divided into hourly intervals. A two-way repeated measure ANOVA and post hoc Least Significant Difference (LSD) were conducted using the statistical package SPSS v. 17 to explore if the average distance travelled per movement differed between species, and if the frequency of movement differed according to time of day or night (among hourly intervals).

3.4 Results

3.4.1 Stonefish Habitat Preference

There was no significant interaction between habitat (complex, simple), species (*S. verrucosa*, *S. horrida*), and/or period within the diel cycle (day, night), in relation to total amount of time spent in each habitat (**Figure 3.3**, species*habitat*diel cycle) [$F_{1,24} = 0.72$, $p = 0.40$]. There was also no significant interaction of the mean time spent in each habitat between the species (species*habitat) [$F_{1,24} = 1.79$, $p = 0.19$]; or significant interaction between substrate type and diel cycle (habitat*diel cycle) [$F_{1,24} = 0.43$, $p = 0.52$]. However, stonefishes spent a significantly larger amount of time in the complex environment (94%, $M = 1016$ min) than in the simple environment (6%, $M = 73$ min) [$F_{1,24} = 566$, $p < 0.001$].

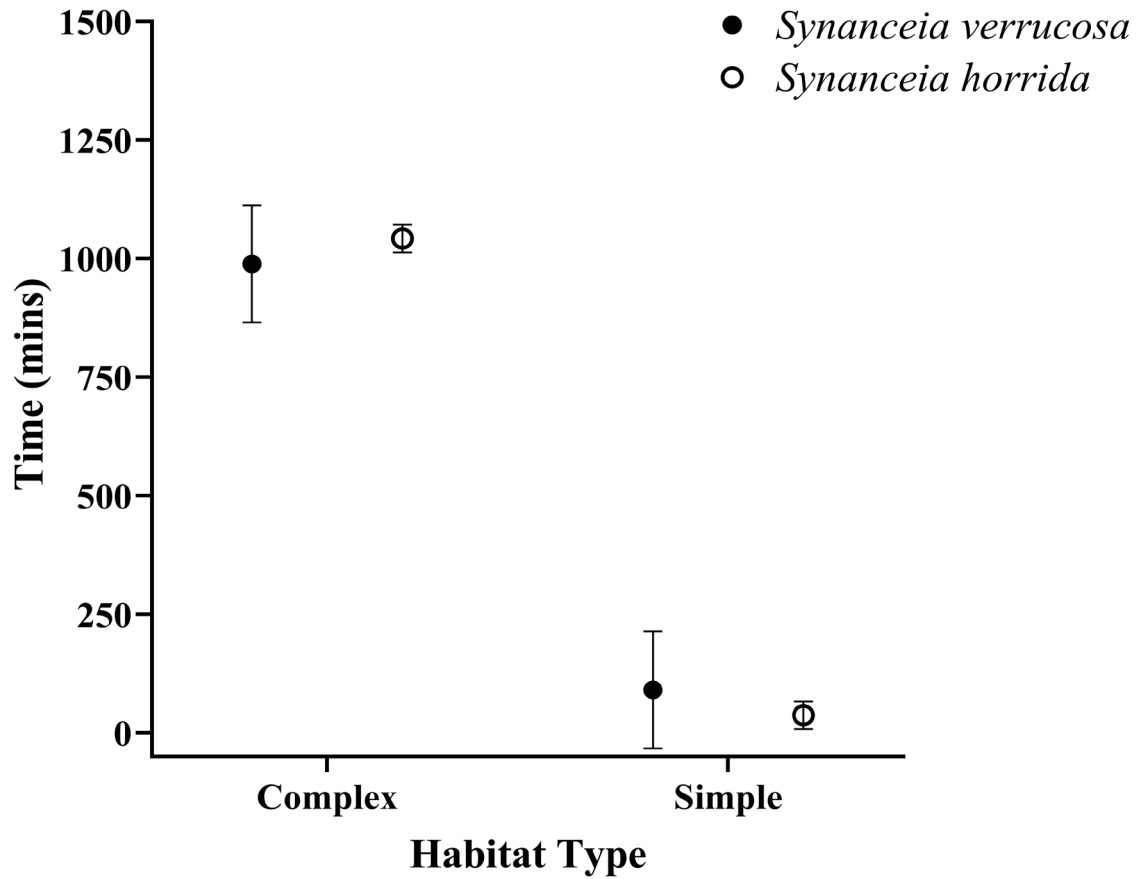


Figure 3.3 Habitat Preferences of Stonefish (*Synanceia* spp.). The mean amount of time (minutes) that *Synanceia verrucosa* (Reef Stonefish, ●), and *Synanceia horrida* (Estuarine Stonefish, ○) spent amongst simple (i.e., sand-only) and complex (i.e., a mixture of sand, coral rubble, and rocks) habitats measured over 18 hours of day/night (between 08:00 – 17:00 hours, and 20:00 – 05:00 hours). Error bars represent 95% CI.

3.4.2 Stonefish Movement Frequency and Distance

There was no significant interaction between the mean frequency of movement in each habitat per hour and species, habitat, or time of diel cycle (**Figure 3.4**) [$F_{1, 22} = 0.13, p = 0.72$]. There was also no significant interaction between the mean number of movements in each habitat type per hour and species (species*habitat) [$F_{1, 22} = 0.64, p = 0.43$]; (habitat*diel cycle) [$F_{1, 22} = 0.65, p = 0.43$]; (species*diel cycle) [$F_{1, 22} = 0.06, p = 0.81$]. However, stonefish in the complex habitat moved less frequently on average (1.6 movements per hour) than in the simple habitat (17.3 movements per hour) [$F_{1, 22} = 45.90, p < 0.001$] (**Figure 3.4A**). There was no significant interaction in the average distance travelled per movement between the two species and the time each movement occurred (species*time) [$F_{13, 712} = 0.91, p = 0.54$]. However, *S. verrucosa* travelled further per movement ($M = 0.94$ m; $SD = 0.68$ m) than *S. horrida* ($M = 0.67$ m; $SD = 0.37$ m), [$F_{1, 712} = 53.20, p < 0.001$] (**Figure 3.4B**).

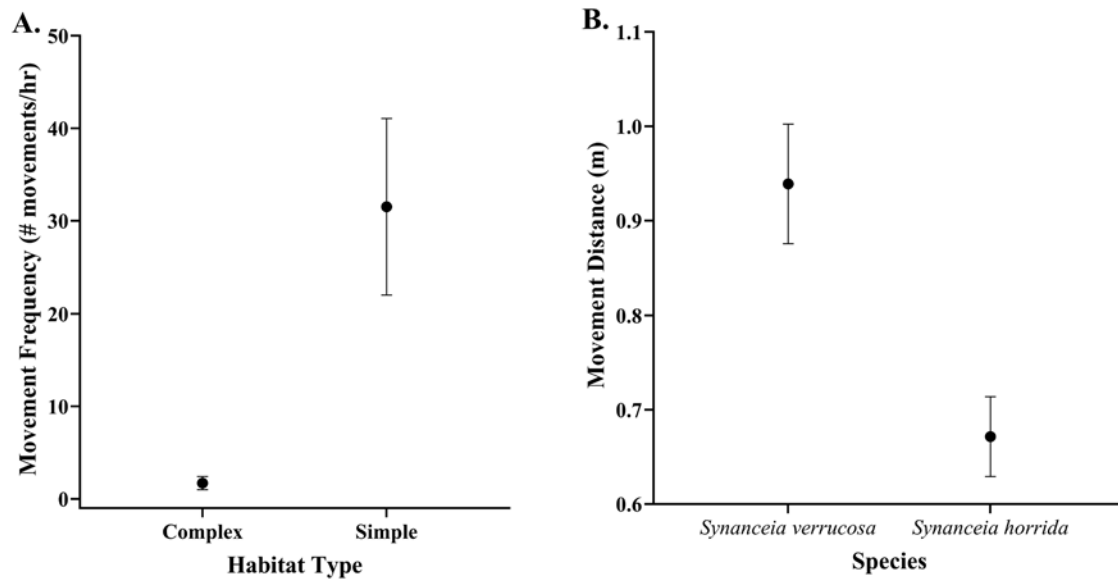


Figure 3.4 Movement Patterns of Stonefish (*Synanceia* spp.). **Panel A:** Mean movement frequency (number of movements per hour) of *Synanceia verrucosa* (Reef Stonefish) and *Synanceia horrida* (Estuarine Stonefish) over 18 hours (08:00 – 17:00 hours, and 20:00 – 05:00 hours) in complex (a mixture of sand, rock, and large coral rubble) and simple (sand-only) habitat types. **Panel B:** Mean movement distance (metres) of *S. verrucosa* and *S. horrida* over 19 hours (08:00 – 17:00 hours, and 20:00 – 05:00 hours) in both complex and simple habitat types. Error bars represent 95% CI.

3.4.3 *Stonefish Diel Movement Trends*

There was a statistically significant effect for the time each movement occurred, and a post hoc analysis (LSD) revealed stonefish travel further per movement during 10:00–10:59 hours ($M = 1.26$ m; $SD = 0.92$ m) and 09:00–09:59 hours (**Figure 3.5**, $M = 1.14$ m; $SD = 0.84$ m). The shortest average distance per movement occurred during 16:00–16:59 hours ($M = 0.63$ m; $SD = 0.44$ m) and 20:00–20:59 hours ($M = 0.65$ m; $SD = 0.43$ m), [$F_{17, 712} = 2.76$, $p < 0.001$]. There was a bimodal distribution corresponding to a decrease in the distance travelled per movement that related to reduced movement during transitional periods between light and dark.

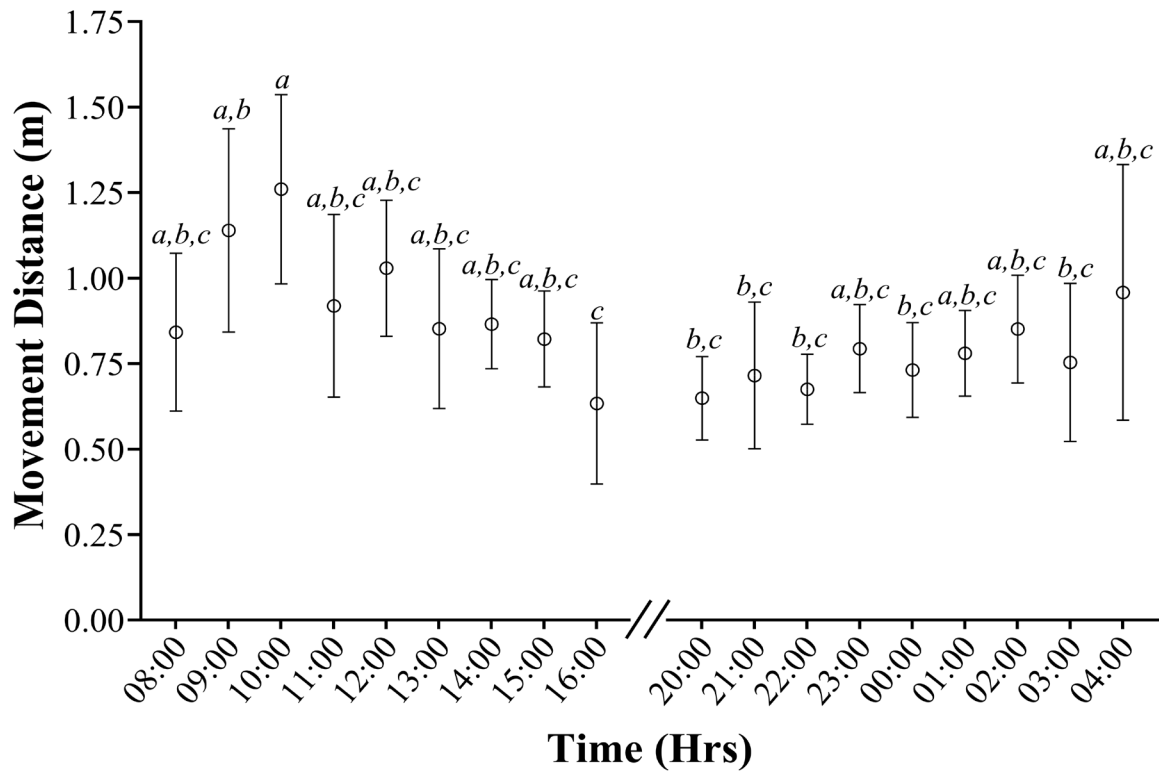


Figure 3.5 Stonefish (*Synanceia* spp.) Diurnal Movement Distance. The mean distance (metres) that *Synanceia verrucosa* (Reef Stonefish) and *Synanceia horrida* (Estuarine Stonefish) travelled within hourly intervals (i.e., 08:00 = 08:00 – 08:59 hours) over day (08:00 – 17:00 hours), and night (20:00 – 05:00 hours) periods. Error bars represent 95% CI. Means sharing the same letter groupings are not significantly different according to Fishers LSD ($\alpha = 0.05$).

3.5 Discussion

A key finding of the current study was that both *S. verrucosa* and *S. horrida* prefer complex (94% of time) rather than simple benthic habitat (6% of time). This trend was not significantly different between the two species or the time of day. As with other ambush predators, stonefishes presumably benefit from increased feeding efficiency and concealment from visual predators in complex habitats (Horinouchi *et al.*, 2009; Schultz *et al.*, 2009; Laurel & Brown, 2006).

The difference in the average distance travelled per movement between species in the current study may be an indication of a real ecological and/or evolutionary difference in the behaviours of these species in the field. For instance, *S. horrida* may rely on the mobility of prey fishes in tidal estuaries where high turbidity prevails, whereas *S. verrucosa* may need to relocate ambush position in relation to site-attached and highly visual reef fishes that become aware of the position of the predator. The differences in movement may also be an artefact of holding these species in close confines in captivity, although, this is unlikely since antagonist interactions were not observed. Alternatively, the small differences in average distance moved by two stonefishes in the current study may be statistically significant but have no functional basis (Underwood, 1990).

Conversely, activity levels were substantially different between habitats with stonefishes moving less in complex (1.6 movements hour⁻¹) than in simple habitats (17.3 movements hour⁻¹). This is congruent with the current understanding of other cryptic animals which tend to stay still to avoid detection (Loannou & Krause, 2009; Zhang & Richardson, 2007). Specifically, the increased probability of predators or prey detecting the presence of stonefishes in simple habitats may explain the increased frequency of movement by stonefishes on sand in the current study.

The diel activity patterns observed of stonefishes in the current study may have a functional, and possibly, an evolutionary basis. Morris Jr and Whitfield (2009) found that the scorpaenid, *Pterois volitans* (Red Lionfish), fed mostly in the early morning between 08:00–11:00 hours. Similarly, in the current study, stonefishes were most active at that time, and were least active during the late afternoon and evening. Reduced activity of stonefishes near sunset may be an adaptation to ambush feeding during the transition from light to dark conditions. Paradoxically, whereas many predatory coral reef fishes become more active in association

with crepuscular feeding (Helfman, 1986), ambush predators, such as stonefishes, may actually move less at that time, enhancing the advantage of crypsis, and heightening the element of surprise for capturing mobile prey. Numerous studies have focussed on the ecological advantages of crypsis in the context of predator-prey interactions (Thery & Casas, 2002; Grobecker, 1983) and recently the synergy of prey crypsis and reduced movement has been shown to confer additional protection to prey than can otherwise be explained by the additive effects of crypsis and movement (Loannou & Krause, 2009) Whether such synergistic benefits are afforded to cryptic, ambush predators remains to be tested.

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3.7 APPENDIX B

Diel Activity Patterns and Habitat Preferences of Stonefish (*Synanceia* spp.) in Captivity.

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Diel activity patterns and habitat preferences of stonefish (*Synanceia* spp.) in captivity

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Keywords:

Habitat use | diel activity | stonefish | *Synanceia* | crypsis | ambush predator.

ABSTRACT

Synanceia verrucosa (reef stonefish) and *Synanceia horrida* (estuarine stonefish) are known as the world's most venomous fishes. However, little is known about their habitat preference and activity patterns. This study aimed to determine the habitat preferences and diel activity patterns of stonefish. The movements of four adults of each species that were offered complex and simple habitats in captivity were recorded by filming during day and night periods. Generally, both species exhibited similar behaviour, though *S. verrucosa* travelled further per movement than *S. horrida*. Collectively, stonefish spent 94% of their time associated with complex habitat and moved significantly less frequently on complex (1.6 movements per hour) relative to simple habitat (17.3 movements per hour). Stonefish were least active around sunset in the late afternoon and evening, which may relate to an active feeding period during the morning or paradoxically to a higher probability of ambush feeding success near sunset. These findings have enhanced our current understanding of the ecology of these species.



Figure 1. Side view of two stonefish species found in Queensland (North-Eastern Australia) waters, *Synanceia horrida* (estuarine stonefish, left) and *Synanceia verrucosa* (reef stonefish, right).

The reef stonefish (*Synanceia verrucosa*) and the estuarine stonefish (*Synanceia horrida*) are members of the Synanceiidae family (Fig. 1) and are known as the world's most venomous fishes (Isbister 2001; Meyers 1999). Stonefish are widely distributed in both the tropical and sub-tropical coastal marine waters of the Indian and Pacific oceans, including around northern Australia (Saggiomo et al. 2021). *S. verrucosa* is found on reef flats, shallow lagoons and in small intertidal pools, while *S. horrida* inhabits estuaries and near shore areas (Williamson et al. 1996; Grobecker 1981; Endean 1961). Stonefish are cryptic ambush predators that remain motionless for extended periods of time (Williamson et al. 1996; Grobecker 1981; Endean 1961), but will engulf nearby prey with explosive speed (Ferry-Graham and Lauder 2001; Grobecker 1981; Endean 1961).

Research conducted on stonefish has primarily focused on their venom and venom delivery system (Isbister 2001; Williamson et al. 1996; Kizer et al. 1985; Endean 1961), particularly their associated

medical significance (Ngo et al. 2009; Brenneke and Hatz 2006). In contrast, apart from Grobecker's paper (1981), scant attention has been directed toward the ecology of these species, particularly their feeding behaviours, habitat preferences and activity patterns. Unlike fast-swimming pursuit predators that rely on speed and agility to capture prey (Juanes et al. 2002), stonefish are slow-swimming ambush predators that mimic benthic microhabitat (Grobecker 1981; Endean, 1961). Stonefish are highly cryptic, a common morphological adaptation to avoid detection by prey and predators that are visually oriented (Hairston et al. 1982; Endler 1981). Furthermore, stonefish can change colour to better match their environment (Loannou and Krause 2009; Grobecker 1981; Endean 1961). Background matching is less effective when animals are in motion; therefore, many cryptic animals tend to stay still (Loannou and Krause 2009; Zhang and Richardson 2007). Evidence also suggests that crypsis and lack of motion form a synergistic relationship, reducing the probability of detection (Loannou and Krause 2009). Additionally, stonefish encourage algal growth on their body surfaces, which enhances their ability to blend into their surrounding environment (Ballantine et al. 2001; Williamson et al. 1996) and may also attract herbivorous fish and invertebrates into the animal's strike range (Grobecker 1981).

Habitat type has proven to be an important factor that contributes not only to background matching, but also to the efficiency of foraging success for many ambush predators (Horinouchi et al. 2009; Schultz et al. 2009; Laurel and Brown 2006; Savino and Stein 1989). Structurally complex environments

have been shown to reduce the efficiency of pursuit predators through reduced mobility and decreased vision (Laurel and Brown 2006; Abrahams 2005). However, the foraging efficiency of ambush predators in structurally complex environments is helped by their mode of predation, where these predators capitalize on their ability to ambush prey (Schultz et al. 2009; Stephens and Krebs 1986). Stonefish are commonly associated with complex environments, though they can also be found buried in the sand of simple environments (Williamson et al. 1996; Grobecker 1981; Endean 1961). It is unknown which habitat type is preferred by stonefish during the day or night. Subsequently, the aims of this study were to determine the habitat preferences and diel activity patterns of *S. verrucosa* and *S. horrida* in captivity to better understand the ecology of these medically important species.

MATERIALS AND METHODS

A total of four *S. verrucosa* and four *S. horrida* were used in the study. Two of each species (330–410 mm LT) were housed in each of two aquaria (dimensions L x W x D: 2.3 x 1.2 x 0.6 m) with substrate comprised of sand (0.1 m deep) in one half (termed simple habitat) and rock and large coral rubble in the other half (termed complex habitat) (Fig. 2). A ten-day acclimation period was provided before filming commenced. The aquariums were exposed to natural light and shielded from direct sunlight. Filming of the aquariums took place

over a total of 72 hours, which was divided into two 9-hour day sessions from 08:00 to 16:50, and two 9-hour night sessions from 20:00 to 04:59. Both aquariums were filmed simultaneously during these four sessions. These time frames were chosen to compare behaviour patterns between light and dark conditions.

We quantified the time that each individual spent occupying each habitat type from film footage. The position of individuals resting on the boundary between two habitat types was classified according to the habitat containing the majority of the fish's body. A three-way analysis of variances (ANOVA) was conducted using the statistical package SPSS v.17 to determine the effect of habitat type (complex, simple), condition (light, dark) and species (*S. verrucosa* and *S. horrida*) on the total amount of time spent in each environment. A further three-way ANOVA was conducted to explore the frequency of movements by each species per hour in relation to habitat type and period in the diel cycle (day, night).

Distance was calculated from film footage by marking the initial position of each stonefish, tracking their movement on a display monitor and measuring the on-screen distance travelled. This measure was then converted to the true scale movement distance ± 0.05 m and movements under 0.1 m were excluded. The time each movement occurred was recorded and rounded to the nearest minute. The movement times were then divided into hourly intervals. Using the statistical package R, a two-way repeated measures ANOVA and post-hoc least significant difference (LSD) was conducted to explore if the average distance travelled per movement differed between species and if the frequency of movement differed according to the time of day or night (among hourly intervals).



Figure 2. View from above of one of the experimental aquariums used in the study. Two substrate types were used; simple (left) comprised of sand-only and complex (right) containing a mixture of sand, rock, and large coral rubble. Arrows indicate the location of stonefish on complex habitat.

RESULTS

Stonefish habitat preference

There was no significant interaction between habitat, species, and/or period in the diel cycle in relation to total amount of time spent in each habitat (species $\times$ habitat $\times$ diel cycle) [$F_{1,24} = 0.72$, $p = 0.40$]. There was also no significant interaction of the mean time spent in each habitat between the species (species $\times$ habitat) [$F_{1,24} = 1.79$, $p = 0.19$]; or significant interaction between substrate type and period in the diel cycle (habitat $\times$ diel cycle) [$F_{1,24} = 0.43$, $p = 0.52$]. However, stonefish spent significantly longer in the complex habitat (94%, $M = 1016$ min) than in the simple habitat (6%, $M = 73$ min) [$F_{1,24} = 566$, $p < 0.001$] (Fig. 3).

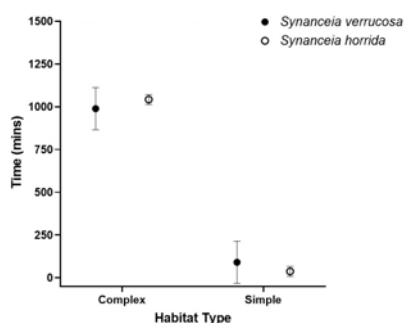


Figure 3. Stonefish (*Synanceia* spp.) habitat preferences. The mean amount of time (minutes) that reef (*S. verrucosa*, ●), and estuarine (*S. horrida*, ○) stonefish spent amongst simple (i.e., sand-only) and complex (i.e., a mixture of sand, coral rubble and rocks) habitats measured over 18 cumulative hours of day and night (i.e., between 08:00 ~ 16:59 hours and 20:00 ~ 04:59 hours). Error bars represent 95% CI.

Stonefish movement frequency and distance

There was no significant interaction between the mean frequency of movement per hour and species, habitat, or period in the diel cycle (species $\times$ habitat $\times$ diel cycle) [$F_{1,22} = 0.13$, $p = 0.72$]. However, stonefish occupying the complex habitat moved significantly less frequently on average (1.6 movements per hour) than in the simple habitat (17.3 movements per hour) [$F_{1,22} = 45.90$, $p < 0.001$] (Fig. 4A). There was no significant interaction between the mean number of movements in each habitat type per hour and species (species $\times$ habitat) [$F_{1,22} = 0.64$,

$p = 0.43$]; habitat and diel cycle (habitat $\times$ diel cycle) [$F_{1,22} = 0.65$, $p = 0.43$]; or species and diel cycle (species $\times$ diel cycle) [$F_{1,22} = 0.06$, $p = 0.81$].

There was no significant interaction in the average distance travelled per movement between the two species and the time each movement occurred (species $\times$ time) [$F_{13,712} = 0.91$, $p = 0.54$]. However, *S. verrucosa* travelled further per movement ($M = 0.94$ m; $SD = 0.68$ m) than *S. horrida* ($M = 0.67$ m; $SD = 0.37$ m), [$F_{1,712} = 53.20$, $p < 0.001$] (Fig. 4B).

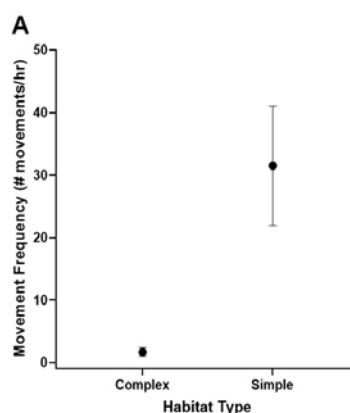


Figure 4. Movement patterns of stonefish. **A** Mean movement frequency (number of movements per hour) of stonefish (both *S. verrucosa* and *S. horrida*) over 18 hours (08:00 ~ 16:59 hours and 20:00 ~ 04:59 hours) in complex (sand, rock, and large coral rubble mixture) and simple (sand-only) habitat types. Error bars represent 95% CI.

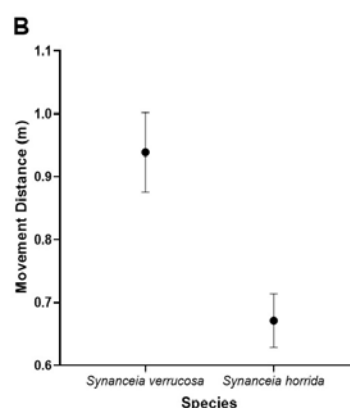


Figure 4. Movement patterns of stonefish. **B** Mean movement distance (metres) of *S. verrucosa* and *S. horrida* over 18 cumulative hours (08:00 ~ 17:00 hours and 20:00 ~ 05:00 hours) in both complex and simple habitat types. Error bars represent 95% CI.

Stonefish diel movement trends

There was a statistically significant effect for the time each movement occurred, and a post-hoc LSD analysis revealed stonefish travel further per movement during 10:00–10:59 hours ($M = 1.26$ m; $SD = 0.92$ m) and 09:00–09:59 hours ($M = 1.14$ m; $SD = 0.84$ m).

The shortest average distance per movement occurred during 16:00–16:59 hours ($M = 0.63$ m; $SD = 0.44$ m) and 20:00–20:59 hours ($M = 0.65$ m; $SD = 0.43$ m), [$F_{17, 712} = 2.76$, $p < 0.001$] (Fig. 5). There was a bimodal distribution corresponding to a decrease in the distance travelled per movement that related to reduced movement during transitional periods between light and dark.

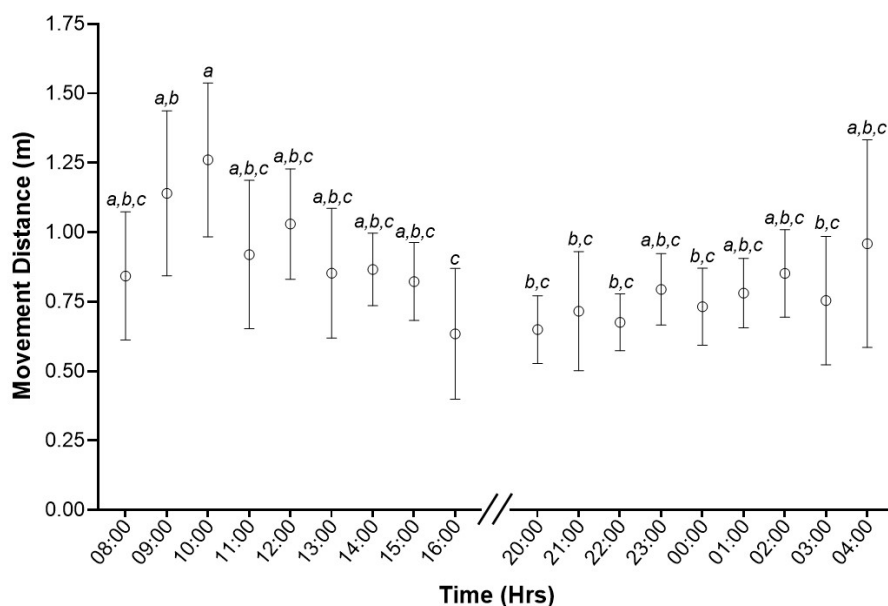


Figure 5. Stonefish diurnal movement distance. The mean distance (metres) that stonefish (both *S. verrucosa* and *S. horrida*) travelled within hourly intervals (i.e., 8:00 = 8:00 ~ 8:59 hours) over day (08:00 ~ 16:59 hours) and night (20:00 ~ 04:59 hours) periods. Error bars represent 95% CI. Means sharing the same letter grouping are not significantly different according to Fishers LSD ($\alpha = 0.05$).

DISCUSSION

A key finding from the study was that both *S. verrucosa* and *S. horrida* exhibited a strong preference for spending time in complex benthic habitats (94%) over simple habitats (6%). This trend was not significantly different between the two species or the time of day. As with other ambush predators, stonefish presumably benefit from increased feeding efficiency and concealment from visual predators in complex habitats (Horinouchi et al. 2009; Schultz et al. 2009; Laurel and Brown 2006).

The difference in average distance travelled per movement between species in the study may indicate a real ecological and/or evolutionary difference in behaviour of these species in the field. For instance, *S. horrida* may rely on mobility of prey fishes in tidal estuaries where high turbidity prevails, whereas *S. verrucosa* may need to relocate ambush position in relation to site-attached and highly visual reef fishes that become aware of a predator's position. The differences in movement may also be an artefact of holding these species in close confines in captivity, although this is unlikely since antagonist interactions were not observed.

Alternatively, the small differences in average distance moved by two stonefish in the study may be statistically significant but have no functional basis (Underwood 1990).

Conversely, activity levels were substantially different between habitat types, with stonefish moving less in complex (1.6 movements per hour) than in simple habitats (17.3 movements per hour). This is consistent with other cryptic animals, which tend to stay still to avoid detection (Loannou and Krause 2009; Zhang and Richardson 2007). Specifically, the increased probability of predators or prey detecting the presence of stonefish in simple habitats may explain the increased frequency of movement on sand by stonefish in the current study. While this study investigated the effect of substrate complexity and time of day on habitat preference and activity patterns in reef and estuarine stonefish species, it is important to consider that various additional factors, such as turbidity, as well as physical substrate attributes like colour, brightness, and texture may also influence these behaviours *in situ*.

The diel activity patterns observed of stonefish in the current study may have a functional and possibly an evolutionary basis. Morris and Whitfield (2009) found that the scorpaenid, *Pterois volitans*, fed mostly in the early morning between 08:00–11:00 hours. Similarly, in this study, stonefish were most active at that time, and were least active during the late afternoon (between 16:00 and 16:59) and night (20:00 to 03:00). Reduced activity of stonefish around sunset may be an adaptation to ambush feeding during the transition from light to dark conditions. Paradoxically, whereas many predatory coral reef fishes become more active in association with crepuscular feeding (Helfman 1986), ambush predators such as stonefish may actually move less at that time, enhancing the advantage of crypsis and heightening the element of surprise for capturing mobile prey. Numerous studies have focused on the ecological advantages of crypsis in the context of predator-prey interactions (Théry and Casas 2002; Grobecker 1981) and recently the synergy between prey crypsis and reduced movement has been shown to confer additional protection to prey than can otherwise be explained by the additive effects of

crypsis and movement (Loannou and Krause 2009). Whether such synergistic benefits are afforded to other cryptic ambush predators remains to be tested. In conclusion, this study suggests that there is an intrinsic link between stonefish movement patterns, time of day, and substrate complexity allowing for a better understanding of the ecology of these species.

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Chapter 4

Geographic Variation in Stonefish (*Synanceia* spp.) Venom

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Statement of Contribution of Others:

D Lennox-Bulow is the principal author of this manuscript and was extensively involved in all aspects of this work under the supervision of J Seymour. R Courtney assisted in collecting stonefish venom, data interpretation, and reviewing earlier versions of this manuscript.

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4.1 Abstract

Stonefish (*Synanceia* spp.) possess a medically significant venom and are widely distributed throughout the Indo-Pacific. Yet, little is known about how the ecology of these animals may influence their venom. The aim of this study was to explore the effect of species and geographic location on stonefish venom composition. We collected the venom of *Synanceia horrida* (Estuarine Stonefish) and *Synanceia verrucosa* (Reef Stonefish) from various locations across Australia (Cairns, Brisbane, Caloundra, and Onslow), and Southeast Asia (Kota Kinabalu, and Cebu) and analysed these samples using SDS-PAGE, FPLC, and HPLC. Stonefish have a complex venom comprised of numerous components. Stonefish venom exhibited both similarities and variations in composition within species between geographically isolated populations, as well as between species in a single location. We speculate that the observed geographic and interspecific trends may be driven by similarities and differences in the selective pressures faced by these animals, particularly those associated with predator dynamics. The findings of this study have furthered our understanding of the ecology of stonefish and their toxins.

4.2 Introduction

Animal toxins, including venoms and poisons, are a form of chemical warfare used by organisms to capture prey and/or defend against predators (McNamee & Cockayne, 2016). To exert their intended effects, toxins must rapidly and potently interfere with the homeostatic processes of their target/s in the most energetically efficient way possible (Casewell *et al.*, 2020; Pintor *et al.*, 2010; Wigger *et al.*, 2002). However, the mechanisms for achieving this vary considerably, with composition playing a key role in optimising the toxin for its intended purpose (Casewell *et al.*, 2013). Toxins, particularly venoms, contain a complex mixture of proteins, peptides, and small molecules that produce a vast array of pharmacological effects (i.e., neurotoxicity, cardiotoxicity, haemotoxicity) on living organisms (McNamee & Cockayne, 2016). This complexity creates potential for compositional variation in toxins.

The physical structure and chemical nature of toxin components, as well as their relative abundance, has been shown to vary both within species (Tasoulis *et al.*, 2020; Schaffrath *et al.*, 2018) and between species (van Thiel *et al.*, 2023; Duran *et al.*, 2020), as well as within and/or between individuals (Dutertre *et al.*, 2014). Research has shown that biotic factors such as predator-prey dynamics (Smith *et al.*, 2023; Chang & Duda, 2016; Daltry *et al.*, 1996), sex (Amorim *et al.*, 2018; Zelanis *et al.*, 2016; Herzig & Hodgson, 2009), and ontogeny (da Silva Aguiar *et al.*, 2020; Chang & Duda, 2016; Underwood & Seymour, 2007), as well as abiotic factors including temperature (Smith *et al.*, 2023; O'Hara *et al.*, 2018), and season (Tasima *et al.*, 2024; Junior *et al.*, 2010) are among some of the drivers of compositional variation in animal toxins, particularly venoms. Anecdotal evidence suggests that salinity may also influence toxin composition (O'Hara *et al.*, 2021; Sachkova *et al.*, 2020). Furthermore, some or all these potential drivers of variation have the capacity to vary geographically (Yu *et al.*, 2020).

The occurrence of geographic variation in venom composition has been reported for numerous species, particularly terrestrial snakes (Smith *et al.*, 2023; van Thiel *et al.*, 2023; Smith & Mackessy, 2021; Davies & Arbuckle, 2019; Sasa, 1999), and to a lesser degree, for some marine species including jellyfish (Winter *et al.*, 2010; Carrette *et al.*, 2002), and cone snails (Weese & Duda, 2019; Phuong *et al.*, 2016). This phenomenon has also been documented at almost all spatial scales, ranging from between microhabitats within a single location (Smith *et al.*, 2023; Abdel-Rahman *et al.*, 2009), to across continents spanning vast distances (Carrette, 2014; Duda *et al.*, 2009). Although geographic variation in venom is widely studied, identifying the ecological factors shaping venom composition is complex and multifaceted, as well as likely

unique to each location. Furthermore, in cases of human envenomation, geographic variation in venom composition may have implications for first aid practices (Armstrong *et al.*, 2023). One medically significant venomous animal that is sorely lacking in research aimed at its toxin ecology are stonefish.

Stonefish (*Synanceia* spp.) are revered as the world's most venomous fish, a title owing to the noxious defensive venom housed in twin glands associated with each of their 13 dorsal spines (Endean, 1961). Very little work has focussed on elucidating the structural and functional characteristics of stonefish venom components. However, what has been identified are components with the ability to form pores in biological membranes, and others with an affinity for binding to nicotinic acetylcholine receptors which function to interrupt neuronal signalling (Harris *et al.*, 2021; Harris & Jenner, 2019; Ellisdon *et al.*, 2015). These combined activities were suggested to induce significant pain to deter predators, and at the same time cause localised paralysis – most likely in the predators' mouths – to improve the fish's chances of a successful escape (Harris *et al.*, 2021; Ellisdon *et al.*, 2015).

While pain is the most common symptom associated with stonefish envenomation in people, clinical presentations can vary significantly, and range from localised pain and swelling, to oedema, cardiac arrest, and in extreme cases, death (Saggiomo *et al.*, 2021; Ellisdon *et al.*, 2015). Given that stonefish are highly cryptic, a strategy used primarily to enhance their success as benthic ambush predators (Cameron & Endean, 1973; Endean, 1961), human envenomation typically occurs when the animal is stood on. In Australia, stonefish envenomation often results in hospitalisation, with reports ranging from nine (Armstrong *et al.*, 2023) to thirty (Pointer & Harrison, 2021; Little & Gairdner, 2012) hospitalisation cases annually. Globally, the frequency of stonefish envenomation is suspected to be much higher, particularly in places like Southeast Asia where fishing practices are a primary source of income in many communities, and stonefish are considered a prized food fish (Yamamoto *et al.*, 2010). Furthermore, emerging case studies suggest that the clinical symptoms associated with stonefish envenomation may vary in severity, as well as in response to treatment between different locations (Armstrong *et al.*, 2023; Hifumi *et al.*, 2020; Poon *et al.*, 2020; Ngo *et al.*, 2009). One contributing factor may be geographic variation in stonefish venom composition.

Stonefish are widely distributed throughout both the tropical and subtropical waters of the Indo-Pacific (Saggiomo *et al.*, 2021; Endean, 1961). Of the five species that comprise the genera *Synanceia*, two of them, the Reef Stonefish (*Synanceia verrucosa*) and the Estuarine

Stonefish (*Synanceia horrida*), have the widest distribution throughout this range (Saggiomo *et al.*, 2021; Endean, 1961). Additionally, these species generally occur in different habitats. For instance, *S. verrucosa* is typically associated with offshore coral or rocky reef ecosystems, whereas *S. horrida* inhabits inshore intertidal areas and estuaries (Endean, 1961). However, the two species are not exclusive to these environments and their distributions, particularly in places like Australia, Kota Kinabalu in Malaysia, and Cebu in the Philippines, can overlap.

Given the environmental heterogeneity across their range, it is reasonable to suspect that stonefish encounter diverse selective pressures. While prior studies acknowledge potential individual and state-dependent (i.e., starvation) variations in stonefish venom composition (Saggiomo *et al.*, 2017), the influence of geographic variation on their venom is currently unknown. Therefore, the aim of this study was to determine whether the composition of stonefish venom varies according to the animals' geographic location. Additionally, this study compares stonefish venom composition between *S. verrucosa* and *S. horrida* inhabiting the same location in attempt to elucidate inter-species geographical trends.

4.3 Materials and Methods

4.3.1 Animal and Toxin Collection

Collecting and Sourcing Stonefish.

Mature (>100 mm body size) *S. horrida* and *S. verrucosa* were hand captured and/or sourced from commercial fish suppliers (Cairns Marine, Reef 2 Aquarium, Jardini International) between January 2019 to 2024. *Synanceia horrida* were sourced from shallow reef or estuarine environments in Cairns, Brisbane, and Caloundra in Queensland Australia, Onslow in Western Australia, Kota Kinabalu in Malaysia, and Cebu in the Philippines. Similarly, *S. verrucosa* were sourced from offshore reefs in Cairns, Kota Kinabalu, and Cebu. *Synanceia verrucosa* were unable to be sourced from South Queensland (Brisbane and Caloundra) and Western Australia (Onslow). Species were visually identified based on unique physiological features (e.g., head shape, eye position). The sexes of the animals were not able to be determined. Venom collection was done in accordance with James Cook University Animal Ethics Committee approval (AEC approval number A2832).

Extracting Stonefish Venom.

The venom of *S. horrida* and *S. verrucosa* was collected and pooled from multiple individuals (three or greater). Collection involved transferring a single live *S. verrucosa* or *S. horrida* to a saltwater-dampened surface. The animals' eyes were covered with a saltwater dampened cloth to reduce stress. Fish were then gently supporting either side of the pectoral fins and venom was aspirated from the dorsal spines using 9 mL no additive vacuette blood collection tubes (Greiner Bio One) under negative pressure. This involved pressing the rubber seals of the vacuette tubes over each dorsal spine, which induced venom sac compression and venom release. Due to the labile nature of stonefish venom (Harris *et al.*, 2021; Gwee *et al.*, 1994), to reduce degradation of the venom components, collected venom was lyophilised and stored at -80°C until required.

4.3.2 Profiling Stonefish Venom

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was performed on *S. horrida* and *S. verrucosa* venom following a similar protocol outlined by Lennox-Bulow *et al.* (2023). In brief, venom samples were centrifuged at 15,000x g, at 4°C for 5 min to remove debris. The concentration of the sample supernatants was determined using Nanodrop One (Thermo Scientific™) set to protein A₂₈₀ nm and adjusted to yield 500 µg/mL (±50 µg). Sample supernatants (10 µL) were then linearised by mixing (1:2)

with 2x Laemmli SDS lysis buffer. This process was catalysed by heating the samples at 95°C for 5 min.

Linearised samples (20 µL) and protein standards (2 µL Precision Plus Protein™ Dual Colour) were loaded on to 12% Bis-Tris mini protein gels (NuPAGE™, 1.0 mm x 12 well) set up in an electrophoresis cell. Gels were run in a discontinuous buffer system with the application of 90 V for ~10 min during the stacking phase, and 150 V for ~45 min during the running phase with a constant amperage of 0.02 A used throughout. The running buffer used was 1x MOPS SDS running buffer (NuPAGE™). Current was applied until the dye marker (bromophenol blue) was within 1 cm of the gel bottom.

Gels were stained by submersion into Imperial™ Protein Stain (Thermo Scientific) for 1 hour, and de-stained using deionised (MilliQ) H₂O until the gel background was clear. Gels were photographed with a Nikon D810 against an illuminated surface. Variation in the banding pattern between samples was determined via visual analysis of the gel images.

Size Exclusion Fast Performance Liquid Chromatography (SE-FPLC).

Absorbance (A_{280}) profiles of *S. horrida* and *S. verrucosa* venom were determined using SE-FPLC following a similar protocol outlined by Lennox-Bulow *et al.* (2023). Lyophilised venom samples were reconstituted in 600 µL of 1x phosphate-buffered saline (PBS, pH 7.0) and centrifuged at 15,000x g for 5 min. The resulting supernatants were then passed through a 0.22 µm syringe filter to remove debris. Cleaned venom samples (500 µL) were then manually injected onto the ÄKTA™ prime system. Separation of the venom components was achieved using a Superdex 200 Increase 10/300 GL Column (8.6 µm, 10 x 300 mm, GE Healthcare). The running buffer used was 1x PBS (pH 7.0) and the system was set to run at 500 µL/min at 8°C for 50 mL. The ultra-violet (UV) absorbance of the eluant was continuously monitored at 280 nm.

Venom profiles were processed using UNICORN™ 7 software (2016, GE Healthcare). To account for variations in sample concentrations, absorbance intensity was normalised for each trace. This was accomplished by multiplying each individual absorbance value by a normalisation factor. The normalisation factor was calculated by dividing the absorbance value of the tallest synchronous peak (observed at 21.5 mL elution volume in all traces) in the reference profile (i.e., the profile with the highest overall absorbance) by the absorbance value of the corresponding peak in the profile being adjusted. Chromatograms were produced using GraphPad Prism 10 for visual comparisons of the venom profiles.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC).

Hydrophobicity profiles of *S. horrida* and *S. verrucosa* venom were produced using RP-HPLC. Lyophilised venom samples ($\sim 25 \text{ mg} \pm 1 \text{ mg}$) were diluted with a 50:50 mixture of solvent A (250 μL H_2O /0.05% trifluoroacetic acid (TFA; Auspep) and solvent B (250 μL 90% ACN (Merck)/10% H_2O /0.045% TFA) and refrigerated overnight. Samples were centrifuged at 15,000x g for 10 min to remove debris. Fractionation used a C_{18} Phenomenex Aeris® PEPTIDE XB liquid chromatography column (150 x 2.10 mm, 3.6 μm , 100 Å) using a gradient of 0 – 60% solvent B in 60 min, 60 – 90% solvent B in 5 min, 90% solvent B for 10 min, and 90 – 0% solvent B in 5 min. The flow rate remained constant at 0.250 mL/min. The concentration of the sample supernatant was determined using Nanodrop One (Thermo Scientific™) under the Protein A_{280} program, and the injection volume of each venom sample was adjusted to achieve a final concentration of 250 μg . Absorbance was monitored at 214 nm. Raw data acquisition and initial analyses were conducted within OpenLAB CDS ChemStation software (v1.8.1). Data was then exported to GraphPad Prism 10 for the generation of chromatograms.

4.4 Results

4.4.1 SDS-PAGE of Stonefish Venom from Different Locations

SDS-PAGE profiles of *S. horrida* and *S. verrucosa* venom revealed both similarities and variations in composition between different locations (**Figure 4.1**). Banding patterns corresponding to smaller molecules (10 – 25 kDa) were highly conserved across all venom samples, regardless of species or origin. Variations in stonefish venom profiles between species and geographic locations arose primarily in bands associated with larger molecules (55 – 100 kDa). A band at ~90 kDa was universally present in all venom samples except for *S. horrida* venom samples from Brisbane and Onslow. *Synanceia horrida* venom samples from Onslow also displayed unique bands at ~70 kDa and ~71 kDa that were absent in all other samples. Furthermore, a distinct band at ~80 kDa was observed exclusively in *S. horrida* venom from Kota Kinabalu, but was a shared characteristic of *S. verrucosa* venom samples from all locations. Conversely, bands at ~55 kDa and 75 kDa were shared by all venom samples, except for *S. horrida* venom from Kota Kinabalu, and *S. verrucosa* venom from Cairns.

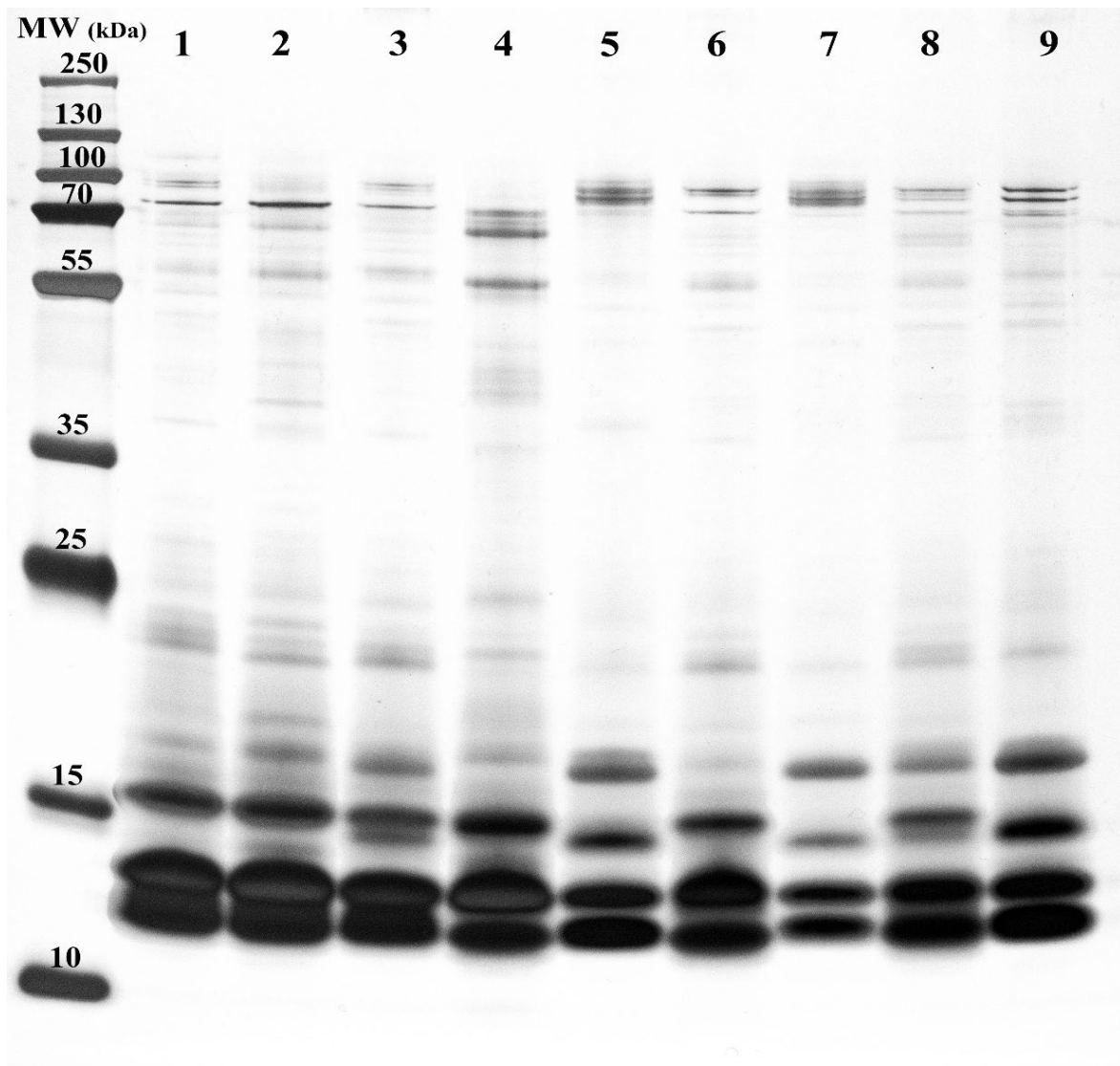


Figure 4.1 SDS-PAGE Profiles of Stonefish (*Synanceia* spp.) Venom from Different Locations. Estuarine Stonefish (*Synanceia horrida*) venom acquired from Cairns (Lane 1), Brisbane (Lane 2), Caloundra (Lane 3), Onslow (Lane 4), Kota Kinabalu (Lane 5), and Cebu (Lane 6) populations, as well as Reef Stonefish (*Synanceia verrucosa*) venom from Cairns (Lane 7), Kota Kinabalu (Lane 8), and Cebu (Lane 9) populations were profiled using gel electrophoresis. Venom samples (10 μ L) were loaded onto a NuPAGE Novex 12% Bis-Tris mini protein gel at a concentration of 500 μ g/mL. Molecular weights (kDa) of venom components were estimated by comparison with Precision Plus Protein™ Dual Colour protein standards.

4.4.2 SE-FPLC of Stonefish Venom from Different Locations

Stonefish venom also showed both similarities and variations between species and geographic locations when analysed using SE-FPLC (**Figure 4.2**). Consistent peak patterns were observed at elution volumes between ~13 and 19 mL and ~21 and 28 mL in *S. horrida* venom from Cairns, Brisbane, Onslow, Caloundra, and Cebu (**Figure 4.2A**), as well as *S. verrucosa* venom from Cairns, Kota Kinabalu, and Cebu (**Figure 4.2B**). However, *S. horrida* venom samples from Kota Kinabalu had a single peak eluting between ~13 and 19 mL that was roughly twice as intense as those seen in *S. horrida* venom from other locations. Furthermore, although both species from all measured locations showed peaks at ~20 mL elution volume, some variation was observed in the peak patterns. A single peak was observed in *S. horrida* venom from Brisbane, Onslow, Caloundra, Kota Kinabalu, and Cebu, and *S. verrucosa* venom from Kota Kinabalu and Cebu. In contrast, a double peak occurred in both species venom from Cairns. Additionally, all stonefish venom profiles showed a peak between ~30 and 33 mL, although there was some slight variation in its elution volume between locations. This peak eluted earlier (~30 mL) in *S. horrida* venom samples from Brisbane, Onslow, and Cebu, compared to venom samples from Cairns, Caloundra, and Kota Kinabalu (~32 mL). *Synanceia verrucosa* venom followed a similar trend where this peak eluted earlier (~30 mL) in samples from Kota Kinabalu and Cebu, than venom samples from Cairns (~32 mL). Both *S. horrida* and *S. verrucosa* venom from Kota Kinabalu also shared a distinct peak at ~13 mL elution volume that was absent from all other locations.

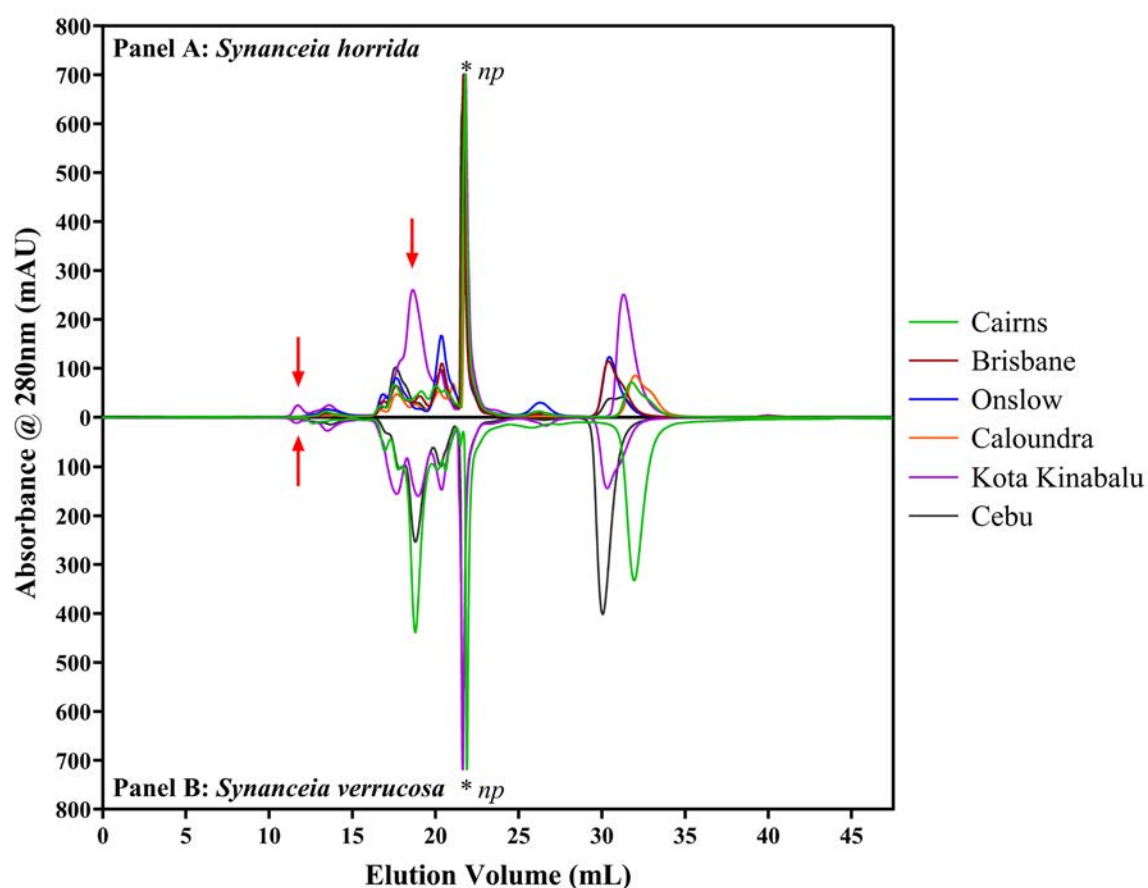


Figure 4.2 SE-FPLC Profiles of Stonefish (*Synanceia* spp.) Venom from Different Locations. **Panel A:** Profiles of Estuarine Stonefish (*Synanceia horrida*) venom from Cairns (—), Brisbane (—), Onslow (—), Caloundra (—), Kota Kinabalu (—), and Cebu (—). **Panel B:** Profiles of Reef Stonefish (*Synanceia verrucosa*) venom from Cairns (—), Kota Kinabalu (—), and Cebu (—). Stonefish venom profiles were compared between species, as well as within species between geographic locations. Absorbance was monitored at 280 nm over 50 mL of elution using an ÄKTA Prime System (GE Healthcare). Chromatograms were normalised for the absorbance intensity of the 21.5 mL peak (marked as ‘np’). Arrows (↓) mark observed intra-species variations between locations.

4.4.3 RP-HPLC of Stonefish Venom from Different Locations

Stonefish venom showed broadscale similarities, and some variation in component hydrophobicity when analysed using RP-HPLC (**Figure 4.3**). The venom of both *S. horrida* (**Figure 4.3A**) and *S. verrucosa* (**Figure 4.3B**) from all locations shared five distinct peaks, with retention times of approximately 42, 43, 47.5, 48, and 49 min (± 0.5 min). *Synanceia horrida* venom from all locations, and *S. verrucosa* venom from Kota Kinabalu, shared an additional peak at 46.5 min, which was absent in *S. verrucosa* venom from Cairns. This peak was also present in *S. verrucosa* venom from Cebu, but at approximately 20x lower intensity than *S. verrucosa* from Kota Kinabalu. Furthermore, *S. verrucosa* venom from all locations, and *S. horrida* venom from Caloundra, Kota Kinabalu, and Cebu, shared peaks between 42 and 43 min that were absent in *S. horrida* venom from Cairns, Brisbane, and Onslow. However, the peak pattern within this range differed between some locations. *Synanceia horrida* venom from Kota Kinabalu and Caloundra, as well as *S. verrucosa* venom from Cairns, had a double peak, whereas both *S. horrida* and *S. verrucosa* venom from Cebu, as well as *S. verrucosa* venom from Kota Kinabalu, had a single peak. *Synanceia horrida* venom from Kota Kinabalu and Caloundra also had a double peak between 44 and 45 min that was absent in *S. horrida* samples from Onslow, Brisbane, and Cairns, but was a shared characteristic of *S. verrucosa* venom from all locations. However, *S. horrida* samples from Cebu also had a single peak that appeared within this range. Furthermore, *S. horrida* venom from Cairns and Onslow, as well as *S. verrucosa* venom from Kota Kinabalu, shared a peak between 45 and 46 min that was absent in all other samples (i.e., both species and all other locations). *Synanceia verrucosa* venom from Kota Kinabalu had a unique peak at ~39.5 min that was absent from all other samples. Similarly, *S. horrida* venom from Cairns also had a unique peak at ~52.5 min that was absent from all other samples.

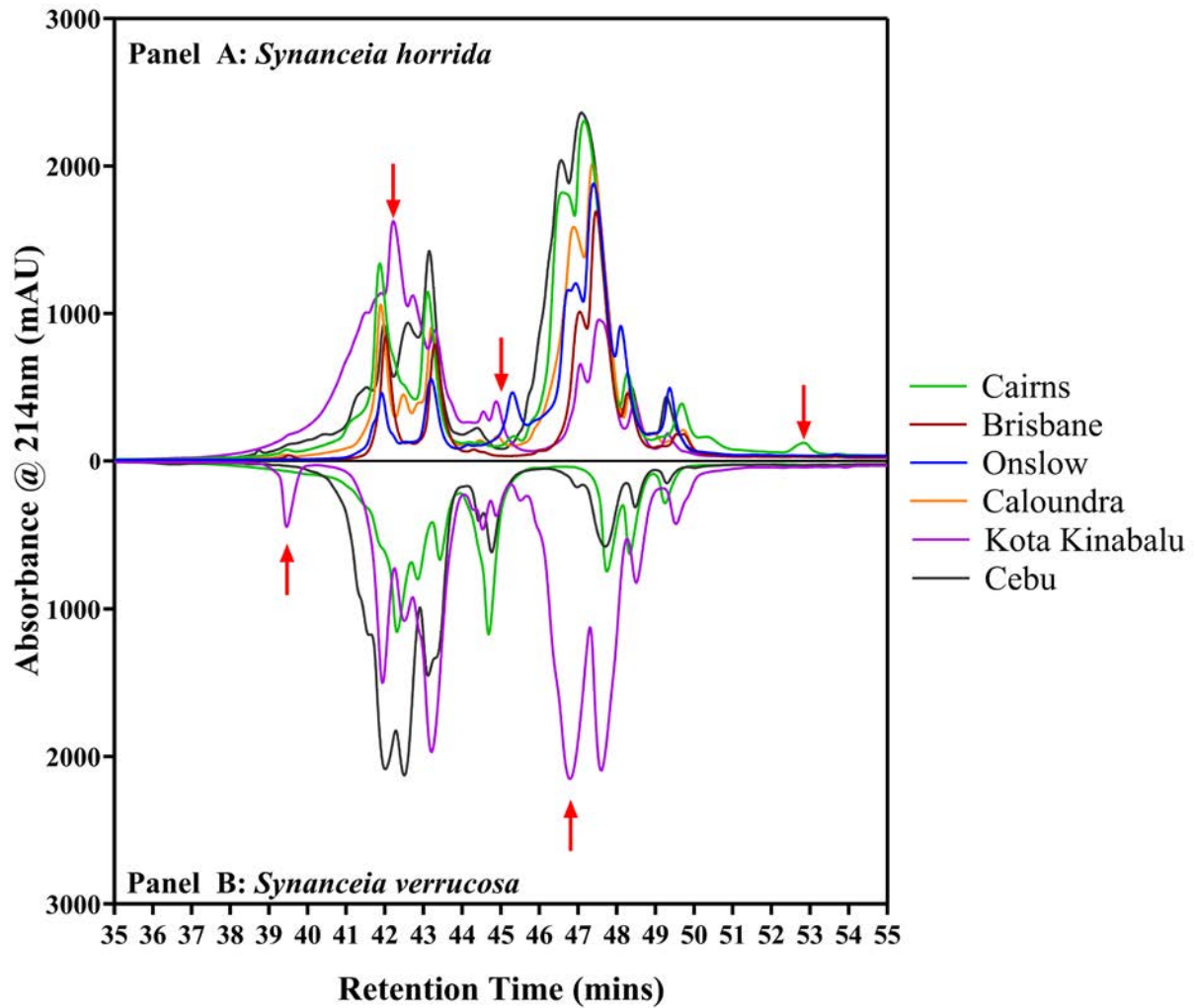


Figure 4.3 RP-HPLC Profiles of Stonefish (*Synanceia* spp.) Venom from Different Locations. **Panel A:** Profiles of Estuarine Stonefish (*Synanceia horrida*) venom from Cairns (—), Brisbane (—), Onslow (—), Caloundra (—), Kota Kinabalu (—), and Cebu (—). **Panel B:** Profiles of Reef Stonefish (*Synanceia verrucosa*) venom from Cairns (—), Kota Kinabalu (—), and Cebu (—). Stonefish venom profiles were compared between species, as well as within species between geographic locations. Venom samples (250 µg total protein) were profiled using a C₁₈ Phenomenex Aeris® PEPTIDE XB liquid chromatography column. Absorbance was monitored at 214 nm using a 1% gradient over 80 min. The absorbance traces for retention times up to the first 34.9 min, and after 55.1 min were omitted from the figure to enhance viewing resolution. Arrows (↓) mark observed intra-species variations between locations.

4.4.4 Comparison of Stonefish Venom between Species and Locations

Stonefish were found to have a complex venom profile comprised of numerous components. Within each species, a large proportion of the venom components exhibited consistent properties across geographic locations. The majority of the observed venom components showed similarities in size (particularly in smaller components between ~10 and 25 kDa), and hydrophobicity (~40 to 50 min retention time), regardless of the animal's geographic origin. These same size and hydrophobicity characteristics were also found to be shared between the two species.

Despite the overall consistency, some variation in venom component size (particularly in the larger components between ~55 and 100 kDa), and hydrophobicity characteristics, were observed. This variation occurred within each stonefish species across different locations, as well as between species found in the same location. For both species, intraspecific venom variation was most pronounced in stonefish venom samples collected from Kota Kinabalu. In *S. horrida* venom samples from Kota Kinabalu, variation was observed in both the larger sized components, as well as within the hydrophobicity profile, with unique peaks appearing at 42 min and 45 min retention times. In contrast, variation in *S. verrucosa* venom samples from Kota Kinabalu were only observed in the hydrophobicity profile, with unique peaks appearing at 39 min and 47 min retention times. Interestingly, both the size and hydrophobicity profiles of *S. horrida* venom from Kota Kinabalu more closely resembled *S. verrucosa* venom from all locations, than *S. horrida* venom from any other location tested in this study. Furthermore, *S. horrida* venom from the west coast of Australia (Onslow) exhibited notable differences in venom composition (i.e., the size and hydrophobicity of some components), compared to *S. horrida* venom from the east coast of Australia (Cairns, Brisbane, and Caloundra).

4.5 Discussion

Mounting evidence suggests the presence of broad-scale similarities in *S. horrida* and *S. verrucosa* venom in the composition of the smaller components (Saggiomo *et al.*, 2021; Madokoro *et al.*, 2011; Garnier *et al.*, 1996), as well as within subunits of their vertebrate-toxic factors verrucotoxin and stonustoxin (Saggiomo *et al.*, 2021; Ueda *et al.*, 2006; Garnier *et al.*, 1997). Similarly, the results of this study show that stonefish venom components are highly conserved in molecule size, and hydrophobicity characteristics, both intra- and inter-specifically, regardless of the animal's geographic origin. There are several suggestions within the literature as to why venom composition may be either entirely or partially conserved within species, and between closely related species. These centre around the animal's evolutionary history, as well as the selective pressures it faces in its environment (See review Casewell *et al.*, 2020).

The retention of some venom components within and between stonefish species is, at least in part, an artefact of their shared and/or close evolutionary history. Whereby, populations of recent common ancestry are likely to produce more similar venoms than populations separated by greater patristic distances (Davies & Arbuckle, 2019; Phuong *et al.*, 2016). As such, the shared ancestry of Scorpaenid fish, and moreover, the close relationship between *Synanceia* species (*S. horrida* and *S. verrucosa*), and individuals, likely translates to shared or similar venom-producing genes, and thus, shared or similar venom components. Furthermore, the retention of some venom components over evolutionary time, such as in the divergence of the two *Synanceia* species, and/or intra-specifically between isolated populations, might suggest that these components are highly successful in their function. The reason behind this being that venom components operating against the intended target effectively would be strongly favoured by natural selection, leading to their retention (Casewell *et al.*, 2020). Conversely, the observed compositional variation in stonefish venom, both inter- and intra-specifically, may be driven by differences in selective pressures in their environment.

Compositional variation in venom between geographically isolated populations has been attributed to a complex interplay of factors, both biotic and abiotic, related to the respective environment/location (Smith & Mackessy, 2021). As such, elucidating the factors shaping stonefish venom composition is both complex, as well as potentially unique to each location. However, the most compelling evidence of strong positive selection shaping venom composition is the occurrence of target-specific compounds (Smith & Mackessy, 2021; Barlow

et al., 2009). Several studies have shown correlations in prey dynamics and venom composition among geographically isolated populations within species. For example, one study found that *Crotalus v. viridis* (Prairie Rattlesnake) populations in northern America produce venom that is dominant in a compound (i.e., myotoxin) with specific toxicity towards mammals, which is non-toxic to lizards (Smith *et al.*, 2023). Conversely, southern populations were found to produce a venom phenotype effective against both lizards and mammals (Smith *et al.*, 2023). Others have highlighted that the relative abundance of certain components within the venom of *Calloselasma rhodostoma* (Malayan Pit Viper) exhibited significant geographic variation, which was hypothesised to be driven by variations in the relative abundances of local prey taxa including amphibians, reptiles, and other endotherms (Daltry *et al.*, 1996). Similarly, venom from *Chironex fleckeri* (Australian Big Box Jellyfish) populations in northern Australia were found to be more cardiotoxic to mammals than Western Australia populations. This was hypothesised to be correlated with a difference in the physical size of the animals between the two locations (i.e., larger animals in Queensland, and smaller animals in Western Australia) (Winter *et al.*, 2010), which potentially influences cnidae composition (Carrette *et al.*, 2002), and thus, feeding ecology (i.e., a vertebrate diet for Queensland populations and an invertebrate diet for Western Australia populations). However, the focus on prey dynamics as the primary force influencing venom composition may not be universally applicable.

In Scorpaenid fish, which include stonefish (*Synanceia* spp.), venom is used exclusively for defence. As such, we propose that the strongest selective force acting upon and shaping stonefish venom composition is most likely predator dynamics. The results of this study show that *S. horrida* venom from Kota Kinabalu displayed a greater resemblance to *S. verrucosa* venom from all locations, than to *S. horrida* venom from animals inhabiting Australia or the Philippines. Similarly, *S. horrida* venom collected on the west coast of Australia (Onslow) contained unique components not found in the venom from east coast locations (Cairns, Brisbane, Caloundra). At a smaller spatial scale, stonefish venom also showed inter-specific compositional variation between typically reef (*S. verrucosa*) and estuary (*S. horrida*) inhabiting species within the same location. The observed variations in *S. horrida* venom composition at both an international and national scale, as well as between stonefish species within a single location may be driven by differences in predator dynamics. Such an adaptation would likely confer a selective advantage for stonefish against variations in the species, abundance, and encounter frequency of natural predators within and between locations. Conversely, similarities in stonefish venom composition between species within a single locale,

such as Kota Kinabalu stonefish populations, as well as between spatially separated populations within a region, such as *S. horrida* from various locations along the east coast of Australia, might suggest that these populations experience common and/or shared predator dynamics. Alternatively, similarities in venom composition between stonefish species in a single location (i.e., Kota Kinabalu stonefish populations) might indicate that these species exist in similar habitats which is unusual for other locations.

Similarities in stonefish venom composition were also observed at all levels, including both within and between species, as well as at all spatial scales from tens of kilometres (i.e., microhabitats), to upwards of tens of thousands of kilometres (i.e., intercontinental). This could suggest that venoms designed exclusively to inflict immediate and intense pain, such as the proposed activity of stonefish venom, might not need to be highly variable to be an effective predator deterrent. This may be because pain pathways are generally conserved throughout the animal kingdom, so the same pain-inducing compounds are likely to be effective against a variety of predators (Sneddon, 2017). Additionally, while little is known about the natural predators of stonefish, some speculate that they likely span a wide range of taxa such as sharks, eels, sea snakes, and stingrays (Harris & Jenner, 2019; Govind, 2013). As such, the complexity of stonefish venom (i.e., the presence of numerous components in the venom profiles), could be driven by the generalist nature of the toxin.

Research suggests that a complex venom concoction is often coupled with greater dietary breadth in some venomous taxa (Lyons *et al.*, 2020; Davies & Arbuckle, 2019; Phuong *et al.*, 2016). It is hypothesised that venoms with high compositional complexity may allow for multiple avenues to disrupt the targets physiology (Lyons *et al.*, 2020; Phuong *et al.*, 2016). While the identified active components in stonefish venom (i.e., stonustoxin and verrucotoxin), are known to create pores in biological membranes (Ellisdon *et al.*, 2015; Ueda *et al.*, 2006; Ghadessy *et al.*, 1996), little is known about the function and activity of the other components that comprise the venom, particularly smaller sized components (Saggiomo *et al.*, 2021). Therefore, the complexity of stonefish venom, coupled with a high degree of conservation of components within and between stonefish species, may translate to having a highly potent generalist venom capable of defending against a wide variety of predator taxa, that also likely encompass diverse physiologies. This would ultimately reduce the need for diverging specialist venom compounds between locations, or species.

Although the primary focus of this study was to expand the current understanding of stonefish venom ecology, it is important to consider that both inter-specific and geographic variation in stonefish venom composition may have implications for medical practices in cases of envenomation. Emerging evidence suggests that there is potential variation in the clinical presentation and severity of stonefish sting cases between locations (Armstrong *et al.*, 2023). For instance, reports indicate that populations in Southeast Asia might experience higher frequencies of sting cases resulting in systemic symptoms (i.e., hypotension, arrhythmia, and pulmonary oedema), compared to those in Australia, Singapore, and Japan (Armstrong *et al.*, 2023; Hifumi *et al.*, 2020; Ngo *et al.*, 2009). Additionally, the efficacy of antivenom in managing pain appears to differ internationally, with studies reporting limited success in Australia (Armstrong *et al.*, 2023), and high efficacy (7 out of 8 cases, or 87.5%) in Hong Kong (Poon *et al.*, 2020). Geographic variation in stonefish venom composition could contribute to the reported diversity of symptoms arising in sting cases, as well as the efficacy of antivenenes in managing pain associated with stonefish envenomation across different locations. However, further structural and functional characterisation of the venom components is required to evaluate the clinical relevance of the observed variations in stonefish venom composition.

In conclusion, the results of this study add to the growing body of evidence suggesting that stonefish venoms are complex and dynamic concoctions. Investigating the influence of species and geographic location on the composition of stonefish venom has provided valuable insights into the toxin ecology of these medically significant species. To further solidify these findings, future research should prioritise the structural and functional characterisation of stonefish venom components. Further research into compositional variation in stonefish venom not only improves our understanding of the ecology and evolutionary history of stonefish and their toxins, but also potentially holds implications for the treatment of envenomation in people.

4.6 References

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4.7 APPENDIX C

Geographic Variation in Stonefish (*Synanceia* spp.) Venom

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Geographic variation in stonefish (*Synanceia* spp.) venom

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ABSTRACT

Stonefish (*Synanceia* spp.) possess a medically significant venom and are widely distributed throughout the Indo-Pacific. Yet, little is known about how the ecology of these animals may influence their venom. The aim of this study was to explore the effect of species and geographic location on stonefish venom composition. We collected the venom of *Synanceia horrida* (Estuarine Stonefish) and *Synanceia verrucosa* (Reef Stonefish) from various locations across Australia (Cairns, Brisbane, Caloundra, and Onslow), and Southeast Asia (Kota Kinabalu, and Cebu) and analysed these samples using SDS-PAGE, FPLC, and HPLC. Stonefish have a complex venom comprised of numerous components. Stonefish venom exhibited both similarities and variations in composition within species between geographically isolated populations, as well as between species in a single location. We speculate that the observed geographic and interspecific trends may be driven by similarities and differences in the selective pressures faced by these animals, particularly those associated with predator dynamics. The findings of this study have furthered our understanding of the ecology of stonefish and their toxins.

1. Introduction

Animal toxins, including venoms and poisons, are a form of chemical warfare used by organisms to capture prey and/or defend against predators (McNamee and Cockayne, 2016). To exert their intended effects, toxins must rapidly and potentially interfere with the homeostatic processes of their target/s in the most energetically efficient way possible (Casewell et al., 2020; Pintor et al., 2010; Wigger et al., 2002). However, the mechanisms for achieving this vary considerably, with composition playing a key role in optimising the toxin for its intended purpose (Casewell et al., 2013). Toxins, particularly venoms, contain a complex mixture of proteins, peptides, and small molecules that produce a vast array of pharmacological effects (i.e., neurotoxicity, cardiotoxicity, haemotoxicity) on living organisms (McNamee and Cockayne, 2016). This complexity creates potential for compositional variation in toxins.

The physical structure and chemical nature of toxin components, as well as their relative abundance, has been shown to vary both within species (Tasoulis et al., 2020; Schaffrath et al., 2018) and between species (van Thiel et al., 2023; Duran et al., 2020), as well as within and/or between individuals (Dutertre et al., 2014). Research has shown that biotic factors such as predator-prey dynamics (Smith et al., 2023; Chang and Duda, 2016; Daltry et al., 1996), sex (Amorim et al., 2018; Zelanis et al., 2016; Herzig and Hodgson, 2009), and ontogeny (da Silva

Aguiar et al., 2020; Chang and Duda, 2016; Underwood and Seymour, 2007), as well as abiotic factors including temperature (Smith et al., 2023; O'Hara et al., 2018), and season (Tasima et al., 2024; Junior et al., 2010) are among some of the drivers of compositional variation in animal toxins, particularly venoms. Anecdotal evidence suggests that salinity (O'Hara et al., 2021; Sachkova et al., 2020) may also influence toxin composition. Furthermore, some or all these potential drivers of variation have the capacity to vary geographically (Yu et al., 2020).

The occurrence of geographic variation in venom composition has been reported for numerous species, particularly terrestrial snakes (Smith et al., 2023; van Thiel et al., 2023; Smith and Mackessy, 2021; Davies and Arbuckle, 2019; Sasa, 1999), and to a lesser degree, for some marine species including jellyfish (Winter et al., 2010; Carrette et al., 2002), and cone snails (Weese and Duda, 2019; Phuong et al., 2016). This phenomenon has also been documented at almost all spatial scales, ranging from between microhabitats within a single location (Smith et al., 2023; Abdel-Rahman et al., 2009), to across continents spanning vast distances (Carrette, 2014; Duda et al., 2009). Although geographic variation in venom is widely studied, identifying the ecological factors shaping venom composition is complex and multifaceted, as well as likely unique to each location. Furthermore, in cases of human envenomation, geographic variation in venom composition may have implications for first aid practices (Armstrong et al., 2023). One medically significant venomous animal that is sorely lacking in research aimed at

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its toxin ecology are stonefish.

Stonefish (*Synanceia* spp.) are revered as the world's most venomous fish, a title owing to the noxious defensive venom housed in twin glands associated with each of their 13 dorsal spines (Endean, 1961). Very little work has focussed on elucidating the structural and functional characteristics of stonefish venom components. However, what has been identified are components with the ability to form pores in biological membranes, and others with an affinity for binding to nicotinic acetylcholine receptors which function to interrupt neuronal signalling (Harris et al., 2021; Harris and Jenner, 2019; Ellisdon et al., 2015). These combined activities were suggested to induce significant pain to deter predators, and at the same time cause localised paralysis – most likely in the predators' mouths – to improve the fish's chances of a successful escape (Harris et al., 2021; Ellisdon et al., 2015).

While pain is the most common symptom associated with stonefish envenomation in people, clinical presentations can vary significantly, and range from localised pain and swelling, to oedema, cardiac arrest, and in extreme cases, death (Saggiomo et al., 2021; Ellisdon et al., 2015). Given that stonefish are highly cryptic, a strategy used primarily to enhance their success as benthic ambush predators (Cameron and Endean, 1973; Endean, 1961), human envenomation typically occurs when the animal is stood on. In Australia, stonefish envenomation often results in hospitalisation, with reports ranging from nine (Armstrong et al., 2023) to thirty (Pointer and Harrison, 2021; Little and Gairdner, 2012) hospitalisation cases annually. Globally, the frequency of stonefish envenomation is suspected to be much higher, particularly in places like Southeast Asia where fishing practices are a primary source of income in many communities, and stonefish are considered a prized food fish (Yamamoto et al., 2010). Furthermore, emerging case studies suggest that the clinical symptoms associated with stonefish envenomation may vary in severity, as well as in response to treatment between different locations (Armstrong et al., 2023; Hifumi et al., 2020; Poon et al., 2020; Ngo et al., 2009). One contributing factor may be geographic variation in stonefish venom composition.

Stonefish are widely distributed throughout both the tropical and subtropical waters of the Indo-Pacific (Saggiomo et al., 2021; Endean, 1961). Of the five species that comprise the genera *Synanceia*, two of them, the Reef Stonefish (*Synanceia verrucosa*) and the Estuarine Stonefish (*Synanceia horrida*), have the widest distribution throughout this range (Saggiomo et al., 2021; Endean, 1961). Additionally, these species generally occur in different habitats. For instance, *S. verrucosa* is typically associated with offshore coral or rocky reef ecosystems, whereas *S. horrida* inhabits inshore intertidal areas and estuaries (Endean, 1961). However, the two species are not exclusive to these environments, and their distributions, particularly in places like Australia, Kota Kinabalu in Malaysia, and Cebu in the Philippines, can overlap.

Given the environmental heterogeneity across their range, it is reasonable to suspect that stonefish encounter diverse selective pressures. While prior studies acknowledge potential individual and state-dependent (i.e., starvation) variations in stonefish venom composition (Saggiomo et al., 2017), the influence of geographic variation on their venom is currently unknown. Therefore, the aim of this study was to determine whether the composition of stonefish venom varies according to the animals' geographic location. Additionally, this study compares stonefish venom composition between *S. verrucosa* and *S. horrida* inhabiting the same location in attempt to elucidate inter-species geographical trends.

2. Materials and methods

2.1. Animal and toxin collection

2.1.1. Collecting and sourcing stonefish

Mature (>100 mm body size) *S. horrida* and *S. verrucosa* were hand captured and/or sourced from commercial fish suppliers (Cairns Marine,

Reef 2 Aquarium, Jardini International) between January 2019 to 2024. *Synanceia horrida* were sourced from shallow reef or estuarine environments in Cairns, Brisbane, and Caloundra in Queensland Australia, Onslow in Western Australia, Kota Kinabalu in Malaysia, and Cebu in the Philippines. Similarly, *S. verrucosa* were sourced from offshore reefs in Cairns, Kota Kinabalu, and Cebu. *Synanceia verrucosa* were unable to be sourced from South Queensland (Brisbane and Caloundra) and Western Australia (Onslow). Species were visually identified based on unique physiological features (e.g., head shape, eye position). The sexes of the animals were not able to be determined. Venom collection was done in accordance with James Cook University Animal Ethics Committee approval (AEC approval number A2832).

2.1.2. Extracting stonefish venom

The venom of *S. horrida* and *S. verrucosa* was collected and pooled from multiple individuals (three or greater). Collection involved transferring a single live *S. verrucosa* or *S. horrida* to a saltwater-dampened surface. The animals' eyes were covered with a saltwater dampened cloth to reduce stress. Fish were then gently supporting either side of the pectoral fins and venom was aspirated from the dorsal spines using 9 mL no additive vacuette blood collection tubes (Greiner Bio One) under negative pressure. This involved pressing the rubber seals of the vacuette tubes over each dorsal spine, which induced venom sac compression and venom release. Due to the labile nature of stonefish venom (Harris et al., 2021; Gwee et al., 1994), to reduce degradation of the venom components, collected venom was lyophilised and stored at -80°C until required.

2.2. Profiling stonefish venom

2.2.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on *S. horrida* and *S. verrucosa* venom following a similar protocol outlined by Lennox-Bulow et al. (2023). In brief, venom samples were centrifuged at $15,000\times g$ at 4°C for 5 min to remove debris. The concentration of the sample supernatants was determined using Nanodrop One (Thermo Scientific™) set to protein A_{280} nm and adjusted to yield $500\text{ }\mu\text{g/mL}$ ($\pm 50\text{ }\mu\text{g}$). Sample supernatants ($10\text{ }\mu\text{L}$) were then linearised by mixing (1:2) with 2x Laemmli SDS lysis buffer. This process was catalysed by heating the samples at 95°C for 5 min.

Linearised samples ($20\text{ }\mu\text{L}$) and protein standards ($2\text{ }\mu\text{L}$ Precision Plus Protein™ Dual Colour) were loaded on to 12% Bis-Tris mini protein gels (NuPAGE™, $1.0\text{ mm}\times 12\text{ well}$) set up in an electrophoresis cell. Gels were run in a discontinuous buffer system with the application of 90 V for ~ 10 min during the stacking phase, and 150 V for ~ 45 min during the running phase with a constant amperage of 0.02 A used throughout. The running buffer used was 1x MOPS SDS running buffer (NuPAGE™). Current was applied until the dye marker (bromophenol blue) was within 1 cm of the gel bottom.

Gels were stained by submersion into Imperial™ Protein Stain (Thermo Scientific) for 1 h, and de-stained using deionised (MilliQ) H_2O until the gel background was clear. Gels were photographed with a Nikon D810 against an illuminated surface. Variation in the banding pattern between venom samples was determined via visual analysis of the gel images.

2.2.2. Size exclusion fast performance liquid chromatography (SE-FPLC)

Absorbance (A_{280}) profiles of stonefish (*S. horrida* and *S. verrucosa*) venom were determined using SE-FPLC following a similar protocol outlined by Lennox-Bulow et al. (2023). Lyophilised venom samples were reconstituted in $600\text{ }\mu\text{L}$ of 1x phosphate-buffered saline (PBS, pH 7.0) and centrifuged at $15,000\times g$ for 5 min. The resulting supernatants were passed through a $0.22\text{ }\mu\text{m}$ syringe filter to remove debris. Cleaned venom samples ($500\text{ }\mu\text{L}$) were then manually injected onto the ÄKTA™ prime system. Separation of the venom components was achieved using

a Superdex 200 Increase 10/300 GL Column (8.6 μ m, 10 $\times$ 300 mm, GE Healthcare). The running buffer used was 1x PBS (pH 7.0) and the system was set to run at 500 μ L/min at 8 $^{\circ}$ C for 50 mL. The ultra-violet (UV) absorbance of the eluant was continuously monitored at 280 nm.

Venom profiles were processed using UNICORNTM 7 software (2016, GE Healthcare). To account for variations in sample concentrations, absorbance intensity was normalised for each trace. This was accomplished by multiplying each individual absorbance value by a normalisation factor. The normalisation factor was calculated by dividing the absorbance value of the tallest synchronous peak (observed at 21.5 mL elution volume in all traces) in the reference profile (i.e., the profile with the highest overall absorbance) by the absorbance value of the corresponding peak in the profile being adjusted. Chromatograms were produced using GraphPad Prism 10 for visual comparisons of the toxin profiles.

2.2.3. Reversed-phase high performance liquid chromatography (RP-HPLC)

Hydrophobicity profiles of *S. horrida* and *S. verrucosa* venom were produced using RP-HPLC. Lyophilised venom samples ($\sim$ 25 mg $\pm$ 1 mg) were diluted with a 50:50 mixture of solvent A (250 μ L H₂O/0.05% trifluoroacetic acid (TFA; Auspep) and solvent B (250 μ L 90% ACN (Merck)/10% H₂O/0.045% TFA) and refrigerated overnight. Samples were centrifuged at 15,000x g for 10 min to remove debris. Fractionation used a C₁₈ Phenomenex Aeris[®] PEPTIDE XB liquid chromatography column (150 $\times$ 2.10 mm, 3.6 μ m, 100 $\text{\AA}$) using a gradient of 0–60% solvent B in 60 min, 60–90% solvent B in 5 min, 90% solvent B for 10 min, and 90–0% solvent B in 5 min. The flow rate remained constant at 0.250 mL/min. The concentration of the sample supernatant was determined using Nanodrop One (Thermo ScientificTM) under the Protein A₂₈₀ program, and the injection volume of each venom sample was adjusted to achieve a final concentration of 250 μ g. Absorbance was monitored at 214 nm. Raw data acquisition and initial analysis were conducted within OpenLAB CDS ChemStation software (v1.8.1). Data was then exported to GraphPad Prism 10 for the generation of chromatograms.

3. Results

3.1. SDS-PAGE of stonefish venom from different locations

The SDS-PAGE profiles of *S. horrida* and *S. verrucosa* venom revealed both similarities and variations in composition between different locations (Fig. 1). Banding patterns corresponding to smaller molecules (10 to 25 kDa) were highly conserved across all venom samples, regardless of species or origin. Variations in stonefish venom profiles between species and geographic locations arose primarily in bands associated with larger molecules (55 to 100 kDa). A band at $\sim$ 90 kDa was universally present in all venom samples except for *S. horrida* venom samples from Brisbane and Onslow. *Synanceia horrida* venom samples from Onslow also displayed unique bands at $\sim$ 70 kDa and $\sim$ 71 kDa that were absent in all other samples. Furthermore, a distinct band at $\sim$ 80 kDa was observed exclusively in *S. horrida* venom from Kota Kinabalu, but was a shared characteristic of *S. verrucosa* venom samples from all locations. Bands at $\sim$ 55 kDa and 75 kDa were shared by all venom samples, except for *S. horrida* venom from Kota Kinabalu, and *S. verrucosa* venom from Cairns.

3.2. SE-FPLC of stonefish venom from different locations

Stonefish venom also showed both similarities and variation between species and geographic locations when analysed using SE-FPLC (Fig. 2). Consistent peak patterns were observed at elution volumes between $\sim$ 13 and 19 mL and $\sim$ 21 and 28 mL in *S. horrida* venom from Cairns, Brisbane, Onslow, Caloundra, and Cebu (Fig. 2A), as well as *S. verrucosa* venom from Cairns, Kota Kinabalu, and Cebu (Fig. 2B). However,

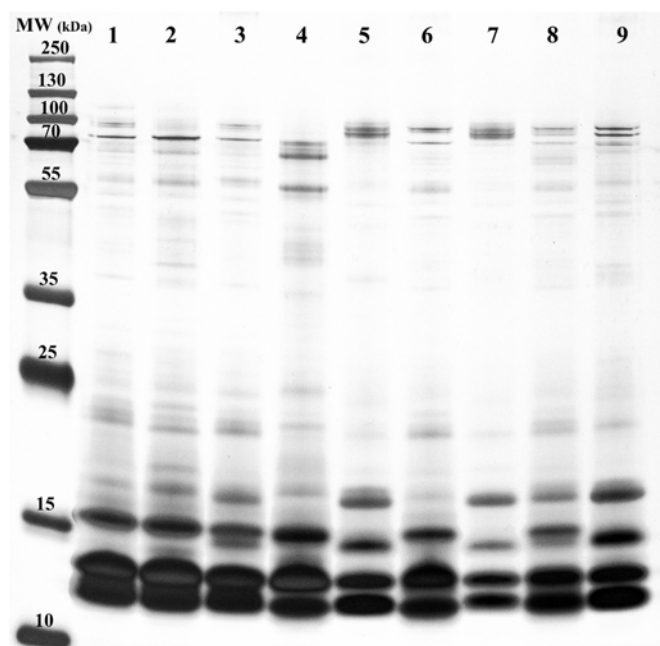


Fig. 1. SDS-PAGE Profiles of Stonefish (*Synanceia* spp.) Venom from Different Locations. Estuarine Stonefish (*Synanceia horrida*) venom acquired from Cairns (lane 1), Brisbane (lane 2), Caloundra (lane 3), Onslow (lane 4), Kota Kinabalu (lane 5), and Cebu (lane 6) populations, as well as Reef Stonefish (*S. verrucosa*) venom from Cairns (lane 7), Kota Kinabalu (lane 8), and Cebu (lane 9) populations were profiled using gel electrophoresis. Venom samples (10 μ L) were loaded onto a NuPAGE Novex 12% Bis-Tris mini protein gel at a concentration of 500 μ g/mL. Molecular weights (kDa) of sample components were estimated by comparison with Precision Plus ProteinTM Dual Colour protein standards.

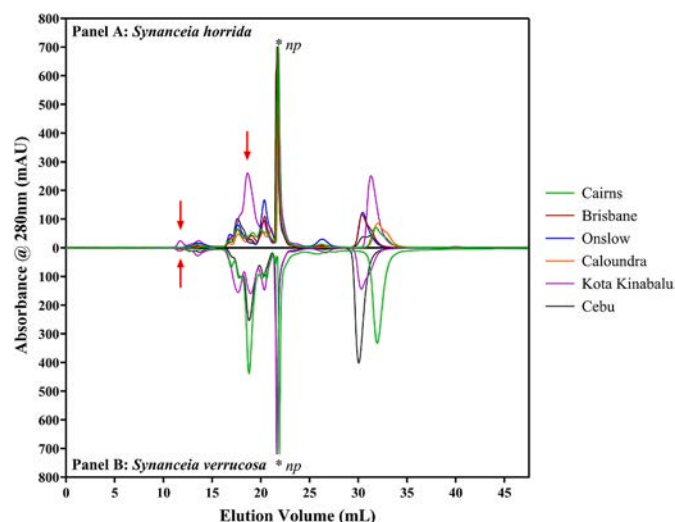


Fig. 2. SE-FPLC Profiles of Stonefish (*Synanceia* spp.) Venom from Different Locations. Estuarine Stonefish (*Synanceia horrida*) venom (Panel A) from Cairns (—), Brisbane (—), Onslow (—), Caloundra (—), Kota Kinabalu (—), and Cebu (—), as well as Reef Stonefish (*Synanceia verrucosa*) venom (Panel B) from Cairns (—), Kota Kinabalu (—), and Cebu (—) were compared within and between species, and across geographic locations. Absorbance was monitored at 280 nm over 50 mL of elution using an ÄKTA Prime System (GE Healthcare). Chromatograms were normalised for the absorbance intensity of the 21.5 mL peak (marked as 'np'). Arrows (↓) mark observed intra-species variations between locations.

S. horrida venom samples from Kota Kinabalu had a single peak eluting between ~13 and 19 mL that was roughly twice as intense as those seen in *S. horrida* venom from other locations. Furthermore, although both species from all measured locations showed peaks at ~21 mL elution volume, some variation was observed in the peak patterns. A single peak was observed in *S. horrida* venom from Brisbane, Onslow, Caloundra, Kota Kinabalu, and Cebu, and *S. verrucosa* venom from Kota Kinabalu and Cebu. In contrast, a double peak occurred in both species venom from Cairns. Additionally, all stonefish venom profiles showed a peak between ~30 and 33 mL, although there was some slight variation in its elution volume between locations. This peak eluted earlier (~30 mL) in *S. horrida* venom samples from Brisbane, Onslow, and Cebu, compared to venom samples from Cairns, Caloundra, and Kota Kinabalu (~32 mL). *Synanceia verrucosa* venom followed a similar trend where this peak eluted earlier (~30 mL) in samples from Kota Kinabalu and Cebu, than venom samples from Cairns (~32 mL). Both *S. horrida* and *S. verrucosa* venom from Kota Kinabalu also shared a distinct peak at ~13 mL elution volume that was absent from all other locations.

3.3. RP-HPLC of stonefish venom from different locations

Stonefish venom showed broadscale similarities, and some variation in component hydrophobicity when analysed using RP-HPLC (Fig. 3). The venom of both *S. horrida* (Fig. 3A) and *S. verrucosa* (Fig. 3B) from all locations shared five distinct peaks, with retention times of approximately 42, 43, 47.5, 48, and 49 min (± 0.5 min). *Synanceia horrida* venom from all locations, and *S. verrucosa* venom from Kota Kinabalu, shared an additional peak at 46.5 min, which was absent in *S. verrucosa* venom from Cairns. This peak was also present in *S. verrucosa* venom from Cebu, but at approximately 20x lower intensity than *S. verrucosa* venom from Kota Kinabalu. Furthermore, *S. verrucosa* venom from all locations, and *S. horrida* venom from Caloundra, Kota Kinabalu, and Cebu, shared

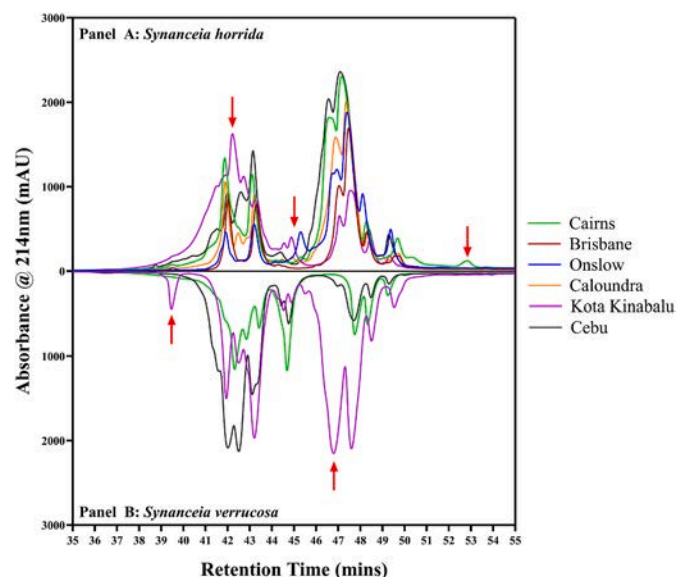


Fig. 3. RP-HPLC Profiles of Stonefish (*Synanceia* spp.) Venom from Different Locations. The venom of Estuarine Stonefish (Panel A: *Synanceia horrida*) from Cairns (—), Brisbane (—), Onslow (—), Caloundra (—), Kota Kinabalu (—), and Cebu (—) and Reef Stonefish (Panel B: *Synanceia verrucosa*) from Cairns (—), Kota Kinabalu (—), and Cebu (—) were compared within and between species, as well as between geographic locations, using chromatograms generated by RP-HPLC. Venom samples (250 μ g total protein) were profiled using a C₁₈ Phenomenex Aeris® PEPTIDE XB liquid chromatography column. Absorbance was monitored at 214 nm using a 1% gradient over 80 min. The absorbance traces for retention times up to the first 34.9 min, and after 55.1 min were omitted from the figure to enhance viewing resolution. Arrows (↓) mark observed intra-species variations between locations.

peaks between 42 and 43 min that were absent in *S. horrida* venom from Cairns, Brisbane, and Onslow. However, the peak pattern within this range differed between some locations. *Synanceia horrida* venom from Kota Kinabalu and Caloundra, as well as *S. verrucosa* venom from Cairns, had a double peak, whereas both *S. horrida* and *S. verrucosa* venom from Cebu, as well as *S. verrucosa* venom from Kota Kinabalu, had a single peak. *Synanceia horrida* venom from Kota Kinabalu and Caloundra also had a double peak between 44 and 45 min that was absent in *S. horrida* samples from Onslow, Brisbane, and Cairns, but was a shared characteristic of *S. verrucosa* venom from all locations. However, *S. horrida* samples from Cebu also had a single peak that appeared within this range. Furthermore, *S. horrida* venom from Cairns and Onslow, as well as *S. verrucosa* venom from Kota Kinabalu, shared a peak between 45 and 46 min that was absent in all other samples (i.e., both species and all other locations). *Synanceia verrucosa* venom from Kota Kinabalu had a unique peak at ~39.5 min that was absent from all other samples. Similarly, *S. horrida* venom from Cairns also had a unique peak at ~52.5 min that was absent from all other samples.

3.4. Comparison of stonefish venom between species and locations

Stonefish were found to have a complex venom profile comprised of numerous components. Within each species, a large proportion of the venom components exhibited consistent properties across geographic locations. The majority of the observed venom components showed similarities in size (particularly in smaller components between ~10 and 25 kDa), and hydrophobicity (~40–50 min retention time), regardless of the animal's geographic origin. These same size and hydrophobicity characteristics were also found to be shared between the two species.

Despite the overall consistency, some variation in venom component size (particularly in larger components between ~55 and 100 kDa), and hydrophobicity characteristics, were observed. This variation occurred within each stonefish species across different locations, as well as between species found in the same location. For both species, intraspecific venom variation was most pronounced in stonefish venom samples collected from Kota Kinabalu. In *S. horrida* venom samples from Kota Kinabalu, variation was observed in both the larger sized components, as well as within the hydrophobicity profile, with unique peaks appearing at 42 min and 45 min retention time. In contrast, variation in *S. verrucosa* venom samples from Kota Kinabalu were only observed in the hydrophobicity profile, with unique peaks appearing at 39 min and 47 min retention time. Interestingly, both the size and hydrophobicity profiles of *S. horrida* venom from Kota Kinabalu more closely resembled *S. verrucosa* venom from all locations, than *S. horrida* venom from any other location tested in this study. Furthermore, *S. horrida* venom from the west coast of Australia (Onslow) exhibited notable differences in venom composition (i.e., the size and hydrophobicity of some components), compared to *S. horrida* venom from the east coast of Australia (Cairns, Brisbane, and Caloundra).

4. Discussion

Mounting evidence suggests the presence of broad-scale similarities in *S. horrida* and *S. verrucosa* venom in the composition of the smaller components (Saggiomo et al., 2021; Madokoro et al., 2011; Garnier et al., 1996), as well as within subunits of their vertebrate-toxic factors verrucotoxin and stonustoxin (Saggiomo et al., 2021; Ueda et al., 2006; Garnier et al., 1997). Similarly, the results of this study show that stonefish venom components are highly conserved in molecule size, and hydrophobicity characteristics, both intra- and inter-specifically, regardless of the animal's geographic origin. There are several suggestions within the literature as to why venom composition may be either entirely or partially conserved within species, and between closely related species. These centre around the animal's evolutionary history, as well as the selective pressures it faces in its environment (See review Casewell et al., 2020).

The retention of some venom components within and between stonefish species is, at least in part, an artefact of their shared and/or close evolutionary history. Whereby, populations of recent common ancestry are likely to produce more similar venoms than populations separated by greater patristic distances (Davies and Arbuckle, 2019; Phuong et al., 2016). As such, the shared ancestry of Scorpaenid fish, and moreover, the close relationship between *Synanceia* species (*S. horrida* and *S. verrucosa*), and individuals, likely translates to shared or similar venom-producing genes, and thus, shared or similar venom components. Furthermore, the retention of some venom components over evolutionary time, such as in the divergence of the two *Synanceia* species, and/or intra-specifically between isolated populations, might suggest that these components are highly successful in their function. The reason behind this being that venom components operating against the intended target effectively would be strongly favoured by natural selection, leading to their retention (Casewell et al., 2020). Conversely, the observed compositional variation in stonefish venom, both inter- and intra-specifically, may be driven by differences in selective pressures in their environment.

Compositional variation in venom between geographically isolated populations has been attributed to a complex interplay of factors, both biotic and abiotic, related to the respective environment/location (Smith and Mackessy, 2021). As such, elucidating the factors shaping stonefish venom composition is both complex, as well as potentially unique to each location. However, the most compelling evidence of strong positive selection shaping venom composition is the occurrence of target-specific compounds (Smith and Mackessy, 2021; Barlow et al., 2009). Several studies have shown correlations in prey dynamics and venom composition among geographically isolated populations within species. For example, one study found that *Crotalus v. viridis* (Prairie rattlesnakes) populations in northern America produce venom that is dominant in a compound (i.e., myotoxin) with specific toxicity towards mammals, which is non-toxic to lizards (Smith et al., 2023). Conversely, southern populations were found to produce a venom phenotype effective against both lizards and mammals (Smith et al., 2023). Others have highlighted that the relative abundance of certain components within the venom of *Calloselasma rhodostoma* (Malayan pit viper) exhibited significant geographic variation, which was hypothesised to be driven by variations in the relative abundances of local prey taxa including amphibians, reptiles, and other endotherms (Daltry et al., 1996). Similarly, venom from *Chironex fleckeri* (Australian big box jellyfish) populations in northern Australia were found to be more cardiotoxic to mammals than Western Australian populations. This was hypothesised to be correlated with a difference in the physical size of the animals between the two locations (i.e., larger animals in Queensland, and smaller animals in Western Australia) (Winter et al., 2010), which potentially influences cnidae composition (Carrette et al., 2002), and thus, feeding ecology (i.e., a vertebrate diet for Queensland populations and an invertebrate diet for Western Australian populations). However, the focus on prey dynamics as the primary force influencing venom composition may not be universally applicable.

In Scorpaenid fish, which include stonefish (*Synanceia* spp.), venom is used exclusively for defence. As such, we propose that the strongest selective force acting upon and shaping stonefish venom composition is most likely predator dynamics. The results of this study show that *S. horrida* venom from Kota Kinabalu displayed a greater resemblance to *S. verrucosa* venom from all locations, than to *S. horrida* venom from animals inhabiting Australia or the Philippines. Similarly, *S. horrida* venom collected on the west coast of Australia (Onslow) contained unique components not found in the venom from east coast locations (Cairns, Brisbane, Caloundra). At a smaller spatial scale, stonefish venom also showed inter-specific compositional variation between typically reef (*S. verrucosa*) and estuary (*S. horrida*) inhabiting species within the same location. The observed variations in *S. horrida* venom composition at both an international and national scale, as well as between stonefish species within a single location may be driven by

differences in predator dynamics. Such an adaptation would likely confer a selective advantage for stonefish against variations in the species, abundance, and encounter frequency of natural predators within and between locations. Conversely, similarities in stonefish venom composition between species within a single locale, such as Kota Kinabalu stonefish populations, as well as between spatially separated populations within a region, such as *S. horrida* from various locations along the east coast of Australia, might suggest that these populations experience common and/or shared predator dynamics. Alternatively, similarities in venom composition between stonefish species in a single location (i.e., Kota Kinabalu stonefish populations) might indicate that these species exist in similar habitats which is unusual for other locations.

Similarities in stonefish venom composition were also observed at all levels, including both within and between species, as well as at all spatial scales from tens of kilometres (i.e., microhabitats), to upwards of tens of thousands of kilometres (i.e., intercontinental). This could suggest that venoms designed to exclusively inflict immediate and intense pain, such as the proposed activity of stonefish venom, might not need to be highly variable to be an effective predator deterrent. This may be because pain pathways are generally conserved throughout the animal kingdom, so the same pain-inducing compounds are likely to be effective against a variety of predators (Sneddon, 2017). Additionally, while little is known about the natural predators of stonefish, some speculate that they likely span a wide range of taxa such as sharks, moray eels, sea snakes, and stingrays (Harris and Jenner, 2019; Govind, 2013). As such, the complexity of stonefish venom (i.e., the presence of numerous components in the venom profiles), could be driven by the generalist nature of the toxin.

Research suggests that a complex venom concoction is often coupled with greater dietary breadth in some venomous taxa (Lyons et al., 2020; Davies and Arbuckle, 2019; Phuong et al., 2016). It is hypothesised that venoms with high compositional complexity may allow for multiple avenues to disrupt the targets physiology (Lyons et al., 2020; Phuong et al., 2016). While the identified active components in stonefish venom (i.e., stonustoxin and verrucotoxin), are known to create pores in biological membranes (Ellisdon et al., 2015; Ueda et al., 2006; Ghadessy et al., 1996), little is known about the function and activity of the other components that comprise the venom, particularly smaller sized components (Saggiomo et al., 2021). Therefore, the complexity of stonefish venom, coupled with a high degree of conservation of components within and between stonefish species, may translate to having a highly potent generalist venom capable of defending against a wide variety of predator taxa, that also likely encompass diverse physiologies. This would ultimately reduce the need for diverging specialist venom compounds between locations, or species.

Although the primary focus of this study was to expand the current understanding of stonefish venom ecology, it is important to consider that both inter-specific and geographic variation in stonefish venom composition may have implications for medical practices in cases of envenomation. Emerging evidence suggests that there is potential variation in the clinical presentation and severity of stonefish sting cases between locations (Armstrong et al., 2023). For instance, reports indicate that populations in Southeast Asia might experience higher frequencies of sting cases resulting in systemic symptoms (i.e., hypotension, arrhythmia, and pulmonary oedema), compared to those in Australia, Singapore, and Japan (Armstrong et al., 2023; Hifumi et al., 2020; Ngo et al., 2009). Additionally, the efficacy of antivenom in managing pain appears to differ internationally, with studies reporting limited success in Australia (Armstrong et al., 2023), and high efficacy (7 out of 8 cases, or 87.5%) in Hong Kong (Poon et al., 2020). Geographic variation in stonefish venom composition could contribute to the reported diversity of symptoms arising in sting cases, as well as the efficacy of antivenenes in managing pain associated with stonefish envenomation across different locations. However, further structural and functional characterisation of the venom components is required to

evaluate the clinical relevance of the observed variations in stonefish venom composition.

In conclusion, the results of this study add to the growing body of evidence suggesting that stonefish venoms are complex and dynamic concoctions. Investigating the influence of species and geographic location on the composition of stonefish venom has provided valuable insights into the toxin ecology of these medically significant species. To further solidify these findings, future research should prioritise the structural and functional characterisation of stonefish venom components. Further research into compositional variation in stonefish venom not only improves our understanding of the ecology and evolutionary history of stonefish and their toxins, but also potentially holds implications for the treatment of envenomation in people.

CRedit authorship contribution statement

Danica Lennox-Bulow: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Robert Courtney:** Writing – review & editing, Conceptualization. **Jamie Seymour:** Writing – review & editing, Supervision, Conceptualization.

Ethical Statement for Solid State Ionics

Hereby, I Danica Lennox-Bulow consciously assure that for the manuscript “Geographic Variation in Stonefish (*Synanceia* spp.) venom” the following is fulfilled:

- 1) This material is the authors’ own original work, which has not been previously published elsewhere.
- 2) The paper is not currently being considered for publication elsewhere.
- 3) The paper reflects the authors’ own research and analysis in a truthful and complete manner.
- 4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
- 5) The results are appropriately placed in the context of prior and existing research.
- 6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.
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I agree with the above statements and declare that this submission follows the policies of Solid State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Chapter 5

Inter-Species Variation in Stonefish (*Synanceia* spp.) Ichthyocrinotoxins; An Ecological Perspective

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Statement of Contribution of Others

D Lennox-Bulow is the principal author of this manuscript and was extensively involved in all aspects of this work under the supervision of J Seymour. M Smout and D Wilson contributed to data collection and analysis, and in reviewing earlier versions of this manuscript.

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5.1 Abstract

Although stonefish (*Synanceia* spp.) are well-known to harbour a highly noxious defensive venom in their dorsal spines, very little is known about the composition and ecological function of the ichthyocrinotoxins that they secrete onto their epidermis. This study profiled *Synanceia verrucosa* (Reef Stonefish) and *Synanceia horrida* (Estuarine Stonefish) ichthyocrinotoxins via electrophoresis, liquid chromatography, and mass spectrometry to visualise and compare the composition of these toxins between the two species. Stonefish ichthyocrinotoxins were found to be multifarious concoctions that exhibited subtle differences between reef and estuarine species. We speculate that these variations and similarities are driven by the different and similar ecology of these fish species. Further research into the activity of the toxins components is now required to better understand their ecological role.

5.2 Introduction

Over 2,900 species of fish possess biological toxins in the form of either a venom or a poison (Harris & Jenner, 2019). Poisons used by fish are generally small molecular oral biotoxins that can be noxious when either consumed or absorbed (Jared *et al.*, 2021). These are produced either by accumulating consumed metabolites in body tissues, or within specialised epithelial cells, which are called club cells or tubercles (Harris & Jenner, 2019; Mahmud *et al.*, 2003). The latter are collectively called ichthyocrinotoxins (Cameron & Endean, 1973; Halstead, 1965).

Approximately 50 species of fish belonging to at least 14 families are known to produce ichthyocrinotoxins, few of which have been extensively studied (Eliahu Kalmanzon & Zlotkin, 2019). A common characteristic of ichthyocrinotoxins is their deterrent, and sometimes fatal effect on other teleost species (Primor & Zlotkin, 1975; Randall *et al.*, 1971; Thomson, 1964). Furthermore, many ichthyocrinotoxic species, such as *Grammistes sexlineatus* (Golden-Striped Soapfish) and *Pardachirus marmoratus* (Red Sea Moses Sole), secrete copious amounts of this toxin in response to threat (Al-Hassan *et al.*, 1985; Clark, 1974; Randall *et al.*, 1971; Boylan & Scheuer, 1967). As such, ichthyocrinotoxins are generally believed to be a defensive substance, primarily acting to deter and/or repel predators (Abdul-Haqq & Shier, 2008; Cameron & Endean, 1973; Randall *et al.*, 1971). One genus of fish that harness ichthyocrinotoxins are stonefish.

Stonefish (*Synanceia* spp.) are a bottom-dwelling fish found throughout the shallow, coastal waters of the Indo-Pacific region (Endean, 1961). Two species of stonefish are found in Queensland waters; *Synanceia verrucosa*, and *Synanceia horrida* (Endean, 1961). One key distinction between these species is their preferred habitat, with *S. horrida* (Estuarine Stonefish) primarily found inshore amongst sheltered bays and estuaries, whereas *S. verrucosa* (Reef Stonefish) are commonly associated with coral reefs (Endean, 1961). Stonefish are one of only a few known animals that harness two distinct types of toxin: an injectable venom which is housed within twin glands associated with 13 dorsal spines, and an ingestible ichthyocrinotoxin secreted by the tubercles that span their epidermis (Saggiomo *et al.*, 2021; Endean, 1961). Stonefish venom is believed to function purely as a predator defence (Saggiomo *et al.*, 2021; Endean, 1961). The deleterious nature of this venom to people has awarded stonefish the title of worlds' most venomous fish (Saggiomo *et al.*, 2021; Cameron & Endean, 1973). As such, research into stonefish toxins is often centred around medical aid to

envenomation which is commonly a result of accidentally standing on the animal (Ziegman *et al.*, 2019; Cameron & Endean, 1973). However, very little is currently known about stonefish ichthyocrinotoxins.

Stonefish exhibit a primarily sedentary lifestyle and lack squamation (Endean, 1961). Thus, it has been suggested that their ichthyocrinotoxin may act to manage biological interactions with their integument, particularly those involving bacteria, parasites, as well as fouling flora and fauna (Cameron *et al.*, 1981; Endean, 1961). However, these interactions may vary between reef and estuarine environments, potentially leading to different ichthyocrinotoxins. Therefore, the aim of this study was to determine if stonefish ichthyocrinotoxins exhibit inter-species variation, and if so, discuss the potential ecological drivers behind it.

5.3 Materials and Methods

5.3.1 Animal and Toxin Collection

Collecting and Sourcing Stonefish.

Mature (>100 mm body size) *S. horrida* and *S. verrucosa* were collected in Cairns, Queensland, Australia by a local commercial fish supplier, Cairns Marine. Species were visually identified based on unique physiological features (e.g., head shape, eye position). The sexes of the animals were not able to be determined.

Extracting Stonefish Ichthyocrinotoxins.

Ichthyocrinotoxin collection was done in accordance with James Cook University Animal Ethics Committee approval (AEC approval number A2832). The ichthyocrinotoxins of *S. verrucosa* and *S. horrida* were collected from multiple animals of both sexes ranging between 143 and 370 mm in body length. To collect the ichthyocrinotoxin, live *S. verrucosa* or *S. horrida* were placed on a surface and secured by covering the animals' eyes with a saltwater dampened cloth to promote calmness while firmly holding either side of the pectoral fins. Ichthyocrinotoxin was then extracted from the skin by massaging the tubercles under a gentle vacuum. This vacuum was created with a custom-built extraction apparatus composed of a 10 mm piece of plastic tubing fastened to a 50 mL syringe. Evacuated toxin was centrifuged at 15,000 rpm for 20 min, or until the white component had pelleted out and the supernatant became clear, prior to use to remove any cell debris or contaminants. The supernatant was transferred to a new 1.5 mL Eppendorf tube and protein concentration was determined using a Nanodrop One (Thermo Scientific™) under the Protein A₂₈₀ program. Ichthyocrinotoxin supernatant was stored at -80°C until required.

5.3.2 Profiling Stonefish Ichthyocrinotoxins

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was performed as an exploratory assessment of the toxins to determine the size range of the protein constituents. This informed the conditions selected for liquid chromatography. Ichthyocrinotoxin supernatant samples were serially diluted to a concentration of 2 mg/mL (± 0.05 mg/mL) and mixed with 2× Laemmli buffer at a ratio of 1:2. The toxin components were then denatured at 95 °C for 5 min. Denatured ichthyocrinotoxin samples (20 μ L) and protein standards (2 μ L, Precision Plus Protein™ Dual Colour, BioRad) were loaded on to a 15% polyacrylamide gel (1.0 mm x 12 well) set up in an electrophoresis

cell. Gels were run in a discontinuous buffer system with the application of 90 V for ~10 min during the stacking phase, and 150 V for ~45 min during the running phase with a constant amperage of 0.02 Å used throughout. The running buffer used was 1× Sodium Dodecyl Sulphate (SDS) at pH 8. Current was applied until the dye marker (bromophenol blue) was within 1 cm of the gel bottom. Gels were stained by submersion into Imperial™ Protein Stain (Thermo Scientific™) for 1 hr, and de-stained using deionised (MilliQ) H₂O until the gel background was clear. Gels were photographed with a Nikon D810 against an illuminated surface. Inter-species variation in the banding pattern was determined via visual analysis of the gel images. Inter-species variation in band intensity was quantified using histogram analysis in Image J. Grey scores were generated for each band that appeared in the species profiles, as well as for the image background which was assigned as maximum brightness. Grey scores were then inverted by subtracting the score of each band from maximum brightness to yield a darkness score. Darkness scores were plotted as percent intensity using GraphPad Prism 9.

Size Exclusion Fast Performance Liquid Chromatography (SE-FPLC).

SE-FPLC on an ÄKTA™ prime system (GE Healthcare) was used to generate the protein profiles and purify the toxin components. Ichthyocrinotoxin supernatant samples (500 µL) were manually injected onto the FPLC system at a concentration of 20 mg/mL (± 0.05 mg/mL). Ichthyocrinotoxin components were separated using a Superdex 75 10/300 (GE Healthcare) GL Column (13 µm, 10 × 300 mm) with 0.25× Phosphate-Buffered Saline (PBS) as a running buffer (500 µL/min for 40 mL at 8 °C). The ultraviolet (UV) absorbance of the subsequent eluant was monitored at 280 nm. Fractions (500 µL) were collected and stored in 1.5 mL 96 deep-well plates (Thermo Scientific™) at 4 °C until the run completion. Toxin profiles were processed and exported using UNICORN™ 7 software (2016, GE Healthcare). Due to the disproportionate nature of absorbance intensity between the chromatograms of each species, raw data was exported to Excel and normalised. Normalisation involved dividing each absolute absorbance value in each chromatogram by the highest absolute absorbance value in the corresponding chromatogram. Chromatograms were visually analysed in GraphPad Prism 9 and fraction pools were determined based on the presence of distinct peaks. A total of five pooled fractions were collated from each species toxin based on the identified peaks (See **Table 5.1**).

Liquid Chromatography/Electrospray Ionisation-Mass Spectrometry (LC/ESI-MS).

The protein composition profiles of crude and SE-FPLC fractionated stonefish ichthyocrinotoxins were determined using a Shimadzu LCMS-2020 mass spectrometer coupled to a Shimadzu Prominence high performance liquid chromatography (HPLC) system (Shimadzu). LC/MS grade solvents (Buffer A: 0.1% formic acid (FA; Sigma-Aldrich)/H₂O; Buffer B: 90% acetonitrile (ACN; Thermo Scientific™)/0.09% FA/H₂O) were delivered via pumps (Shimadzu LC-20AD) at a flow rate of 0.250 mL/min. Ichthyocrinotoxin samples were injected via an autosampler (Shimadzu SIL-20AC_{HT}) onto a Phenomenex Kinetex® C₈ liquid chromatography column (50 × 2.1 mm, 5.0 µm, 100 Å) and eluted with a 1% gradient of Buffer B (0–80%, 80 min; 80–90%, 5 min; 90%, 10 min; 80–0%, 5 min; 0%, 10 min). SE-FPLC fractions included a 10 min de-salting wash of Buffer A prior to commencing the gradient. The UV absorbance was monitored at 280 nm using a Shimadzu SPD-20 A detector. Mass spectra were collected in positive and negative ion mode over a scan range of 160–2000 *m/z* with a detector voltage of 1.35 kV, nebulizing gas flow of 1.5 L/min, and drying gas flow of 3 L/min. Data were collected and analysed using Shimadzu LabSolutions v5.96 software. Spectra across the total ion current (TIC) peaks were averaged and the multi-charge ion analysis tool was used to reconstruct the ion series in the spectra to provide the compound mass. Raw data was exported to GraphPad Prism 9 to generate chromatograms. **Reversed-Phase High Performance Liquid Chromatography (RP-HPLC).**

RP-HPLC of stonefish ichthyocrinotoxins was performed using a C₁₈ Phenomenex Aeris® PEPTIDE XB liquid chromatography column (250 × 10 mm, 5 µm, 100 Å). Ichthyocrinotoxin samples (500 µL) were mixed with solvent A (500 µL H₂O/0.05% trifluoroacetic acid (TFA; Auspep) and centrifuged at 15,000 rpm for 5 min. Samples were manually loaded onto the RP-HPLC system. Fractionation of the sample used a gradient of 0–60% solvent B (90% ACN (Merck)/10% H₂O/0.045% TFA) in 120 min, 60–90% solvent B in 5 min, 90% solvent B for 10 min, and 90–0% solvent B in 5 min. The flow rate throughout was 3 mL/min and the absorbance was monitored at 280 nm. Fractions (30 s) were automatically collected into 2.0 mL 96 well deep-well plates (Axygen). Raw data was collected and analysed within OpenLAB CDS ChemStation software (v1.8.1) and exported to GraphPad Prism 9 to generate chromatograms. Chromatograms were simplified for visual comparisons by plotting the negated absorbance values for *S. horrida* ichthyocrinotoxin against the raw absorbance values of *S. verrucosa* ichthyocrinotoxin.

5.4 Results

5.4.1 SDS-PAGE Profiles of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins

SDS-PAGE of *S. verrucosa* and *S. horrida* ichthyocrinotoxins revealed that there were distinct differences in the pattern and intensity of the bands between the profiles (**Figure 5.1**). The bulk of the profile for both species fell within the ~15 to 18 kDa range. The banding pattern within this range was similar between the two species (**Figure 5.1A**). However, there was a distinct difference in the intensity of the bands, where *S. verrucosa* ichthyocrinotoxin had a darker banding pattern than *S. horrida* (**Figure 5.1B**). Similarly, there was a faint double band at ~33 kDa in both profiles that appeared more intense in *S. verrucosa* ichthyocrinotoxin than *S. horrida*. Additionally, there was some variation in the banding pattern between the toxin profiles, particularly in the lower and upper size ranges. *S. horrida* ichthyocrinotoxin, for example, had a double band that appeared around ~13 kDa which was absent in the profile of *S. verrucosa*. Conversely, *S. verrucosa* ichthyocrinotoxin had a band that appeared around ~22 kDa that was absent in the profile of *S. horrida*. Thus, the mass range of the components slightly differed between the two species profiles, with *S. horrida* ranging between ~13 and 33 kDa, and *S. verrucosa* ranging between ~14 and 35 kDa.

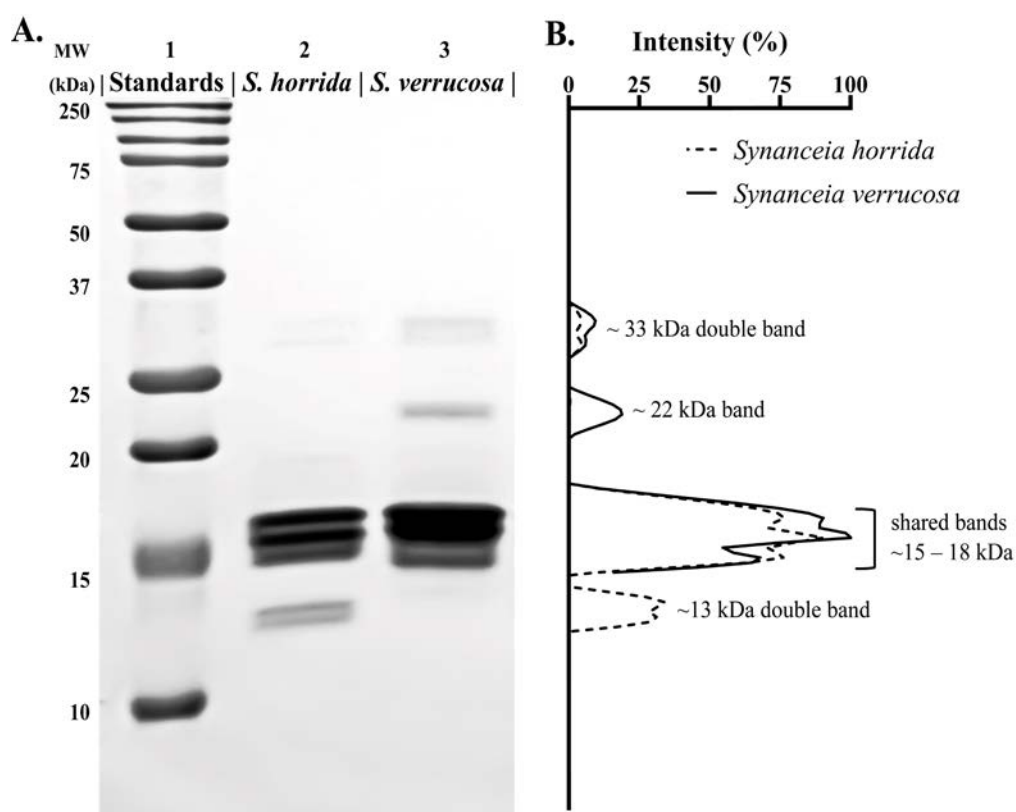


Figure 5.1 SDS-PAGE Gel Profiles and Band Intensity Plot of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins. **Panel A:** SDS-PAGE gel image of *Synanceia horrida* (Estuarine Stonefish, Lane 2), and *Synanceia verrucosa* (Reef Stonefish, Lane 3) ichthyocrinotoxins. Protein standards (Precision Plus Protein™ Dual Colour, Lane 1) were used to estimate the molecular weight (kDa) of the components within each species ichthyocrinotoxin. **Panel B:** Band intensity plot of stonefish ichthyocrinotoxins. The intensity (%) of the bands that appeared in the gel electrophoresis profile of *S. verrucosa* (—), and *S. horrida* (---) ichthyocrinotoxins were quantified using an inverted grey scale. Grey values were generated using histogram analysis in ImageJ.

5.4.2 SE-FPLC Profiles of Stonefish (Synanceia spp.) Ichthyocrinotoxins

SE-FPLC chromatograms varied between *S. verrucosa* and *S. horrida* ichthyocrinotoxins in both peak intensity and occurrence (**Figure 5.2**). In general, most peaks in the chromatogram of *S. horrida* had a greater absorbance intensity than those appearing in *S. verrucosa*. One peak (c) that eluted around 16 mL in both profiles was approximately ten times more intense in the profile of *S. horrida* compared to *S. verrucosa*. There were also peaks that appeared where others were absent. In the profile of *S. verrucosa* ichthyocrinotoxin, a peak appeared between 7.5 and 10 mL (x) that was absent in the profile of *S. horrida*. Similarly, there was a peak between 30 and 32.5 mL (y) in the profile of *S. horrida* that were absent from *S. verrucosa*. A total of five distinct peaks were visually identified in the FPLC chromatograms of *S. verrucosa* and *S. horrida* ichthyocrinotoxins (Letters a – e) which were collected and pooled for each species (**Table 5.1**).

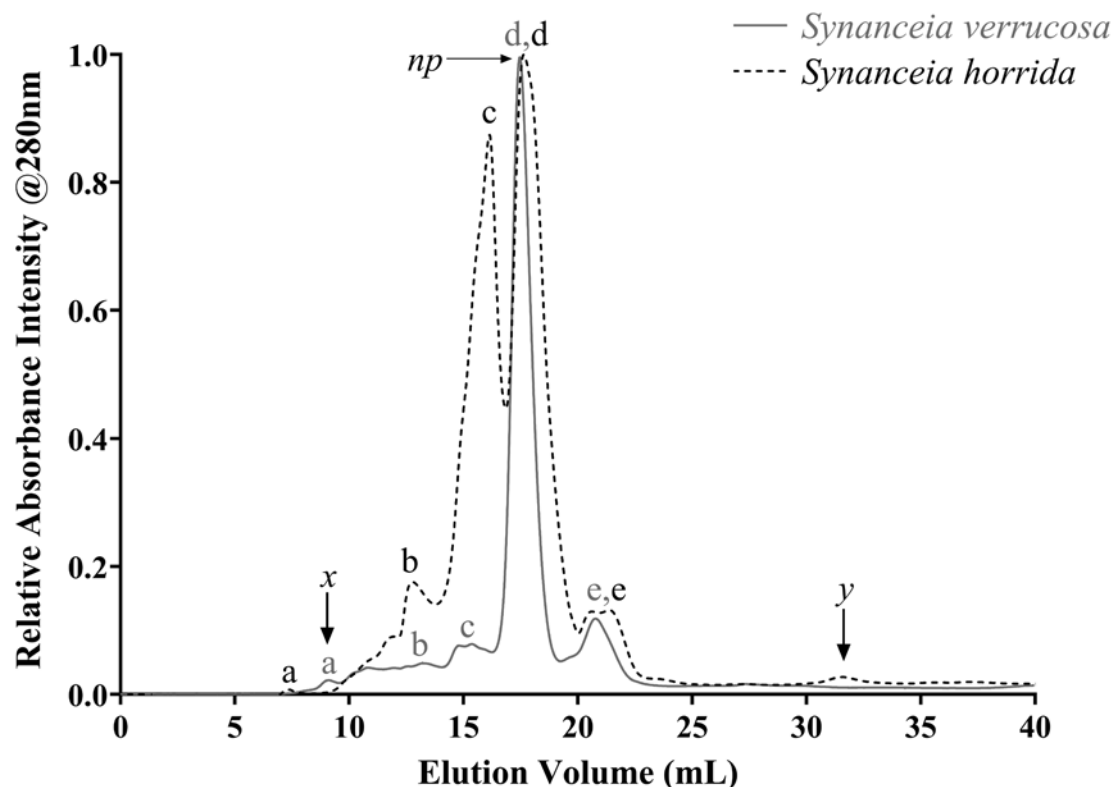


Figure 5.2 SE-FPLC Chromatograms of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins. The components within *Synanceia verrucosa* (Reef Stonefish, —), and *Synanceia horrida* (Estuarine Stonefish, - - -) ichthyocrinotoxins were separated by size using a Superdex 75 10/300 (Tricorn) GL Column (13µm, 10 x 300 mm) with 0.25x PBS as a running buffer (500 µL/min). Absorbance (280 nm) was monitored for 40 mL of the eluant. Chromatograms were normalised for the absorbance intensity of 17.5 mL peak (marked as 'np'). Letter designations 'x' and 'y' mark specific points of interest. Letters (a – e) mark visually identified distinct peaks which correspond to the pooled fraction designations listed in **Table 5.1**.

Table 5.1 Peak Designations of SE-FPLC Fractionated Stonefish (*Synanceia* spp.) Ichthyocrinotoxins. *Synanceia verrucosa* (Reef Stonefish) and *Synanceia horrida* (Estuarine Stonefish) ichthyocrinotoxins were fractionated (0.5 mL fractions) using SE-FPLC over 40 mL elution volume. Chromatographic peaks were visually identified, the corresponding fractions were pooled and given a letter designation (a – e).

Fraction Name	Sub-Fractions (Elution volume, mL)	
	<i>Synanceia verrucosa</i>	<i>Synanceia horrida</i>
a	7 – 10	7 – 8
b	10 – 14	8 – 14
c	14 – 16	14 – 16
d	16 – 19	16 - 19
e	19 – 23	19 – 23, 30 – 33

5.4.3 RP-HPLC Profiles of Stonefish (Synanceia spp.) Ichthyocrinotoxins

RP-HPLC chromatograms of *S. verrucosa* and *S. horrida* ichthyocrinotoxins revealed relatively complex chromatograms, with components predominantly eluting between 20 and 35 min (**Figure 5.3**). While the profiles for both species were similar, several differences were observed. A greater abundance of components eluted between 2.5 and 20 min in the *S. horrida* ichthyocrinotoxin sample. Additionally, there were components eluting after 35 min in the profile of *S. horrida* that were absent in the profile of *S. verrucosa*. Similarly, there were components eluting between 33 and 34 min in *S. verrucosa* that were either completely absent or in low abundance in the profile of *S. horrida*.

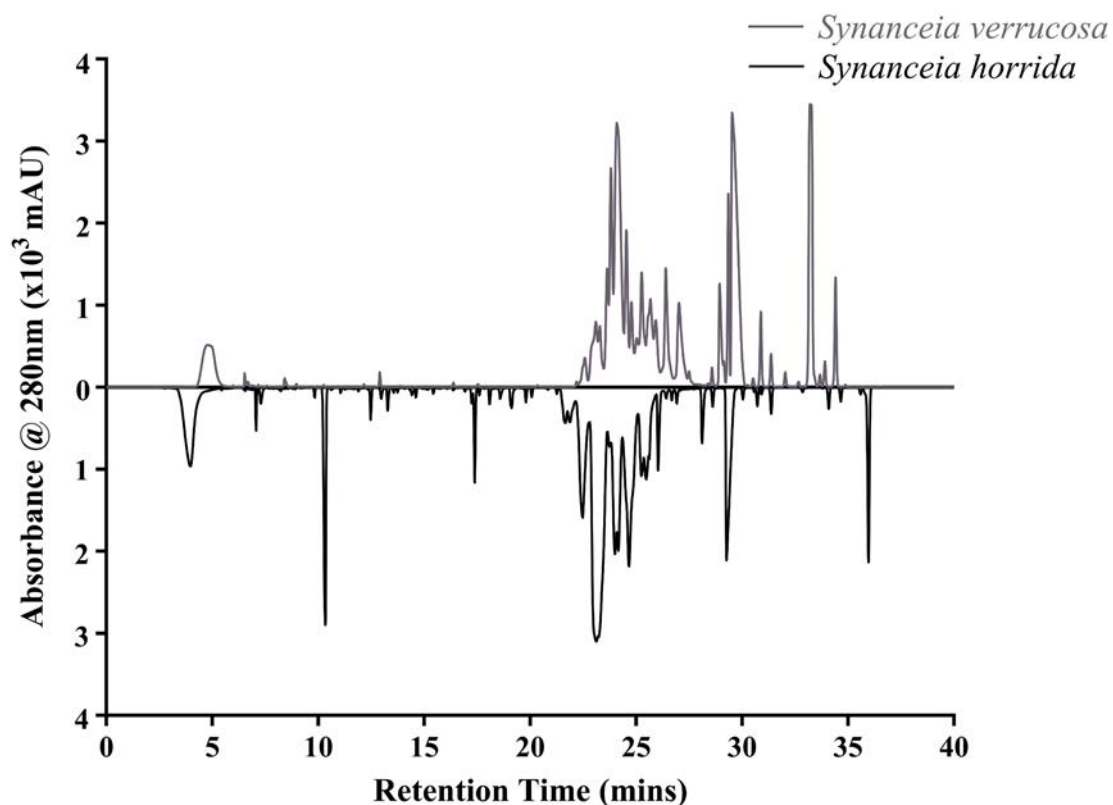


Figure 5.3 RP-HPLC Profiles of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins. Semi-Preparative RP-HPLC of *Synanceia verrucosa* (Reef Stonefish, —) and *Synanceia horrida* (Estuarine Stonefish, —) ichthyocrinotoxins used a C₁₈ Phenomenex Aeris® PEPTIDE XB liquid chromatography column (250 x 10 mm, 5 µm, 100 Å) with a gradient of 0-60% solvent B (Solvent A: H₂O/0.05% TFA; Solvent B: 90% ACN/10% H₂O/0.045% TFA) over 40 min at a flow rate of 3 mL/min. Absorbance was monitored at 280 nm. Chromatograms were simplified for visual comparisons between species by mirroring the absorbance values for *S. horrida* ichthyocrinotoxin.

5.4.4 LC/ESI-MS

LC/ESI-MS revealed that the masses and relative abundance of proteins within stonefish ichthyocrinotoxins varied between the two species (**Figure 5.4**). Protein masses ranged from 11.8 to 35.9 kDa in *S. verrucosa* ichthyocrinotoxin (**Figure 5.4A**) and 11.1 to 32 kDa in *S. horrida* ichthyocrinotoxin (**Figure 5.4B**). Furthermore, double the number of proteins with unique masses were identified in *S. verrucosa* ichthyocrinotoxin compared to *S. horrida* ichthyocrinotoxin. However, *S. horrida* ichthyocrinotoxin had a higher abundance of lower mass (11–12 kDa) uniquely sized proteins, which is consistent with the bands observed in the SDS-PAGE gel results.

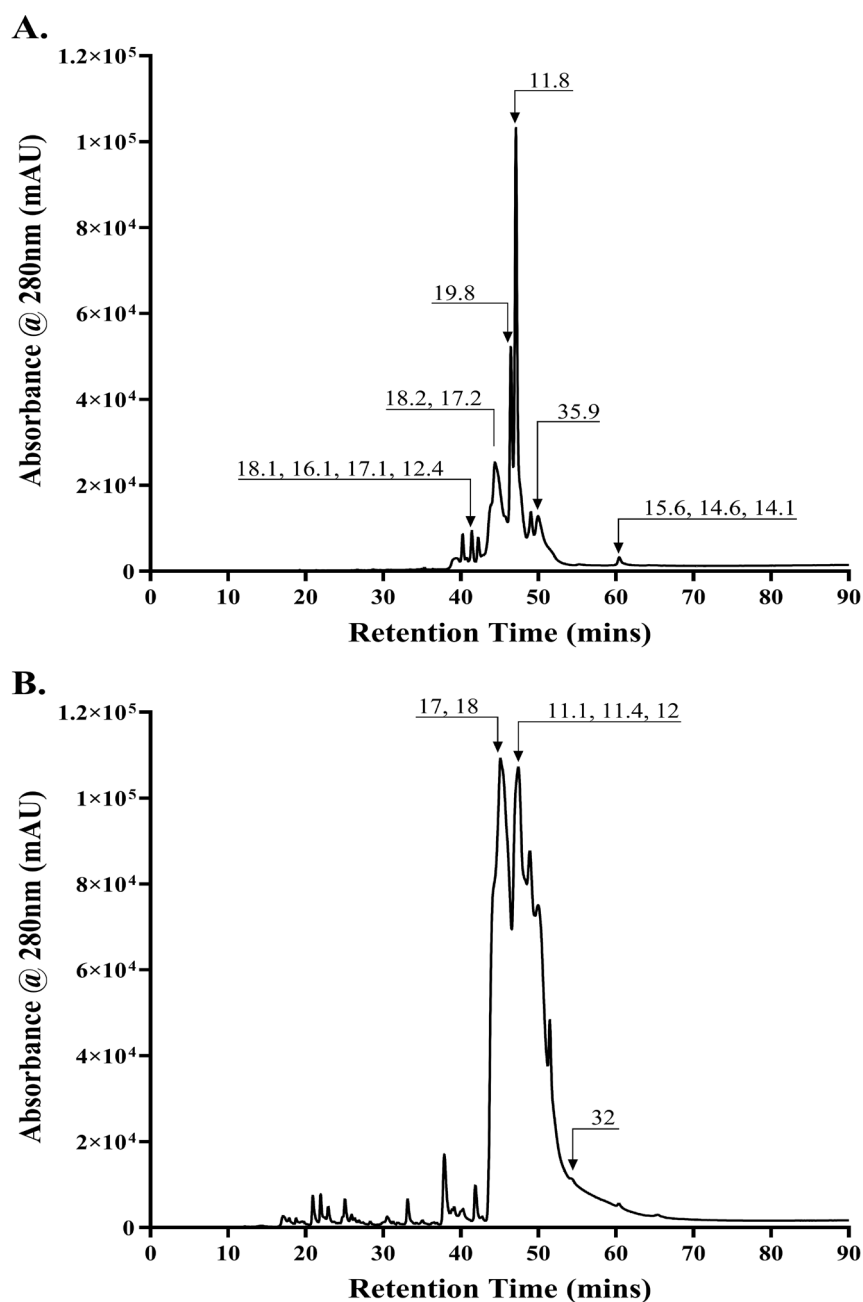


Figure 5.4 LC/ESI-MS Protein Fingerprint of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins. *Synanceia verrucosa* (Reef Stonefish, Panel A) and *Synanceia horrida* (Estuarine Stonefish, Panel B) ichthyocrinotoxins were fractionated using a Phenomenex Kinetex[®] C₈ liquid chromatography column (50 x 2.1 mm, 5.0 µm, 100 Å) with a 1% gradient of Buffer B (0 – 80% Buffer B, 80 min; Buffer A: 0.1% FA/H₂O; Buffer B: 90% ACN/0.09% FA/H₂O) at a flow rate of 0.250 mL/min. UV absorbance was monitored at 280 nm. Mass spectra of the elution was collected in positive and negative ion mode over a scan range of 160 – 2000 *m/z*. TIC spectra were analysed for proteins, and masses (kDa) are labelled on the A₂₈₀ UV chromatograms.

5.5 Discussion

The results of this study revealed that *S. verrucosa* and *S. horrida* ichthyocrinotoxins are multifarious concoctions that have a similar mass distribution (between ~15 and 20 kDa) in their primary components. However, there were some differences between the two species toxins in the diversity of the components within this mass range. LCMS analysis showed that *S. verrucosa* ichthyocrinotoxin has a greater variety of components than *S. horrida*. However, components in the lower (10 to 15 kDa) mass range also differed between the two species toxins, with *S. horrida* ichthyocrinotoxins containing a greater variety than *S. verrucosa*, which supports the presence of the lower bands observed in the *S. horrida* SDS-PAGE gel results. Additionally, higher intensity bands in SDS-PAGE coupled with larger peak intensity in SE-FPLC chromatograms may indicate that *S. horrida* ichthyocrinotoxins contain a higher concentration of components than *S. verrucosa*. However, as NanoDrop A₂₈₀ readings may have been impacted by the presence of small molecules, this requires further investigation.

Previous studies conducted on the ichthyocrinotoxins of numerous fish taxa, including Soapfish (*Grammistid* spp.), Flatfish (*Pardachirus* spp.), and Boxfish (*Ostracion* spp.) hypothesise that the primary function of ichthyocrinotoxin is to deter predators (Abdul-Haqq & Shier, 2008; E. Kalmanzon *et al.*, 1999; Randall *et al.*, 1971). Therefore, perhaps one role of stonefish ichthyocrinotoxins may be predator defence. However, unlike the ichthyocrinotoxins of the other taxa, preliminary toxicity studies revealed that stonefish ichthyocrinotoxins demonstrated minimal ichthyo- and haemo-toxic activity (Cameron *et al.*, 1981). In addition, stonefish harness a venom that may be better suited as a predator defence (Ziegman & Alewood, 2015). As such, we suggest the primary function of the skin toxin is not aimed at predator avoidance.

Stonefish are primarily sedentary, bottom-dwelling animals that lack squamation (Endean, 1961). This renders them highly susceptible to a range of benthic organisms such as bacteria, algae, parasites, as well as the larvae of encrusting and/or fouling animals, most of which are detrimental to the integrity of their integument (Cameron & Endean, 1973). As such, one plausible explanation for harnessing a multifarious skin toxin may be to prevent, and/or potentially facilitate interactions with some or all of these benthic organisms. Interestingly, to date, only four species of internal parasites have been formally identified to occur within, and are unique to, *Synanceia* (Miller *et al.*, 2018). As such, one of the functions of stonefish ichthyocrinotoxins may be to prevent parasitic invasion; perhaps specifically targeting those

capable of breaching the epidermis. However, as parasite assemblages are unlikely to differ considerably between reef and estuarine environments in the tropics, it is unlikely that the observed variation between *S. verrucosa* and *S. horrida* ichthyocrinotoxins is a function of its potential anti-parasitic qualities. Instead, it may reflect the similarities observed in the toxins of the two species.

Given that the types of algae that settle on the integument differ between *S. verrucosa* and *S. horrida*, we propose that selective fouling may be the primary driver of variation between the two species ichthyocrinotoxins. Early observations elucidated that the integument of both *S. verrucosa* and *S. horrida* is covered in algae that is reportedly always the same colour, and type as that found in their immediate surroundings (Endean, 1961). Therefore, another potential function of stonefish ichthyocrinotoxins may be to facilitate the growth and/or attachment of different species of algae, which would likely be crustose coralline algae for *S. verrucosa*, and phaeophytes for *S. horrida*. Algal recruitment onto the integument may act to enhance the animals camouflage to other fish, thereby increasing its efficacy as an ambush predator, whilst simultaneously luring in potential food.

This study has expanded the current knowledge on the components within these ichthyocrinotoxins, identifying for the first time the protein masses present and the differences between the two species. We speculate that these variations and similarities are driven by the different and similar ecology of these fish species. The task now is to design and run studies that will allow researchers to test these speculations.

5.6 References

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5.7 APPENDIX D

Inter-Species Variation in Stonefish (*Synanceia* spp.) Ichthyocrinotoxins; An Ecological Perspective.

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Inter-species variation in stonefish (*Synanceia* spp.) ichthyocrinotoxins; an ecological perspective

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ABSTRACT

Although stonefish (*Synanceia* spp.) are well-known to harbour a highly noxious defensive venom in their dorsal spines, very little is known about the composition and ecological function of the ichthyocrinotoxins that they secrete onto their epidermis. This study profiled reef (*Synanceia verrucosa*) and estuarine (*Synanceia horrida*) stonefish ichthyocrinotoxins via electrophoresis, liquid chromatography, and mass spectrometry to visualise and compare the composition of these toxins between the two species. Stonefish ichthyocrinotoxins were found to be multifarious concoctions that exhibited subtle differences between reef and estuarine species. We speculate that these variations and similarities are driven by the different and similar ecology of these fish species. Further research into the activity of the toxins components is now required to better understand their ecological role.

1. Introduction

Over 2900 species of fish possess biological toxins in the form of either a venom or a poison (Harris and Jenner, 2019). Poisons used by fish are generally small molecular oral biotoxins that can be noxious when either consumed or absorbed (Jared et al., 2021). These are produced either by accumulating consumed metabolites in body tissues, or within specialised epithelial cells which are called club cells or tubercles (Harris and Jenner, 2019; Mahmud et al., 2003). The latter are collectively called ichthyocrinotoxins (Cameron and Endean, 1973; Halstead, 1988).

Approximately 50 species of fish belonging to at least 14 families are known to produce ichthyocrinotoxins, few of which have been extensively studied (Eliahu Kalmanson and Zlotkin, 2019). A common characteristic of ichthyocrinotoxins is their deterrent, and sometimes fatal effect on other teleost species (Primor and Zlotkin, 1975; Randall et al., 1971; Thomson, 1964). Furthermore, many ichthyocrinotoxic species, such as *Grammistes sexlineatus* (golden-striped soapfish) and *Pardachirus marmoratus* (Red Sea Moses sole), secrete copious amounts of this toxin in response to threat (Al-Hassan et al., 1985; Boylan and Scheuer, 1967; Clark, 1983; Randall et al., 1971). As such, ichthyocrinotoxins are generally believed to be a defensive substance, primarily acting to deter and/or repel predators (Abdul-Haq and Shier, 2008; Cameron and

Endean, 1973; Randall et al., 1971). One genus of fish that harness ichthyocrinotoxins are *Synanceia* (stonefish).

Stonefish (*Synanceia* spp.) are a bottom-dwelling fish found throughout the shallow, coastal waters of the Indo-Pacific region (Endean, 1961). Two species of stonefish are found in Queensland waters; *Synanceia verrucosa*, and *Synanceia horrida* (Endean, 1961). One key distinction between these species is their preferred habitat, with *S. horrida* (estuarine stonefish) primarily found inshore amongst sheltered bays and estuaries, whereas *S. verrucosa* (reef stonefish) are commonly associated with coral reefs (Endean, 1961). Stonefish are one of the very few animals that harness two distinct types of toxin: an injectable venom which is housed within twin glands associated with 13 dorsal spines, and an ingestible ichthyocrinotoxin secreted by the tubercles that span their epidermis (Endean, 1961; Saggiomo et al., 2021). Stonefish venom is believed to function purely as a predator defence (Endean, 1961; Saggiomo et al., 2021). The deleterious nature of this venom to people has awarded stonefish the title of worlds' most venomous fish (Cameron and Endean, 1973; Saggiomo et al., 2021). As such, research into stonefish toxins is often centred around medical aid to envenomation which is commonly a result of accidentally standing on the animal (Cameron and Endean, 1973; Ziegman et al., 2019). However, very little is currently known about stonefish ichthyocrinotoxins.

Stonefish exhibit a primarily sedentary lifestyle and lack squamation

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(Endean, 1961). Thus, it has been suggested that their ichthyocrinotoxin may act to manage biological interactions with their integument, particularly those involving bacteria, parasites, as well as fouling flora and fauna (Cameron et al., 1981; Endean, 1961). However, these interactions may vary between reef and estuarine environments, leading to potentially different ichthyocrinotoxins. Therefore, the aim of this study was to determine if stonefish ichthyocrinotoxins exhibit inter-species variation, and if so, discuss the potential ecological drivers behind it.

2. Materials and Methods

2.1. Ichthyocrinotoxin collection and preparation

The ichthyocrinotoxins of reef (*S. verrucosa*) and estuarine (*S. horrida*) stonefish were collected from multiple animals of both sexes ranging between 143 and 370 mm in body length. To collect ichthyocrinotoxin, live *S. verrucosa* or *S. horrida* were placed on a surface and secured by covering the animals' eyes with a saltwater dampened cloth to promote calmness while firmly holding either side of the pectoral fins. Ichthyocrinotoxin was then extracted from the skin by massaging the tubercles under a gentle vacuum. This vacuum was created with a custom-built extraction apparatus composed of a 10 mm piece of plastic tubing fastened to a 50 mL syringe. Evacuated toxin was centrifuged at 15,000 rpm for 20 min, or until the white component had pelleted out and the supernatant became clear, prior to use to remove any cell debris or contaminants. The supernatant was transferred to a new 1.5 mL Eppendorf tube and protein concentration was determined using a Nanodrop One (Thermo Scientific™) under the Protein A₂₈₀ program. Ichthyocrinotoxin supernatant was stored at −80 °C until required.

2.2. Visualising and quantifying inter-species variation in stonefish ichthyocrinotoxins

To explore inter-species variation between *S. verrucosa* and *S. horrida* ichthyocrinotoxins, numerous protein/peptide separation techniques were used to visualise, quantify, and purify the toxin components.

2.2.1. Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as an exploratory assessment of the toxins to determine the size range of the protein constituents. This informed the conditions selected for liquid chromatography. Ichthyocrinotoxin supernatant samples were serially diluted to a concentration of 2 mg/mL (± 0.05 mg/mL) and mixed with 2× Laemmli buffer at a ratio of 1:2. The toxin components were then denatured at 95 °C for 5 min. Denatured ichthyocrinotoxin samples (20 μ L) and protein standards (2 μ L, Precision Plus Protein™ Dual Colour, BioRad) were loaded on to a 15% polyacrylamide gel (1.0 mm x 12 well) set up in an electrophoresis cell. Gels were run in a discontinuous buffer system with the application of 90 V for ~10 min during the stacking phase, and 150 V for ~45 min during the running phase with a constant amperage of 0.02 Å used throughout. The running buffer used was 1× Sodium Dodecyl Sulphate (SDS) at pH 8. Current was applied until the dye marker (bromophenol blue) was within 1 cm of the gel bottom. Gels were stained by submersion into Imperial™ Protein Stain (Thermo Scientific™) for 1 h, and de-stained using deionised (MilliQ) H₂O until the gel background was clear. Gels were photographed with a Nikon D810 against an illuminated surface. Inter-species variation in the banding pattern was determined via visual analysis of the gel images. Inter-species variation in band intensity was quantified using histogram analysis in Image J. Grey scores were generated for each band that appeared in the species profiles, as well as for the image background which was assigned as maximum brightness. Grey scores were then inverted by subtracting the score of each band from maximum brightness to yield a darkness score. Darkness scores were plotted as percent intensity using GraphPad Prism 9.

2.2.2. Size exclusion fast performance liquid chromatography (SE-FPLC)

SE-FPLC on an ÄKTA™ prime system (GE Healthcare) was used to generate the protein profiles and purify the toxin components. Ichthyocrinotoxin supernatant samples (500 μ L) were manually injected onto the FPLC system at a concentration of 20 mg/mL (± 0.05 mg/mL). Ichthyocrinotoxin components were separated using a Superdex 75 10/300 (GE Healthcare) GL Column (13 μ m, 10 × 300 mm) with 0.25× phosphate-buffered saline (PBS) as a running buffer (500 μ L/min for 40 mL at 8 °C). The UV absorbance of the subsequent eluant was monitored at 280 nm. Fractions (500 μ L) were collected and stored in 1.5 mL 96 deep-well plates (Thermo Scientific™) at 4 °C until the run completion. Toxin profiles were processed and exported using UNICORN™ 7 software (2016, GE Healthcare). Due to the disproportionate nature of absorbance intensity between the chromatograms of each species, raw data was exported to Excel and normalised. Normalisation involved dividing each absolute absorbance value in each chromatogram by the highest absolute absorbance value in the corresponding chromatogram. Chromatograms were visually analysed in GraphPad Prism 9 and fraction pools were determined based on the presence of distinct peaks. A total of five pooled fractions were collated from each species toxin based on the identified peaks (Table 1).

2.2.3. Liquid chromatography/electrospray ionisation-mass spectrometry (LC/ESI-MS)

The protein composition profiles of crude and SE-FPLC fractionated stonefish ichthyocrinotoxins were determined using a Shimadzu LCMS-2020 mass spectrometer coupled to a Shimadzu Prominence high performance liquid chromatography (HPLC) system (Shimadzu). LC/MS grade solvents (Buffer A: 0.1% formic acid (FA; Sigma-Aldrich)/H₂O; Buffer B: 90% acetonitrile (ACN; Thermo Scientific™)/0.09% FA/H₂O) were delivered via pumps (Shimadzu LC-20AD) at a flow rate of 0.250 mL/min. Ichthyocrinotoxin samples were injected via an autosampler (Shimadzu SIL-20AC_{HT}) onto a Phenomenex Kinetex® C₈ liquid chromatography column (50 × 2.1 mm, 5.0 μ m, 100 Å) and eluted with a 1% gradient of Buffer B (0–80%, 80 min; 80–90%, 5 min; 90%, 10 min; 80–0%, 5 min; 0%, 10 min). SE-FPLC fractions included a 10 min de-salting wash of Buffer A prior to commencing the gradient. The UV absorbance was monitored at 280 nm using a Shimadzu SPD-20 A detector. Mass spectra were collected in positive and negative ion mode over a scan range of 160–2000 *m/z* with a detector voltage of 1.35 kV, nebulizing gas flow of 1.5 L/min, and drying gas flow of 3 L/min. Data were collected and analysed using Shimadzu LabSolutions v5.96 software. Spectra across the total ion current (TIC) peaks were averaged and the multi-charge ion analysis tool was used to reconstruct the ion series in the spectra to provide the compound mass. Raw data was exported to GraphPad Prism 9 to generate chromatograms.

2.2.4. Reversed-phase high performance liquid chromatography (RP-HPLC)

RP-HPLC of stonefish ichthyocrinotoxins was performed using a C₁₈ Phenomenex Aeris® PEPTIDE XB liquid chromatography column (250 × 10 mm, 5 μ m, 100 Å). Ichthyocrinotoxin samples (500 μ L) were mixed with solvent A (500 μ L H₂O/0.05% trifluoroacetic acid (TFA; Auspep) and centrifuged at 15,000 rpm for 5 min. Samples were manually loaded

Table 1

Pooled fraction designations of SE-FPLC sub-fractions of purified reef (*S. verrucosa*) and estuarine (*S. horrida*) stonefish ichthyocrinotoxins.

Fraction Name	Sub-fractions (elution volume, mL)	
	<i>Synanceia verrucosa</i>	<i>Synanceia horrida</i>
a	7–10	7–8
b	10–14	8–14
c	14–16	14–16
d	16–19	16–19
e	19–23	19–23, 30–33

onto the RP-HPLC system. Fractionation of the sample used a gradient of 0–60% solvent B (90% ACN (Merck)/10% H₂O/0.045% TFA) in 120 min, 60–90% solvent B in 5 min, 90% solvent B for 10 min, and 90–0% solvent B in 5 min. The flow rate throughout was 3 mL/min and the absorbance was monitored at 280 nm. Fractions (30 s) were automatically collected into 2.0 mL 96 well deep-well plates (Axygen). Raw data was collected and analysed within OpenLAB CDS ChemStation software (v1.8.1) and exported to GraphPad Prism 9 to generate chromatograms. Chromatograms were simplified for visual comparisons by plotting the negated absorbance values for *S. horrida* ichthyocrinotoxin against the raw absorbance values of *S. verrucosa* ichthyocrinotoxin.

3. Results

3.1. SDS-PAGE

SDS-PAGE of *S. verrucosa* and *S. horrida* ichthyocrinotoxins revealed that there were distinct differences in the pattern and intensity of the bands between the profiles (Fig. 1). The bulk of the profile for both species fell within the ~15–18 kDa range. The banding pattern within this range was similar between the two species (Fig. 1A). However, there was a distinct difference in the intensity of the bands, where *S. verrucosa* ichthyocrinotoxin had a darker banding pattern than *S. horrida* (Fig. 1B). Similarly, there was a faint double band at ~33 kDa in both profiles that appeared more intense in *S. verrucosa* ichthyocrinotoxin than *S. horrida*. Additionally, there was some variation in the banding pattern between

the toxin profiles, particularly in the lower and upper size ranges. *S. horrida* ichthyocrinotoxin, for example, had a double band that appeared around ~13 kDa which was absent in the profile of *S. verrucosa*. Conversely, *S. verrucosa* ichthyocrinotoxin had a band that appeared around ~22 kDa that was absent in the profile of *S. horrida*. Thus, the mass range of the components slightly differed between the two species profiles, with *S. horrida* ranging between ~13 and 33 kDa, and *S. verrucosa* ranging between ~14–35 kDa.

3.2. SE-FPLC

The SE-FPLC chromatograms varied between *S. verrucosa* and *S. horrida* ichthyocrinotoxins in both peak intensity and occurrence (Fig. 2). In general, most peaks in the chromatogram of *S. horrida* had a greater absorbance intensity than those appearing in *S. verrucosa*. One peak (c) that eluted around 16 mL in both profiles was approximately ten times more intense in the profile of *S. horrida* compared to *S. verrucosa*. There were also peaks that appeared where others were absent. In the profile *S. verrucosa* ichthyocrinotoxin, a peak appeared between 7.5 and 10 mL (x) that was absent in the profile of *S. horrida*. Similarly, there was a peak between 30 and 32.5 mL (y) in the profile of *S. horrida* that was absent from *S. verrucosa*. A total of five distinct peaks were visually identified in the FPLC chromatograms of *S. verrucosa* and *S. horrida* ichthyocrinotoxins (letters a-e, Fig. 2), which were collected and pooled for each species (Table 1).

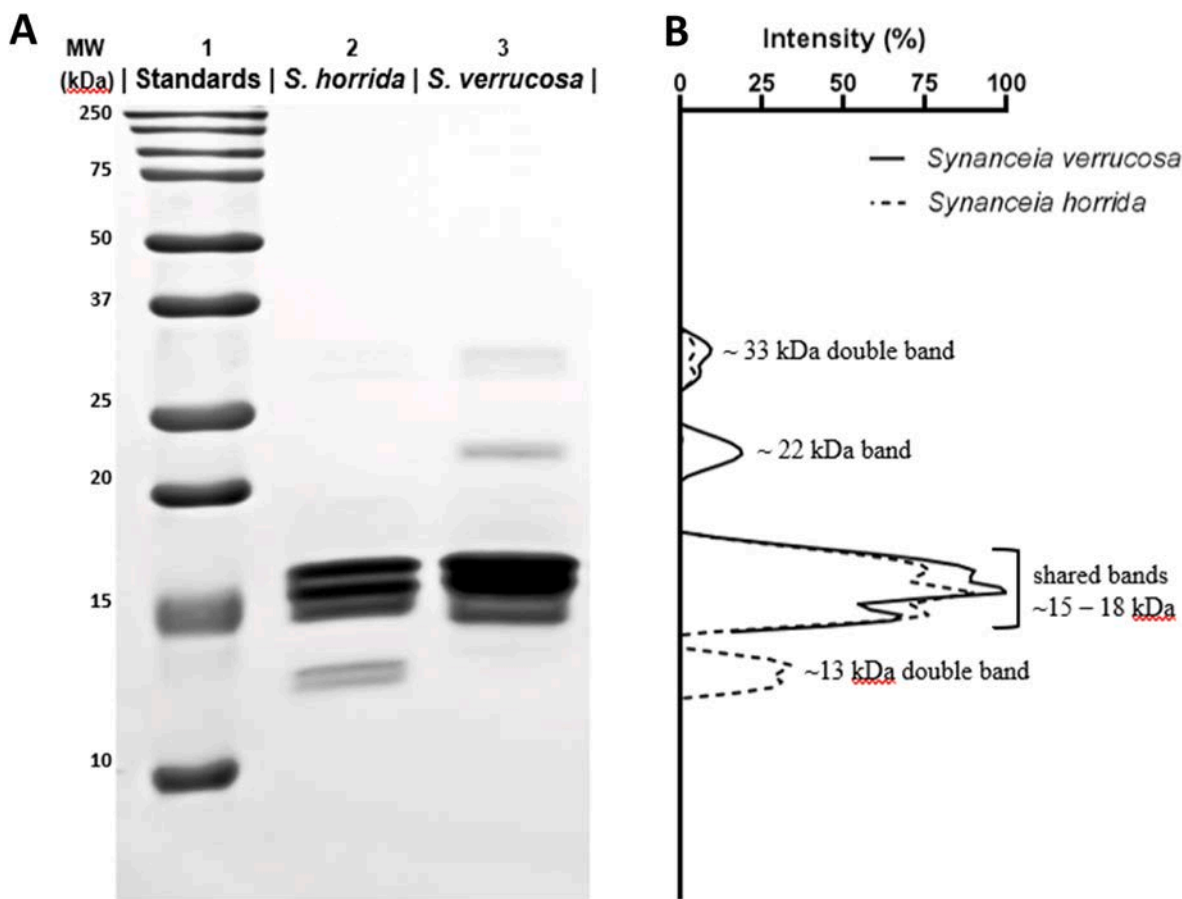


Fig. 1. Panel A: SDS-PAGE Gel Image of Stonefish Ichthyocrinotoxins. The components within reef (*Synanceia verrucosa*, Lane 3) and estuarine (*Synanceia horrida*, Lane 2) stonefish ichthyocrinotoxins were separated in a discontinuous buffer system using a 15% polyacrylamide gel and 1× SDS as a running buffer. Protein standards (Precision Plus Protein™ Dual Colour, Lane 1) were used to estimate the molecular weight (kDa) of the components within each species toxin. **Panel B: Band Intensity Plot of Stonefish Ichthyocrinotoxins.** The intensity (%) of the bands that appeared in the gel electrophoresis profile of reef (*Synanceia verrucosa*, —) and estuarine (*Synanceia horrida*, ---) stonefish ichthyocrinotoxins was quantified using an inverted grey scale. Grey values were generated using histogram analysis in ImageJ.

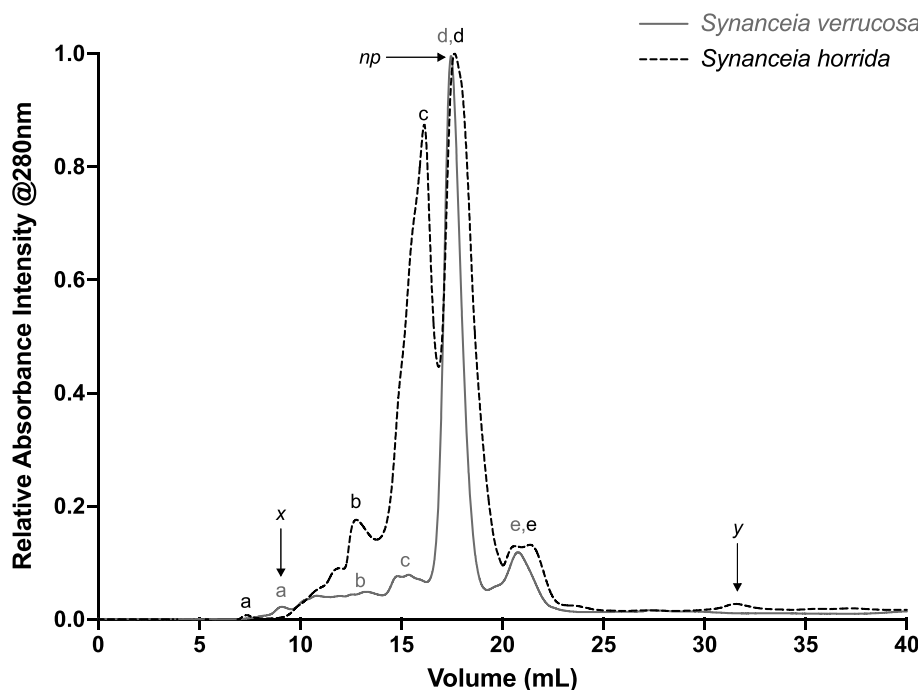


Fig. 2. FPLC Chromatogram of Stonefish Ichthyocrinotoxins. The components within reef (*Synanceia verrucosa*, —) and estuarine (*Synanceia horrida*, ---) stonefish ichthyocrinotoxins were separated by size using a Superdex 75 10/300 (Tricorn) GL Column (13 μ m, 10 $\times$ 300 mm) with 0.25 $\times$ PBS as a running buffer (500 μ L/min). Absorbance (280 nm) was monitored for 40 mL of the eluant. Chromatograms were normalised for the absorbance intensity of the 17.5 mL peak (marked as 'np'). Letter designations x and y mark specific points of interest. Letters a – e mark visually identified distinct peaks which correspond to the pooled fraction designations listed in Table 1.

3.3. RP-HPLC

RP-HPLC chromatograms of *S. verrucosa* and *S. horrida* ichthyocrinotoxins revealed relatively complex chromatograms, with components predominantly eluting between 20 and 35 min (Fig. 3). While the profiles for both species were similar, several differences were observed. A greater abundance of components eluted between 2.5 and 20 min in the *S. horrida* ichthyocrinotoxin sample. Additionally, there were components eluting after 35 min in the profile of *S. horrida* that were absent in the profile of *S. verrucosa*. Similarly, there were components eluting between 33 and 34 min in *S. verrucosa* that were either completely absent or in low abundance in the profile of *S. horrida*.

3.4. LC/ESI-MS

LC/ESI-MS revealed the presence of protein masses ranging from 11.8 to 35.9 kDa in *S. verrucosa* ichthyocrinotoxin and from 11.1 to 32 kDa for *S. horrida* ichthyocrinotoxin (Fig. 4). The masses and relative abundance of the proteins were also determined to vary between *S. verrucosa* and *S. horrida*. Double the number of proteins with unique masses were identified in *S. verrucosa* ichthyocrinotoxin compared to *S. horrida*. However, *S. horrida* ichthyocrinotoxin had a higher abundance of lower mass (11–12 kDa) uniquely sized proteins, which is consistent with the bands observed in the SDS-PAGE gel results.

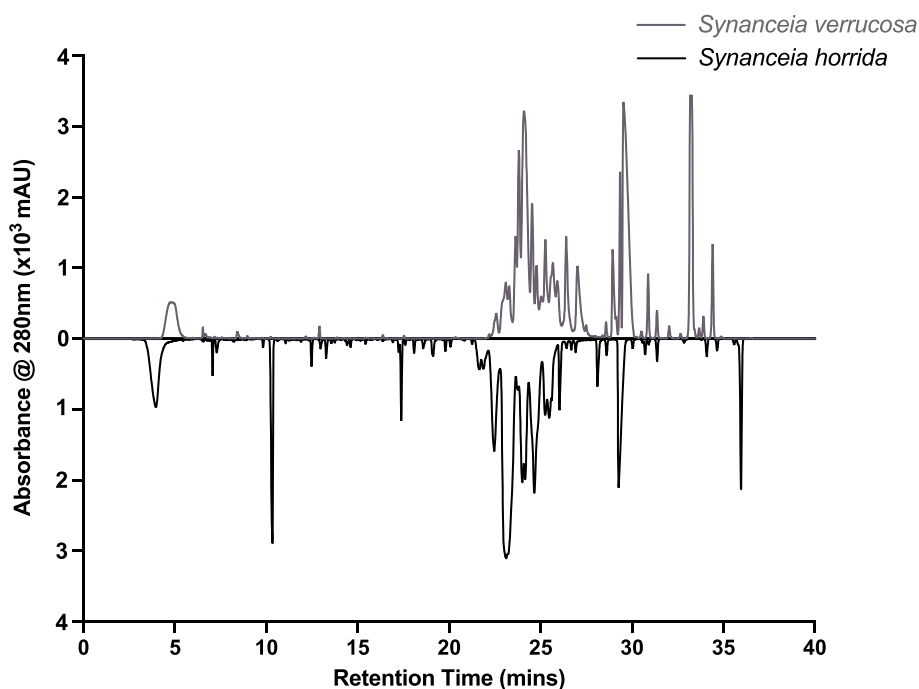


Fig. 3. RP-HPLC Chromatogram of Stonefish Ichthyocrinotoxins. Semi-preparative RP-HPLC of reef (*Synanceia verrucosa*, —) and estuarine (*Synanceia horrida*, ---) stonefish ichthyocrinotoxins used a C₁₈ Phenomenex Aeris® PEPTIDE XB liquid chromatography column (250 $\times$ 10 mm, 5 μ m, 100 Å) with a gradient of 0–60% solvent B (Solvent A: H₂O/0.05% TFA; Solvent B: 90% ACN/10% H₂O/0.045% TFA) over 40 min at a flow rate of 3 mL/min. The absorbance was monitored at 280 nm. Chromatograms were simplified for visual comparisons by mirroring the absorbance values for *S. horrida* ichthyocrinotoxin.

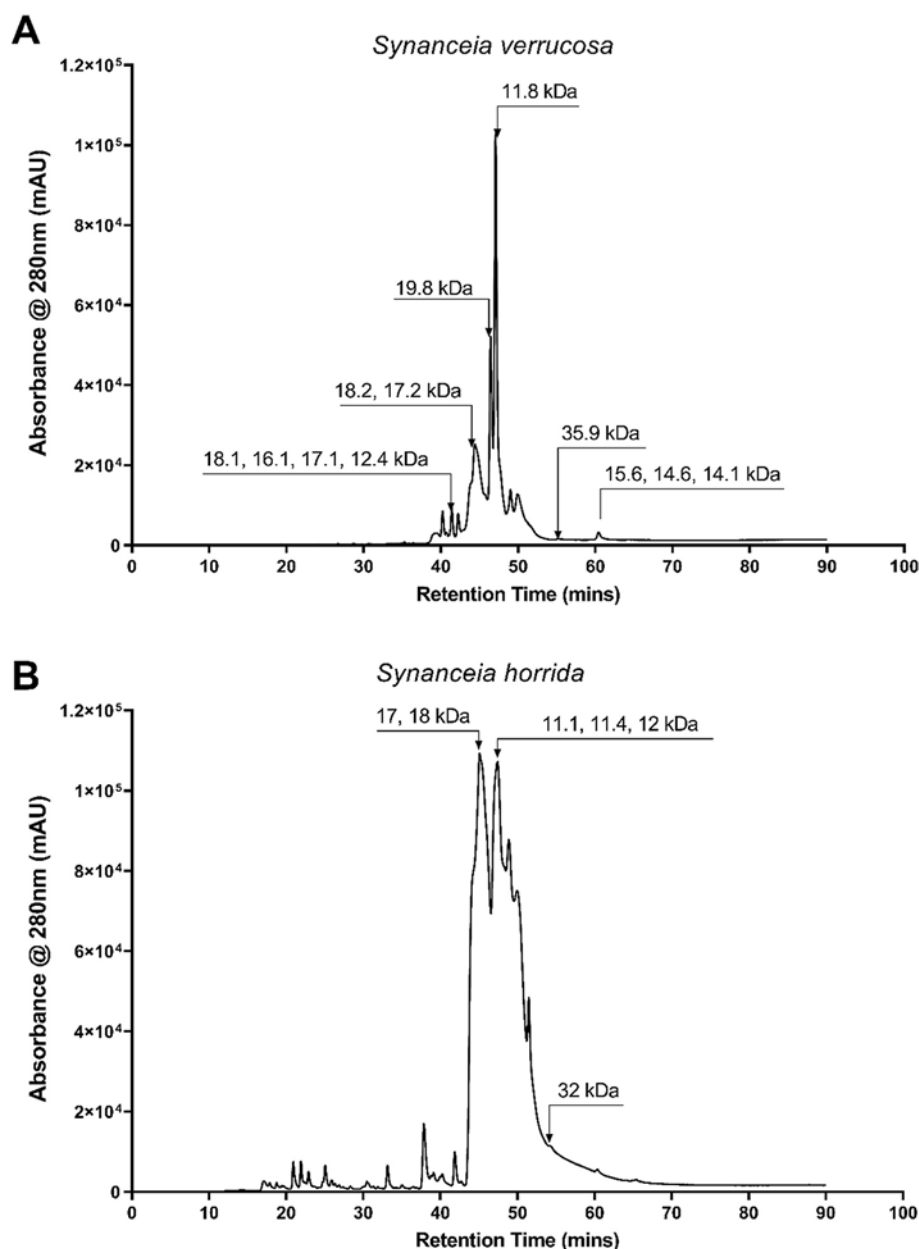


Fig. 4. LC/ESI-MS Protein Fingerprint of Stonefish Ichthyocrinotoxins. LC/ESI-MS fractionation of reef (*Synanceia verrucosa*, Panel A) and estuarine (*Synanceia horrida*, Panel B) stonefish ichthyocrinotoxins using a Phenomenex Kinetex® C₈ liquid chromatography column (50 × 2.1 mm, 5.0 μm, 100 Å) with a 1% gradient of Buffer B (0–80% Buffer B, 80 min; Buffer A: 0.1% FA/H₂O; Buffer B: 90% ACN/0.09% FA/H₂O) at a flow rate of 0.250 mL/min. UV absorbance was monitored at 280 nm. Mass spectra of the elution was collected in positive and negative ion mode over a scan range of 160–2000 *m/z*. TIC spectra were analysed for proteins, and masses (kDa) are labelled on the A₂₈₀ UV chromatograms.

4. Discussion

The results of this study revealed that *S. verrucosa* and *S. horrida* ichthyocrinotoxins are multifarious concoctions that have a similar mass distribution (between ~15 and 20 kDa) in their primary components. However, there were some differences between the two species' toxins in the diversity of the components within this mass range. LCMS analysis showed that *S. verrucosa* ichthyocrinotoxin has a greater variety of components than *S. horrida*. However, components in the lower (10–15 kDa) mass range also differed between the two species' toxins, with *S. horrida* ichthyocrinotoxins containing a greater variety than *S. verrucosa*, which supports the presence of the lower bands observed in the *S. horrida* SDS-PAGE gel results. Additionally, higher intensity bands in SDS-PAGE coupled with larger peak intensity in SE-FPLC chromatograms may indicate that *S. horrida* ichthyocrinotoxins contain a higher concentration of components than *S. verrucosa*. However, as NanoDrop A₂₈₀ readings may have been impacted by the presence of small molecules, this requires further investigation.

Previous studies on the ichthyocrinotoxins of numerous taxa,

including soapfish (*Grammistid* spp.), flatfish (*Pardachirus* spp.), and boxfish (*Ostracion* spp.) hypothesise that the primary function of ichthyocrinotoxin is to deter predators (Abdul-Haqq and Shier, 2008; Kalmanzon et al., 1999; Randall et al., 1971). Therefore, perhaps one potential role of stonefish ichthyocrinotoxins may be predator defence. However, unlike the ichthyocrinotoxins of the other taxa, preliminary toxicity studies revealed that stonefish ichthyocrinotoxins demonstrated minimal ichthyo- and haemo-toxic activity (Cameron et al., 1981). In addition, stonefish harness a venom that may be better suited as a predator defence (Ziegman and Alewood, 2015). As such, we suggest the primary function of the skin toxin is not aimed at predator avoidance.

Stonefish are primarily sedentary, bottom-dwelling animals that lack squamation (Endean, 1961). This renders them highly susceptible to a range of benthic organisms such as bacteria, algae, parasites, as well as the larvae of encrusting and/or fouling animals, most of which are detrimental to the integrity of their integument (Cameron and Endean, 1973). As such, one plausible explanation for harnessing a multifarious skin toxin may be to prevent, and/or potentially facilitate interactions with some or all these benthic organisms. Interestingly, to date, only

four species of internal parasites have been formally identified to occur within, and are unique to, *Synanceia* (Miller et al., 2018). As such, one of the functions of stonefish ichthyocrotoxins may be to prevent parasitic invasion; perhaps specifically targeting those capable of breaching the epidermis. However, as parasite assemblages are unlikely to differ considerably between reef and estuarine environments in the tropics, it is unlikely that the observed variation between *S. verrucosa* and *S. horrida* ichthyocrotoxins is a function of its potential anti-parasitic qualities. Instead, it may reflect the similarities between the toxins of the two species.

Given that the types of algae that settle on the integument differ between *S. verrucosa* and *S. horrida*, we propose that selective fouling may be the primary driver of variation between the two species ichthyocrotoxins. Early observations elucidated that the integument of both *S. verrucosa* and *S. horrida* is covered in algae that is reportedly always the same colour, and type as those found in their immediate surroundings (Endean, 1961). Therefore, another potential function of stonefish ichthyocrotoxins may be to facilitate the growth and/or attachment of different species of algae, which would likely be crustose coralline algae for *S. verrucosa*, and phaeophytes for *S. horrida*. Algal recruitment onto the integument may act to enhance the animals camouflage to other fish, thereby increasing its efficacy as an ambush predator, whilst simultaneously luring in potential food.

This study has expanded the current knowledge on the components and molecules within these ichthyocrotoxins, identifying for the first time the protein masses present and the differences between the two species. We speculate that these variations and similarities are driven by the different and similar ecology of these fish species. The task now is to design and run studies that will allow researchers to test these speculations.

Credit author statement

Danica Lennox-Bulow: Conceptualisation, Investigation, Formal analysis, Writing – original draft, Writing – Editing and Review, Visualisation **Michael Smout:** Methodology, Supervision, Investigation, Writing – Editing and Review **David Wilson:** Methodology, Supervision, Investigation, Writing – Editing and Review **Jamie Seymour:** Supervision, Conceptualisation, Resources, Formal analysis, Writing – Editing and Review

Ethical Statement for Solid State Ionics

Hereby, I Danica Lennox-Bulow consciously assure that for the manuscript “Interspecies Variation in Stonefish (*Synanceia* spp.) Ichthyocrotoxins; An Ecological Perspective” the following is fulfilled:

- 1) This material is the authors’ own original work, which has not been previously published elsewhere.
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- 4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
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I agree with the above statements and declare that this submission follows the policies of Solid State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Chapter 6

Geographic Variation in Ichthyocrinotoxins from the Estuarine Stonefish (*Synanceia horrida*)

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D Lennox-Bulow is the principal author of this manuscript and was extensively involved in all aspects of this work under the supervision of J Seymour. R Courtney assisted with animal husbandry, toxin collection, and in reviewing earlier versions of this manuscript.

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6.1 Abstract

Geographic variation in the composition of animal toxins is well documented for venomous taxa, and to a lesser degree, for some poisonous taxa that secrete toxins obtained from their diet. However, very little is known about animals that synthesise their poisons *de novo*, such as stonefish and their secreted ichthyocrinotoxins. Stonefish are widely distributed throughout the Indo-Pacific, however, the effect of geographic location on the composition of their ichthyocrinotoxin is currently unknown. This study aimed to determine whether the composition of ichthyocrinotoxins from *Synanceia horrida* (Estuarine Stonefish) varied between three geographically isolated Australian populations including Cairns in far north Queensland, Caloundra in southeast Queensland, and Exmouth in Western Australia. The composition of ichthyocrinotoxins from *S. horrida* were largely conserved across the three locations, with the size of most of the components falling between 14 and 25 kDa. However, unique components were identified in *S. horrida* ichthyocrinotoxins from Caloundra and Exmouth populations. Caloundra populations contained unique smaller components (8 and 12 kDa) that were hydrophobic. In contrast, Exmouth populations contained unique larger components (60 and 150 kDa) that exhibited no difference in hydrophobicity. We speculate that the composition of ichthyocrinotoxins produced by *S. horrida* are likely influenced by benthic flora and fauna communities, particularly those that are likely to interact with the animal's skin, such as fouling flora and fauna, as well as parasites. These findings further add to the growing body of evidence underscoring the complexity and compositional diversity of ichthyocrinotoxins produced by stonefish.

6.2 Introduction

It has been well established that toxins produced by animals, which include venoms and poisons, are often complex, dynamic, and diverse concoctions (Warrell, 2020; Suranse *et al.*, 2018). Animal toxins are generally comprised of a multifarious mixture of proteins, peptides, and small molecules (Bolon *et al.*, 2023; Warrell, 2020). The composition and complexity of these concoctions can vary both within species (Tasoulis *et al.*, 2020; Schaffrath *et al.*, 2018) and between species (van Thiel *et al.*, 2023; Duran *et al.*, 2020), as well as within and/or between individuals (Dutertre *et al.*, 2014). The ecological factors shaping the composition of animal toxins is a complex evolutionary question and is often attributed to a combination of biotic factors such as sex (Amorim *et al.*, 2018; Zelanis *et al.*, 2016; Herzig & Hodgson, 2009), ontogeny (da Silva Aguiar *et al.*, 2020; Underwood & Seymour, 2007), diet (Albuquerque-Pinna *et al.*, 2024; Weese & Duda, 2019; Phuong *et al.*, 2016), and community dynamics (Smith *et al.*, 2023; Chang & Duda, 2016; Daltry *et al.*, 1996), as well as abiotic factors such as season (Tasima *et al.*, 2024; Junior *et al.*, 2010), and other physical/chemical environmental parameters (Smith *et al.*, 2023; O'Hara *et al.*, 2021; Sachkova *et al.*, 2020). Furthermore, many of these ecological drivers of variation in animal toxins can vary with, and/or can be unique to, the organism's geographic location (Yu *et al.*, 2020).

Compositional variation in animal toxins between geographically isolated populations is widely documented across venomous taxa, particularly terrestrial snakes (Smith *et al.*, 2023; van Thiel *et al.*, 2023; Smith & Mackessy, 2021; Davies & Arbuckle, 2019; Sasa, 1999), and although to a much lesser extent, some poisonous taxa, such as poison dart frogs (Albuquerque-Pinna *et al.*, 2024; Daly *et al.*, 2008; Daly *et al.*, 1994), and pufferfish (Ito *et al.*, 2023). This phenomenon has also been documented at almost all spatial scales, ranging from between microhabitats within a single location (Smith *et al.*, 2023; Abdel-Rahman *et al.*, 2009), to across continents spanning vast distances (Carrette, 2014; Duda *et al.*, 2009). While the ecological factors influencing venom composition between geographically isolated populations are intricate, the primary selective force often centres around the venoms intended target (Smith & Mackessy, 2021; Barlow *et al.*, 2009). Interestingly, this does not appear to be the case for animal poisons. Poisons sequestered by animals are commonly comprised of only a few peptides and/or small molecules that have been acquired through the animal's diet (Harris & Jenner, 2019; Mahmud *et al.*, 2003). Although these toxins often serve exclusively as a defence against predators or injury, numerous studies have attributed intraspecific compositional variation in animal poisons to geographic variation in the animal's diet (Alto, 2011; Daly *et al.*,

2008; Daly *et al.*, 1994). However, virtually nothing is known about poisonous taxa that synthesise their toxins *de novo* within specialised glands, such as stonefish.

Stonefish (*Synanceia* spp.) are a bottom-dwelling fish found throughout both the tropical and subtropical waters of the Indo-Pacific region (Endean, 1961). Stonefish are one of only a few known animals that harness two distinct types of toxin: a potent defensive venom which is housed within twin glands associated with each of their 13 dorsal spines, and an ichthyocrinotoxin that is secreted by specialised glands (i.e., tubercles) that span their epidermis (Saggiomo *et al.*, 2021; Endean, 1961). Stonefish venom is widely recognised as the most potent of any fish due to its highly deleterious nature to people (Saggiomo *et al.*, 2021; Cameron & Endean, 1973). As a result, research on these animals has largely concentrated on medical interventions for envenomation (Ziegman *et al.*, 2019; Cameron & Endean, 1973). However, very little is currently known about stonefish ichthyocrinotoxins.

Stonefish are devoid of scales and readily bury themselves into the substratum (Endean, 1961). Consequently, the skin of these animals is presumably highly susceptible to a variety of benthic fauna, particularly invertebrate taxa such as parasites and fouling animals, and potentially certain flora, including filamentous, slime, and encrusting algae. It has been previously suggested that stonefish ichthyocrinotoxins may act to manage biological interactions with their integument, particularly those involving bacteria, parasites, as well as fouling flora and fauna (Lennox-Bulow *et al.*, 2023a; Barnett, 2014; Cameron *et al.*, 1981; Endean, 1961). However, these dynamics are likely to vary geographically, potentially leading to different ichthyocrinotoxins. Therefore, the aim of this study is to determine whether the composition of ichthyocrinotoxin from *Synanceia horrida* (Estuarine Stonefish) varies intraspecifically between geographically isolated Australian populations, and if so, discuss the potential ecological drivers behind it.

6.3 Materials and Methods

6.3.1 Animal and Toxin Collection

Collecting and Sourcing Stonefish.

Synanceia horrida were hand captured and/or sourced from commercial fish suppliers (Cairns Marine, Reef 2 Aquarium, Jardini International) between January 2019 to 2024. *Synanceia horrida* were found amongst inshore rocky reef and/or estuarine environments in Cairns in far north Queensland Australia, Caloundra in southeast Queensland Australia, and Exmouth in Western Australia. The sexes of each individual were not able to be determined. The use of *S. horrida* in this study was done in accordance with James Cook University Animal Ethics Committee approval (AEC approval number A2832).

Extracting Ichthyocrinotoxin.

Ichthyocrinotoxin from *S. horrida* were extracted following a protocol outlined by Lennox-Bulow *et al.* (2023b). In brief, an individual stonefish was transferred to a saltwater-dampened surface and secured by gently holding either side of the pectoral fins. A saltwater-dampened cotton cloth was placed over the animal's eyes to minimise stress throughout the process. Ichthyocrinotoxin was then extracted from the skin tubercles using targeted mechanical massage under a controlled vacuum. This vacuum was generated by a purpose-built extraction apparatus comprised of a 100 mm segment of clear vinyl tubing affixed to a 50 mL syringe. To account for potential individual variation in toxin composition and activity, *S. horrida* ichthyocrinotoxin was extracted and pooled from three to five mature individuals. Pooled ichthyocrinotoxin samples were stored in 1.5 mL Eppendorf tubes at -80°C until required.

6.3.2 Profiling Stonefish Ichthyocrinotoxin

Gel electrophoresis (SDS-PAGE), Size Exclusion Fast Performance Liquid Chromatography (SE-FPLC), and Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) were performed on *S. horrida* ichthyocrinotoxins following the protocol outlined by Lennox-Bulow *et al.* (2023b). The following provides a brief description of the methods used for each technique:

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Gel electrophoresis of *S. horrida* ichthyocrinotoxin first involved centrifuging the samples at 15, 000x g at 4°C for 5 min to remove debris. The concentration of the sample

supernatants were determined using Nanodrop One (Thermo Scientific™) set to protein A₂₈₀ nm and adjusted to yield 500 µg/mL (±50 µg). Sample supernatants (10 µL) were then linearised by mixing (1:2) with either 2x Laemmli SDS lysis buffer (for Bis-Tris gels) or 2x Tricine SDS lysis buffer (for Tricine gels). This process was catalysed by heating the samples at 95°C for 5 min for Laemmli buffer sample mixtures, and 85°C for 2 min for Tricine buffer sample mixtures.

Linearised samples (20 µL) and protein standards (2 µL Precision Plus Protein™ Dual Colour for Bis-Tris gels, or 4 µL Spectra™ Multicolour Low Range Protein Ladder for Tricine gels) were loaded on to gels set up in an electrophoresis cell. Ichthyocrinotoxin samples were initially run on 12% Bis-Tris mini protein gels (NuPAGE™, 1.0 mm x 12 well, 10 kDa to 260 kDa separation resolution). To enhance the resolution of banding patterns for smaller molecules (40 to 2 kDa), ichthyocrinotoxin samples were additionally run on a 10 to 20% Tricine mini protein gel (Novex™, 1.0 mm x 12 well, 2 kDa to 200 kDa separation resolution). Gels were run in a discontinuous buffer system with the application of 90 V for ~10 min during the stacking phase, and 150 V for ~45 min during the running phase with a constant amperage of 0.02 Å used throughout. The running buffer used was 1x MOPS SDS running buffer (NuPAGE™) for Bis-Tris gels, or 1x Tricine SDS Running buffer (Novex™) for Tricine gels. Current was applied until the dye marker (bromophenol blue) was within 1 cm of the gel bottom.

Gels were stained by submersion into Imperial™ Protein Stain (Thermo Scientific) for 1 hour, and de-stained using deionised (MilliQ) H₂O until the gel background was clear. Gels were photographed with a Nikon D810 against an illuminated surface. Variation in the banding pattern between samples was determined via visual analysis of the gel images.

Size Exclusion Fast Performance Liquid Chromatography (SE-FPLC).

SE-FPLC of *S. horrida* ichthyocrinotoxin first involved cleaning the toxin to remove debris. To do this, crude ichthyocrinotoxin samples (50 µL) were diluted with 500 µL of 1x PBS, then centrifuged at 15,000x g at 8°C for 5 min. The resulting supernatants were passed through a 0.22 µm syringe filter. Cleaned toxin samples (500 µL) were then manually injected onto the ÄKTA™ prime system. The separation of ichthyocrinotoxin components used a Superdex 75 10/300 (GE Healthcare) GL Column (13 µm, 10 x 300 mm, 3 kDa to 70 kDa fractionation range for globular proteins). The running buffer used was 1x PBS and the system

was set to run at 500 $\mu\text{L}/\text{min}$ at 8°C for 50 mL. The ultra-violet (UV) absorbance of the eluant was continuously monitored at 280 nm.

The toxin profiles were processed using UNICORNTM 7 software (2016, GE Healthcare). Due to the uneven absorbance intensity observed among chromatograms, raw data was normalised by multiplying each individual absorbance value recorded during fractionation by the ratio of the tallest synchronous peak which, for stonefish ichthyocrotoxin, occurred at an elution volume of 19.6 mL. Chromatograms were produced using GraphPad Prism 10 for visual comparisons of the toxin profiles.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

To perform RP-HPLC on *S. horrida* ichthyocrotoxin, crude toxin samples (50 μL) were diluted with a 50:50 mixture of solvent A (250 μL $\text{H}_2\text{O}/0.05\%$ trifluoroacetic acid (TFA; Auspep) and solvent B (250 μL 90% ACN (Merck)/10% $\text{H}_2\text{O}/0.045\%$ TFA) and refrigerated overnight. Samples were centrifuged at $15,000\times g$ at 8°C for 10 min to remove debris. Fractionation used a C_{18} Phenomenex Aeris[®] PEPTIDE XB liquid chromatography column (150 x 2.10 mm, 3.6 μm , 100 $\text{\AA}$) using a gradient of 0 – 60% solvent B in 60 min, 60 – 90% solvent B in 5 min, 90% solvent B for 10 min, and 90 – 0% solvent B in 5 min. The flow rate remained constant at 0.250 mL/min. The concentration of the sample supernatant was determined using Nanodrop One (Thermo ScientificTM) under the Protein A₂₈₀ program and the injection volume of each toxin sample was adjusted to achieve a final concentration of ~250 μg . The ultra-violet absorbance of the eluant was monitored at 214 nm. Raw data acquisition and initial analysis were conducted within OpenLAB CDS ChemStation software (v1.8.1), with subsequent exportation to GraphPad Prism 10 for the generation of chromatograms.

6.4 Results

6.4.1 SDS-PAGE Profiles of Estuarine Stonefish (*Synanceia horrida*) Ichthyocrinotoxins from Different Australian Locations

SDS-PAGE profiles of *S. horrida* ichthyocrinotoxins exhibited considerable variation between geographically isolated Australian populations (**Figure 6.1**). Bis-Tris gel separation showed that proteins within a molecular weight range of approximately 10 to 25 kDa were shared across all three locations. (**Figure 6.1A**). However, *S. horrida* ichthyocrinotoxin from Exmouth populations (Lane 3) showed banding patterns corresponding to large constituents with molecular weights of approximately 60 kDa and 150 kDa, that were absent in *S. horrida* ichthyocrinotoxins from Cairns (Lane 1) and Caloundra (Lane 2). Furthermore, *S. horrida* ichthyocrinotoxin from Caloundra and Exmouth had a band appearing at approximately 12 kDa that was absent in *S. horrida* ichthyocrinotoxins from Cairns.

Tris-Tricine gel separation was also performed to improve the resolution of *S. horrida* ichthyocrinotoxin proteins within the molecular weight range of 14 to 18 kDa (**Figure 6.1B**). In contrast to Bis-Tris gel profiles, Tris-Tricine gel profiles revealed three distinct bands in *S. horrida* ichthyocrinotoxins from Caloundra (Lane 2), corresponding to components with molecular weights of approximately 8, 11, and 18 kDa. These components were also found to be absent in *S. horrida* ichthyocrinotoxins from Cairns (Lane 1) and Exmouth (Lane 2). *Synanceia horrida* ichthyocrinotoxins from Cairns and Caloundra also shared a band at approximately 28 kDa which was less prominent in samples of *S. horrida* ichthyocrinotoxin from Exmouth. The most abundant shared components, indicated by a greater band intensity, were between approximately 13 and 15 kDa. All profiles also shared 11 kDa and 25 kDa components.

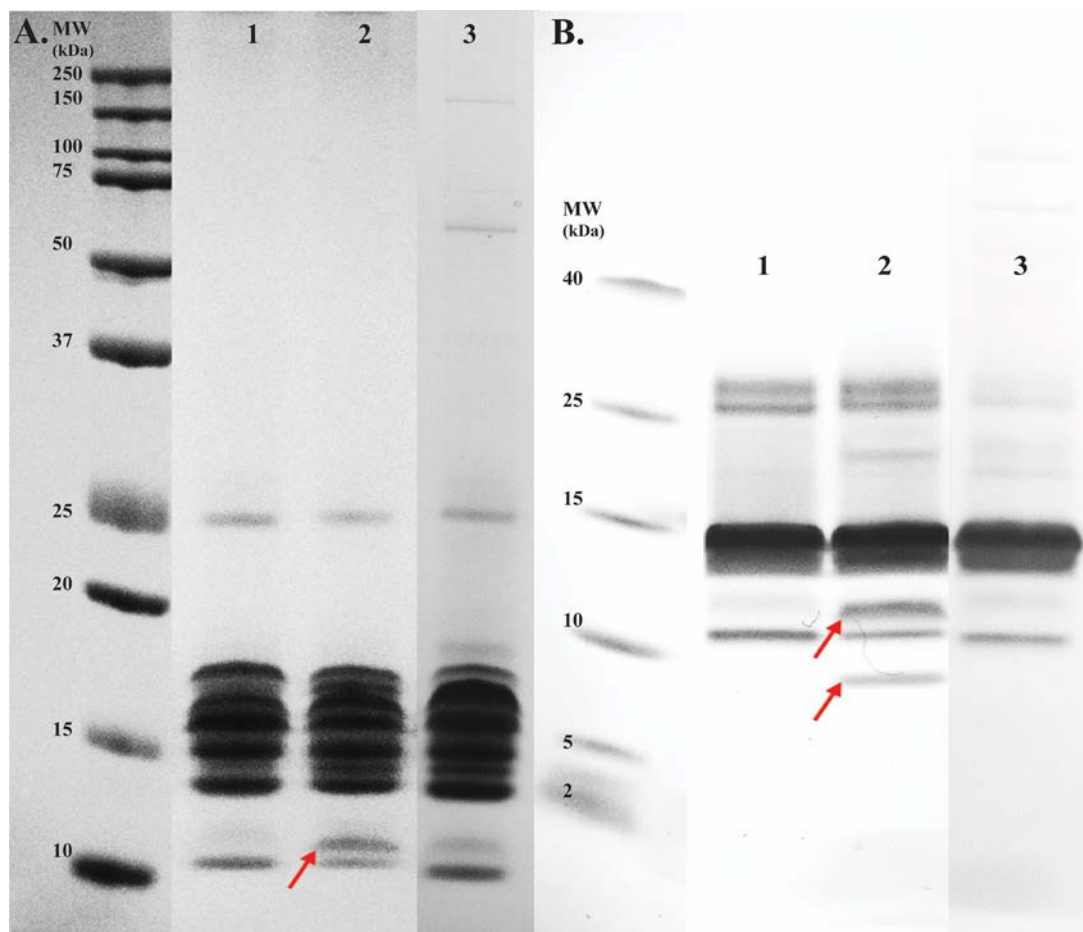


Figure 6.1 SDS-PAGE Profiles of Estuarine Stonefish (*Synanceia horrida*) Ichthyocrinotoxin from Different Australian Locations. *Synanceia horrida* ichthyocrinotoxin collected from Cairns (Lane 1), Caloundra (Lane 2), and Exmouth (Lane 3) populations were profiled using both NuPAGE™ 12% Bis-Tris (**Panel A**), and NuPAGE™ 10 – 20% Tris-Tricine (**Panel B**) mini polyacrylamide gels. Molecular weights (kDa) of sample components were estimated by comparison with either Precision Plus Protein™ Dual Colour (Panel A) or Spectra™ Multicolour Low Range protein standards (Panel B). Arrows (↓) mark observed variations in banding patterns.

6.4.2 SE-FPLC Profiles of Estuarine Stonefish (Synanceia horrida) Ichthyocrinotoxins from Different Australian Locations

SE-FPLC profiles of *S. horrida* ichthyocrinotoxin exhibited both similarities and variations in composition (**Figure 6.2**). *Synanceia horrida* ichthyocrinotoxin samples from all three locations shared peaks 4 (13.1 to 14 mL elution volume), 5 (14.1 to 17.9 mL elution volume), 7 (19.5 to 20 mL elution volume), 10 (22.6 to 31 mL elution volume), and 11 (31.1 to 25 mL elution volume). However, *S. horrida* ichthyocrinotoxin samples from Caloundra and Exmouth shared peaks 2 (9.1 to 11 mL elution volume), 3 (11.1 to 13 mL elution volume), and 9 (20.6 to 22.5 mL elution volume), which were absent in ichthyocrinotoxin samples from Cairns. Furthermore, ichthyocrinotoxin samples from each location had a peak that was unique to that sample. For instance, peak 1 (7.5 to 9 mL elution volume) was only present in ichthyocrinotoxin samples from Exmouth, peak 6 (18 to 19.4 mL elution volume) was unique to ichthyocrinotoxin samples from Caloundra, and peak 8 (20.1 to 20.5 mL elution volume) was solely observed in ichthyocrinotoxin samples from Cairns.

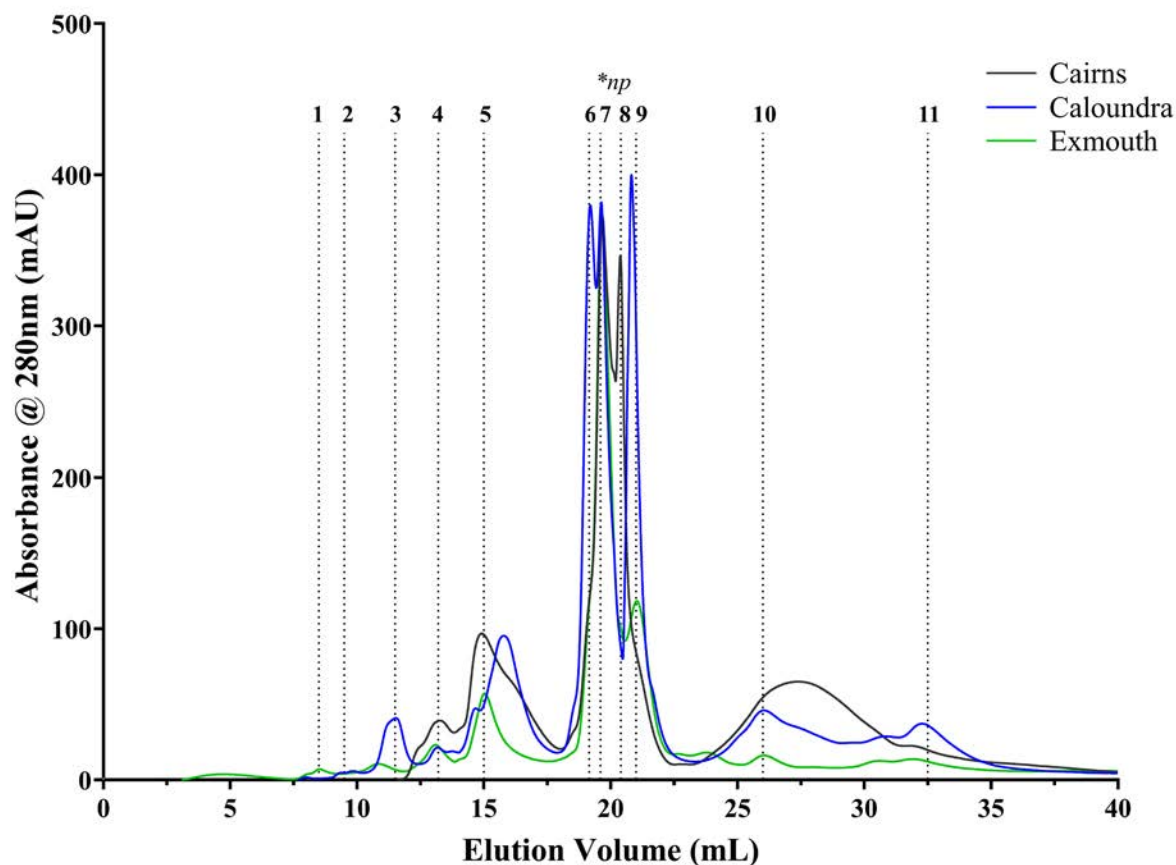


Figure 6.2 SE-FPLC Profiles of Estuarine Stonefish (*Synanceia horrida*) Ichthyocrinotoxins from Different Australian Locations. *Synanceia horrida* ichthyocrinotoxin from Cairns (—), Caloundra (—), and Exmouth (—) populations were profiled using an ÄKTA™ Prime system. Absorbance (280 nm) was monitored for 40 mL of the eluant. Chromatograms were normalised for the absorbance intensity of the 19.6 mL peak (marked as ‘np’). Numbers 1 to 11 mark identified distinct chromatographic peaks.

6.4.3 RP-HPLC Profiles of Estuarine Stonefish (Synanceia horrida) Ichthyocrinotoxin from Different Australian Locations

RP-HPLC profiles revealed little difference between *S. horrida* ichthyocrinotoxins from Cairns, Caloundra, and Exmouth populations (**Figure 6.3**). *Synanceia horrida* ichthyocrinotoxin from Caloundra and Exmouth shared a single peak at approximately 64 min that was absent in the profile of Cairns animals. All other peaks in the ichthyocrinotoxin profiles were shared among all three locations.

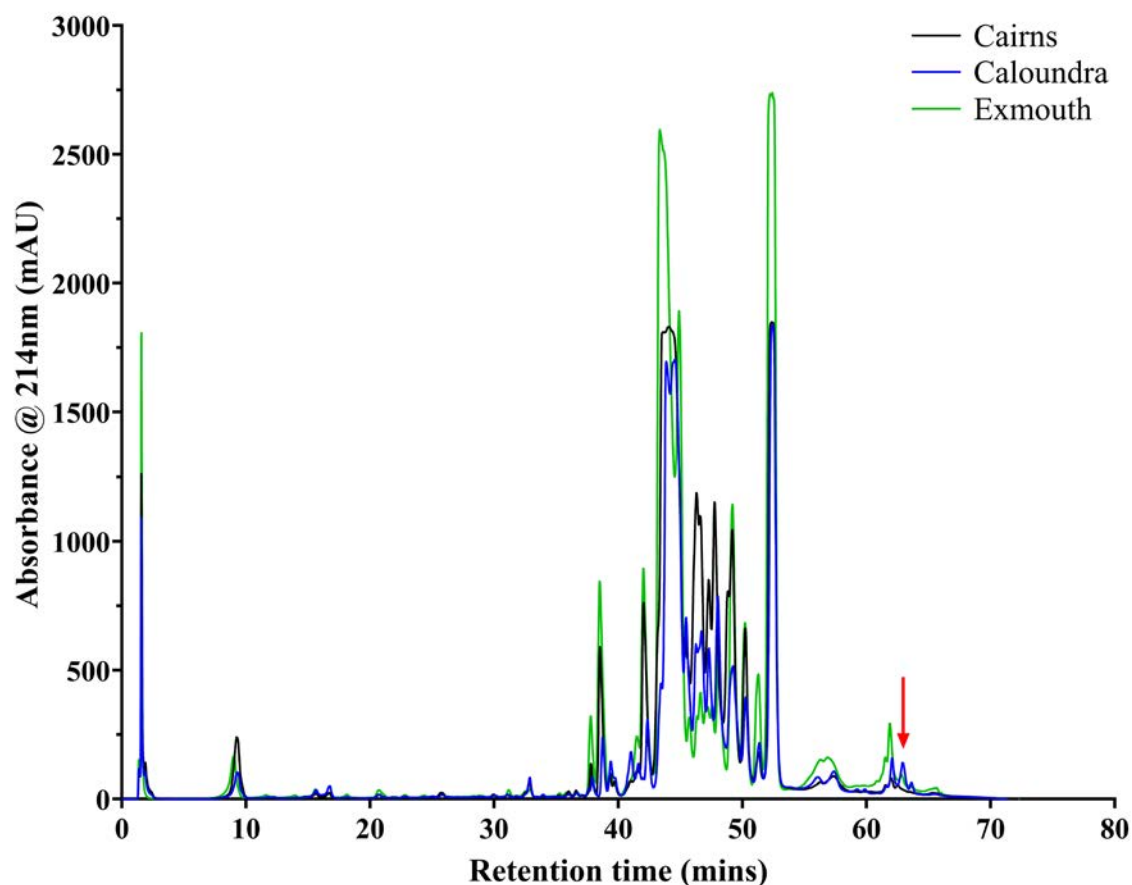


Figure 6.3 RP-HPLC Profiles of Estuarine Stonefish (*Synanceia horrida*) Ichthyocrinotoxins from Different Australian Locations. *Synanceia horrida* ichthyocrinotoxins from Cairns (—), Caloundra (—), and Exmouth (—) populations were compared. Absorbance was monitored at 214 nm. Arrows (↓) mark observed variations in peak patterns.

6.5 Discussion

A previous study highlighted similarities and variations in the composition of stonefish ichthyocrinotoxins between two species (*S. horrida* and *S. verrucosa*) from far north Queensland (Cairns), Australia (Lennox-Bulow *et al.*, 2023b). The results of this study show that the composition of ichthyocrinotoxins from *S. horrida* also exhibit intraspecific commonalities and variations between geographically isolated Australian populations. A large proportion of the components within *S. horrida* ichthyocrinotoxins that were visible by the applied profiling techniques were shared amongst all three locations. SDS-PAGE revealed that the sizes of most components within *S. horrida* ichthyocrinotoxins ranged between approximately 14 and 25 kDa. However, unique components were identified in *S. horrida* ichthyocrinotoxins from Caloundra and Exmouth populations. In Caloundra populations, the unique components were small (8 and 12 kDa as shown by SDS-PAGE, and given that smaller proteins generally elute later with SE-FPLC, peak 6 in the SE-FPLC chromatograms), and hydrophobic (approximately 62 min retention time as determined by RP-HPLC). In contrast, the unique components in *S. horrida* ichthyocrinotoxin from Exmouth populations were large (approximately 60 and 150 kDa as shown by SDS-PAGE, and given that larger proteins generally elute earlier with SE-FPLC, peak 1 in the SE-FPLC chromatograms), with no discernible difference in hydrophobicity. To our knowledge, this is the first documentation of geographic variation in ichthyocrinotoxins produced by fish. These findings further add to the growing body of evidence underscoring the complexity and compositional diversity of ichthyocrinotoxins produced by stonefish. Geographic variation in the composition of poisons harnessed by animals that secrete toxins acquired from their diet is well-established across numerous taxa, such as poison dart frogs (Albuquerque-Pinna *et al.*, 2024; Daly *et al.*, 2008; Daly *et al.*, 1994), and pufferfish (Ito *et al.*, 2023). While the effect of dietary composition on stonefish toxins has not been directly tested, one study found that the rate at which stonefish replenished their venom after discharge was directly tied to their food intake (Saggiomo *et al.*, 2017). However, the composition of the venom was found to remain unchanged regardless of satiety (Saggiomo *et al.*, 2017). Although diet and/or food intake might also influence the composition of stonefish ichthyocrinotoxins, these factors are unlikely to be the primary drivers of the observed variation. This is because unlike well-studied poisonous taxa that sequester toxins acquired from their diet, stonefish likely synthesise their ichthyocrinotoxins *de novo* within specialised glands (i.e., tubercles). Additionally, akin to their defensive venom, stonefish

ichthyocrinotoxins likely do not aid in prey capture, which suggests that they are not under a selective pressure to adapt to prey dynamics.

Although little is known about compositional variation in poisons or ichthyocrinotoxins that are produced *de novo*, numerous biological and environmental factors have been identified to influence the composition of venoms. These include both biotic factors such as sex (Amorim *et al.*, 2018; Zelanis *et al.*, 2016; Herzig & Hodgson, 2009), ontogeny (da Silva Aguiar *et al.*, 2020; Underwood & Seymour, 2007), diet (Albuquerque-Pinna *et al.*, 2024; Weese & Duda, 2019; Phuong *et al.*, 2016), and community dynamics (Smith *et al.*, 2023; Chang & Duda, 2016; Daltry *et al.*, 1996), as well as abiotic factors such as season (Tasima *et al.*, 2024; Junior *et al.*, 2010), and other physiochemical environmental parameters (Smith *et al.*, 2023; O'Hara *et al.*, 2021; Sachkova *et al.*, 2020). Untangling the ecological drivers of compositional variation in toxins is complex and likely unique to each location. However, the most compelling evidence of strong positive selection shaping toxin composition is the occurrence of target-specific compounds (Smith & Mackessy, 2021; Barlow *et al.*, 2009). At present, virtually nothing is known about the ecological function of stonefish ichthyocrinotoxins, however, it has been suggested that they may serve a multifunctional role in managing interactions between certain organisms and the animal's integument (Lennox-Bulow *et al.*, 2023a). This would suggest that ichthyocrinotoxins secreted by stonefish potentially select for and/or against multiple targets.

Preliminary investigations suggest that one of the roles of stonefish ichthyocrinotoxins may be to facilitate the growth and/or attachment of algae and/or plants on to their integument (Barnett, 2014). As such, one potential driver of variation in the composition of stonefish ichthyocrinotoxins may be differences in benthic flora communities between locations. Directly surveying the benthic flora communities within stonefish collection sites was outside the scope of this study. However, previously published benthic surveys from the generalised areas in which the stonefish used in this study were collected suggest that the dominant algal communities may differ between the locations. For instance, the Trinity Inlet in Cairns is comprised of turbid estuarine waters, a muddy/silty substrate, and is abundant in erect macrophyte and filamentous brown (Phaeophyceae) algae (McKenna *et al.*, 2013). Similarly, the arid tropical Exmouth Gulf comprises a mixed substrate (i.e., sand and mud) benthos, with varying turbidity, however, the area also features a fringing coral reef (Ningaloo reef) and numerous turfing and macroalgal species (Cartwright *et al.*, 2023). In contrast, Caloundra's subtropical estuary habitat has generally clearer waters and a sandy substrate that hosts seagrass meadows (Hyland, 1989), and weedy *Sargassum* beds (Phillips & Blackshaw, 2011).

While the epidermis of stonefish is unlikely to host seagrasses and macroalgae, the dominant slime, filamentous, and encrusting algal species also likely differ between these locations. Therefore, it is plausible that the observed intraspecific differences in *S. horrida* ichthyocrinotoxin composition may be influenced by the unique recruitment and/or fertilisation requirements of the dominant algae in each location. Alternatively, the observed shared compositional traits might suggest that the resources required to recruit flora on to the epidermis, and/or accelerate its growth are the same or similar regardless of the species of algae. By facilitating algal attachment and/or growth on the skin, the animal could benefit from enhanced crypsis, reducing its visibility to both predators and prey. Furthermore, the algal covering could be perceived as a resource by herbivorous fish which, in turn, could draw in potential prey items within striking distance. Furthermore, although the epidermis of stonefish is reportedly covered in the same colour and type of algae as what is found in their immediate surroundings (Endean, 1961), it does not appear to host fouling fauna. Additionally, despite being highly susceptible to incursion by parasites, particularly species that have a skin-burrowing life stage, an unusually low diversity of endoparasite taxa appear to be associated with stonefish (Miller *et al.*, 2018). As such, it has been suggested that stonefish ichthyocrinotoxins might also provide a defensive barrier for the skin against the detrimental attachment and/or invasion of fouling or parasitic invertebrates (Lennox-Bulow *et al.*, 2023b). While the dynamics of parasitic and fouling invertebrates are likely similar between reef and estuarine environments in a single location in the tropics (Lennox-Bulow *et al.*, 2023b), there would likely be some variation between the tropical (i.e., Cairns), arid tropical (i.e., Exmouth), and subtropical (i.e., Caloundra) regions of Australia. We speculate that these differences may also exert some influence on the composition of ichthyocrinotoxins from *S. horrida*. For instance, variations in the species assemblages or abundance of parasitic or fouling invertebrate taxa between locations may necessitate different components to provide a successful defence for the integument. Alternatively, *S. horrida* ichthyocrinotoxin may already provide a highly effective defence against numerous invertebrate taxa, which could explain the high degree of conservation observed in *S. horrida* ichthyocrinotoxin composition between locations.

In conclusion, this study supports to the growing body of evidence suggesting that stonefish ichthyocrinotoxins are compositionally both complex and diverse concoctions. While many components within the ichthyocrinotoxins of *S. horrida* were shared across all three Australian populations, unique components were observed. While little is currently known about the ecological function of stonefish ichthyocrinotoxins, we speculate that the strongest

forces acting on its composition are likely benthic flora and invertebrate communities. To truly understand the ecological significance of the compositional diversity of stonefish ichthyocrinotoxins, future investigations should work towards characterising the components within these toxins. Targeted studies exploring the role of these toxins in algal growth/recruitment and invertebrate defence are also required to gain a better understanding of the underlying biological and environmental drivers of the compositional diversity of stonefish ichthyocrinotoxins.

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6.7 APPENDIX E

Geographic Variation in Ichthyocrinotoxin from the Estuarine Stonefish (*Synanceia horrida*).

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Geographic variation in ichthyocrinotoxin from the Estuarine Stonefish (*Synanceia horrida*)

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ABSTRACT

Geographic variation in the composition of animal toxins is well documented for venomous taxa, and to a lesser degree, for some poisonous taxa that secrete toxins obtained from their diet. However, very little is known about animals that synthesise their poisons *de novo*, such as stonefish and their secreted ichthyocrinotoxins. Stonefish are widely distributed throughout the Indo-Pacific, however, the effect of location on the composition of their ichthyocrinotoxin is currently unknown. This study aimed to determine whether the composition of ichthyocrinotoxins from *Synanceia horrida* (Estuarine Stonefish) varied between three geographically isolated Australian populations including Cairns in far north Queensland, Caloundra in southeast Queensland, and Exmouth in Western Australia. The composition of ichthyocrinotoxins from *S. horrida* were largely conserved across the three locations, with the size of most of the components falling between 14 and 25 kDa. However, unique components were identified in *S. horrida* ichthyocrinotoxins from Caloundra and Exmouth populations. Caloundra populations contained unique smaller components (8 and 12 kDa) that were hydrophobic. In contrast, Exmouth populations contained unique larger components (60 and 150 kDa) with no difference in hydrophobicity. We speculate that the composition of ichthyocrinotoxins produced by *S. horrida* are likely influenced by benthic flora and fauna communities, particularly those that are likely to interact with the animal's skin, such as fouling flora and fauna, as well as parasites. These findings further add to the growing body of evidence underscoring the complexity and compositional diversity of ichthyocrinotoxins produced by stonefish.

1. Introduction

It has been well established that toxins produced by animals, which include venoms and poisons, are often complex, dynamic, and diverse concoctions (Suranase et al., 2018; Warrell, 2020). Animal toxins are generally comprised of a multifarious mixture of proteins, peptides, and small molecules (Bolon et al., 2023; Warrell, 2020). The composition and complexity of these concoctions can vary both within species (Schaffrath et al., 2018; Tasoulis et al., 2020) and between species (Duran et al., 2020; van Thiel et al., 2023), as well as within and/or between individuals (Dutertre et al., 2014). The ecological factors shaping the composition of animal toxins is a complex evolutionary question and is often attributed to a combination of biotic factors such as sex (Amorim et al., 2018; Herzig and Hodgson, 2009; Zelanis et al., 2016), ontogeny (da Silva Aguiar et al., 2020; Underwood and Seymour, 2007), diet (Albuquerque-Pinna et al., 2024; Phuong et al., 2016; Weese and Duda, 2019), and community dynamics (Chang and Duda, 2016; Daltry et al., 1996; Smith et al., 2023), as well as abiotic factors such as

season (Junior et al., 2010; Tasima et al., 2024), and other physico-chemical environmental parameters (O'Hara et al., 2021; Sachkova et al., 2020; Smith et al., 2023). Many of these factors can vary with, and/or are likely unique to, the individual's geographic location (Yu et al., 2020).

Compositional variation in animal toxins between geographically isolated populations is widely documented across venomous taxa, particularly terrestrial snakes (Davies and Arbuckle, 2019; Sasa, 1999; Smith and Mackessy, 2021; Smith et al., 2023; van Thiel et al., 2023), and although to a much lesser extent, some poisonous taxa, such as poison dart frogs (Albuquerque-Pinna et al., 2024; Daly et al., 1994, 2008), and pufferfish (Ito et al., 2023). This phenomenon has also been documented at almost all spatial scales, ranging from between micro-habitats within a single location (Abdel-Rahman et al., 2009; Smith et al., 2023), to across continents spanning vast distances (Carrette, 2014; Duda et al., 2009). While the ecological factors influencing venom composition between geographically isolated populations are intricate, the primary selective force often centres around the venoms intended

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target (Barlow et al., 2009; Smith and Mackessy, 2021). Interestingly, this does not appear to be the case for animal poisons. Poisons sequestered by animals are generally comprised of a few peptides and/or small molecules that have been acquired through the animal's diet (Harris and Jenner, 2019; Mahmud et al., 2003). Although these toxins often serve exclusively as a defence against predators or injury, numerous studies have attributed intraspecific compositional variation in animal poisons to geographic variation in the animal's diet (Alto, 2011; Daly et al., 1994, 2008). However, virtually nothing is known about poisonous taxa that synthesise their toxins *de novo* within specialised glands, such as stonefish.

Stonefish (*Synanceia* spp.) are a bottom-dwelling fish found throughout both the tropical and subtropical waters of the Indo-Pacific region (Endean, 1961). Stonefish are one of very few animals that harness two distinct types of toxin: a venom which is housed within twin glands associated with each of their 13 dorsal spines, and an ichthyocrinotoxin that is secreted by specialised glands (i.e., tubercles) that span their epidermis (Endean, 1961; Saggiomo et al., 2021). Stonefish venom is believed to function purely as a predator defence (Endean, 1961; Saggiomo et al., 2021). The deleterious nature of this venom to people has awarded stonefish the title of worlds' most venomous fish (Cameron and Endean, 1973; Saggiomo et al., 2021). Resultingly, research into stonefish toxins is heavily centred around medical aid to envenomation which often occurs from accidentally standing on the animal (Cameron and Endean, 1973; Ziegman et al., 2019). However, very little is currently known about stonefish ichthyocrinotoxins.

Stonefish are devoid of scales and readily bury themselves into the substratum (Endean, 1961). Consequently, their skin is presumably highly susceptible to a variety of benthic fauna, particularly invertebrate taxa such as parasites and fouling animals, as well as certain flora, including filamentous, slime, and encrusting algae. As such, it has been previously suggested that stonefish ichthyocrinotoxins may act to manage biological interactions with their integument, particularly those involving bacteria, parasites, as well as fouling flora and fauna (Barnett, 2014; Cameron et al., 1981; Endean, 1961; Lennox-Bulow et al., 2023a, b). However, these dynamics are likely to vary geographically, potentially leading to different ichthyocrinotoxins. Therefore, the aim of this study is to determine whether the composition of ichthyocrinotoxin from *Synanceia horrida* (Estuarine Stonefish) varies intraspecifically between geographically isolated Australian populations, and if so, discuss the potential ecological drivers behind it.

2. Materials and methods

2.1. Animal and toxin collection

2.1.1. Collecting and sourcing stonefish

Synanceia horrida were hand captured and/or sourced from commercial fish suppliers (Cairns Marine, Reef 2 Aquarium, Jardini International) between January 2019 to 2024. *Synanceia horrida* were found amongst inshore rocky reef and/or estuarine environments in Cairns in far north Queensland Australia, Caloundra in southeast Queensland Australia, and Exmouth in Western Australia. The sexes of each individual were not able to be determined.

2.1.2. Extracting stonefish ichthyocrinotoxin

Ichthyocrinotoxin collection was done in accordance with James Cook University Animal Ethics Committee approval (AEC approval number A2832). Ichthyocrinotoxin from *S. horrida* were extracted following a protocol outlined by Lennox-Bulow et al., 2023a,b. In brief, individual stonefish were transferred to a saltwater-dampened surface and secured by gently holding either side of the pectoral fins. A saltwater-dampened cotton cloth was also placed over the animal's eyes to minimise stress throughout the process. Ichthyocrinotoxin was then extracted from the skin tubercles using targeted mechanical massage under a controlled vacuum. This vacuum was generated by a

purpose-built extraction apparatus comprised of a 100 mm segment of clear vinyl tubing affixed to a 50 mL syringe. To account for potential individual variation in toxin composition, *S. horrida* ichthyocrinotoxin was extracted and pooled from three to five mature individuals. Pooled ichthyocrinotoxin samples were stored in 1.5 mL Eppendorf tubes at -80°C until required.

2.2. Profiling stonefish toxins

Gel electrophoresis (SDS-PAGE), Size Exclusion Fast Performance Liquid Chromatography (SE-FPLC) and Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) were performed on *S. horrida* ichthyocrinotoxins following the protocol outlined by Lennox-Bulow et al., 2023a,b. The following provides a brief description of the methods used for each technique.

2.2.1. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis of *S. horrida* ichthyocrinotoxins first involved centrifuging the samples at $15,000\times g$ at 4°C for 5 min to remove debris. The concentration of the sample supernatants was determined using Nanodrop One (Thermo Scientific™) set to protein $A_{280\text{ nm}}$ and adjusted to yield $500\text{ }\mu\text{g/mL}$ ($\pm 50\text{ }\mu\text{g}$). Sample supernatants ($10\text{ }\mu\text{L}$) were then linearised by mixing (1:2) with either 2x Laemmli SDS lysis buffer (for Bis-Tris gels) or 2x Tricine SDS lysis buffer (for Tricine gels). This process was catalysed by heating the samples at 95°C for 5 min for Laemmli buffer sample mixtures, and 85°C for 2 min for Tricine buffer sample mixtures.

Linearised samples ($20\text{ }\mu\text{L}$) and protein standards ($2\text{ }\mu\text{L}$ Precision Plus Protein™ Dual Colour for Bis-Tris gels, or $4\text{ }\mu\text{L}$ Spectra™ Multicolour Low Range Protein Ladder for Tricine gels) were loaded on to gels set up in an electrophoresis cell. Ichthyocrinotoxin samples were initially run on 12 % Bis-Tris mini protein gels (NuPAGE™, $1.0\text{ mm} \times 12\text{ well}$, 10 kDa – 260 kDa separation resolution). To enhance the resolution of banding patterns for smaller molecules (40 – 2 kDa), ichthyocrinotoxin samples were additionally run on a 10–20 % Tricine mini protein gel (Novex™, $1.0\text{ mm} \times 12\text{ well}$, 2 kDa – 200 kDa separation resolution). Gels were run in a discontinuous buffer system with the application of 90 V for $\sim 10\text{ min}$ during the stacking phase, and 150 V for $\sim 45\text{ min}$ during the running phase with a constant amperage of 0.02 A used throughout. The running buffer used was 1x MOPS SDS running buffer (NuPAGE™) for Bis-Tris gels, or 1x Tricine SDS Running buffer (Novex™) for Tricine gels. Current was applied until the dye marker (bromophenol blue) was within 1 cm of the gel bottom.

Gels were stained by submersion into Imperial™ Protein Stain (Thermo Scientific) for 1 h, and de-stained using deionised (MilliQ) H_2O until the gel background was clear. Gels were photographed with a Nikon D810 against an illuminated surface. Variation in the banding pattern between samples was determined via visual analysis of the gel images.

2.2.2. Size Exclusion Fast Performance Liquid Chromatography (SE-FPLC)

SE-FPLC of *S. horrida* ichthyocrinotoxins first involved cleaning the toxin to remove debris. To do this, crude ichthyocrinotoxin samples ($50\text{ }\mu\text{L}$) were diluted with $500\text{ }\mu\text{L}$ of 1x PBS, centrifuged at $15,000\times g$ at 8°C for 5 min, and the supernatants were passed through a $0.22\text{ }\mu\text{m}$ syringe filter. Cleaned toxin samples ($500\text{ }\mu\text{L}$) were then manually injected onto the ÄKTA™ prime system. The separation of ichthyocrinotoxin components used a Superdex 75 10/300 (GE Healthcare) GL Column ($13\text{ }\mu\text{m}$, $10 \times 300\text{ mm}$, 3 kDa – 70 kDa fractionation range for globular proteins). The running buffer used was 1x PBS and the system was set to run at $500\text{ }\mu\text{L/min}$ at 8°C for 50 mL . The ultra-violet absorbance of the eluant was continuously monitored at 280 nm .

The toxin profiles were processed using UNICORN™ 7 software (2016, GE Healthcare). Due to the uneven absorbance intensity observed among chromatograms, raw data was normalised by multiplying each

individual absorbance value recorded during fractionation by the ratio of the tallest synchronous peak which, for stonefish ichthyocrinotoxin, occurred at an elution volume of 19.6 mL. Chromatograms were produced using GraphPad Prism 10 for visual comparisons of the toxin profiles.

2.2.3. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

To perform RP-HPLC on *S. horrida* ichthyocrinotoxins, crude ichthyocrinotoxin samples (50 μ L) were diluted with a 50:50 mixture of solvent A (250 μ L H₂O/0.05 % trifluoroacetic acid (TFA; Auspep) and solvent B (250 μ L 90 % ACN (Merck)/10 % H₂O/0.045 % TFA) and refrigerated overnight. Samples were centrifuged at 15,000x g at 8 °C for 10 min to remove debris. Fractionation used a C₁₈ Phenomenex Aeris® PEPTIDE XB liquid chromatography column (150 $\times$ 2.10 mm, 3.6 μ m, 100 Å) using a gradient of 0–60 % solvent B in 60 min, 60–90 % solvent B in 5 min, 90 % solvent B for 10 min, and 90–0 % solvent B in 5 min. The flow rate remained constant at 0.250 mL/min. The concentration of the sample supernatant was determined using Nanodrop One (Thermo Scientific™) under the Protein A₂₈₀ program and the injection volume of each toxin sample was adjusted to achieve a final concentration of ~250 μ g. The ultra-violet absorbance of the eluant was monitored at 214 nm. Raw data acquisition and initial analysis were conducted within

OpenLAB CDS ChemStation software (v1.8.1), with subsequent exportation to GraphPad Prism 10 for the generation of chromatograms.

3. Results

3.1. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Estuarine Stonefish (*Synanceia horrida*) ichthyocrinotoxin from different Australian locations

Synanceia horrida ichthyocrinotoxin exhibited considerable variation between geographically distinct locations in Australia as shown by gel electrophoresis (SDS-PAGE, Fig. 1). *Synanceia horrida* ichthyocrinotoxin from Exmouth (Fig. 1A, Lane 3) showed banding patterns corresponding to large constituents with molecular weights of approximately 60 kDa and 150 kDa, that were absent in *S. horrida* ichthyocrinotoxins from Cairns (Fig. 1A, Lane 1) and Caloundra (Fig. 1A, Lane 2). Furthermore, *S. horrida* ichthyocrinotoxin from Caloundra and Exmouth had a band appearing at approximately 12 kDa that was absent in *S. horrida* ichthyocrinotoxins from Cairns. Because *S. horrida* ichthyocrinotoxin constituents primarily fell between approximately 10 to 25 kDa under 12 % Bis-Tris gel separation, with most shared constituents appearing between approximately 14 to 18 kDa, a Tris-Tricine gel (Fig. 1B) was used to improve the resolution of the toxin components within this range. In

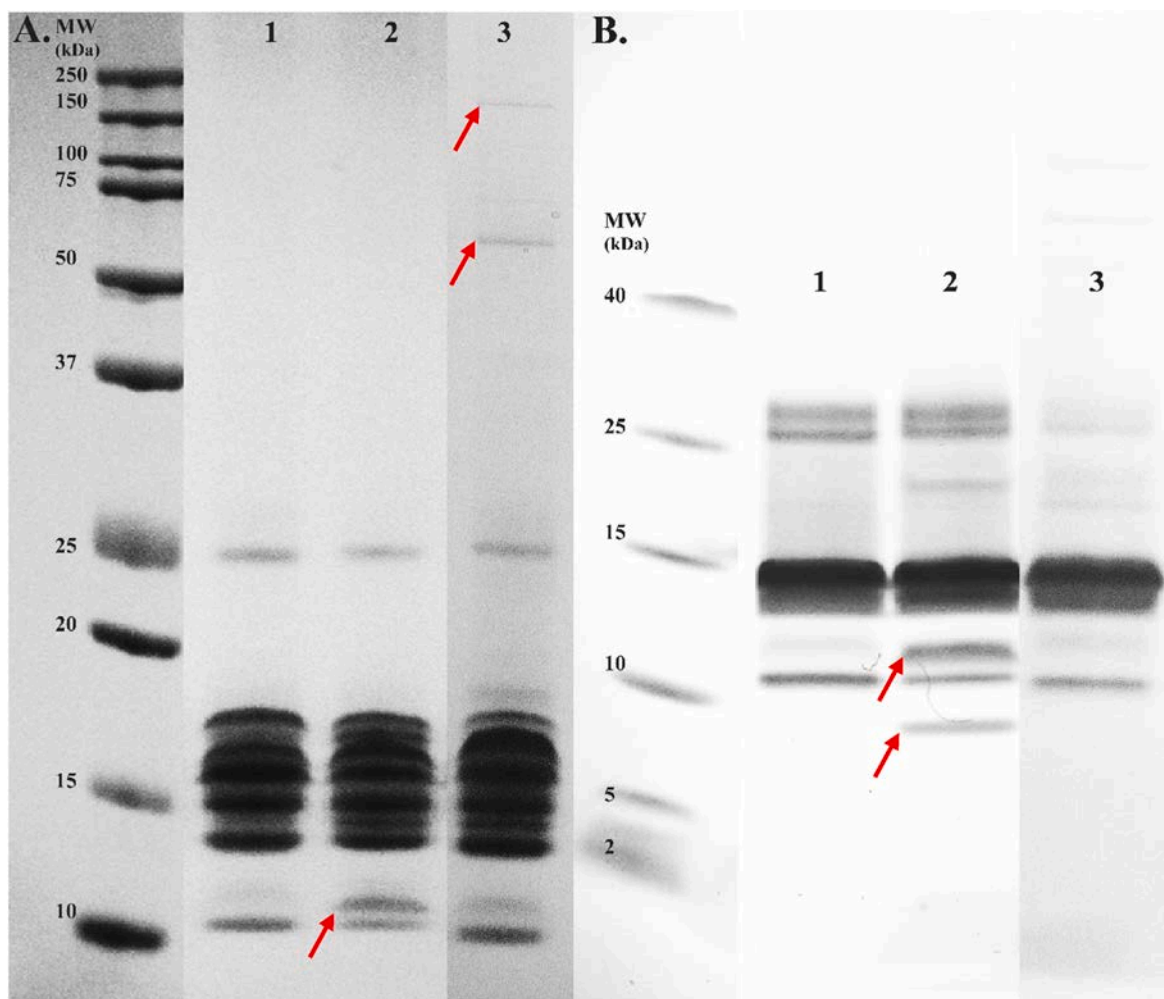


Fig. 1. Gel Electrophoresis of Estuarine Stonefish (*Synanceia horrida*) ichthyocrinotoxin from different Australian locations. *Synanceia horrida* ichthyocrinotoxin collected from Cairns (Lane 1), Caloundra (Lane 2), and Exmouth (Lane 3) populations were profiled using both NuPAGE™ 12 % Bis-Tris (Panel A), and NuPAGE™ 10–20 % Tris-Tricine (Panel B) mini polyacrylamide gels. Molecular weights (kDa) of sample components were estimated by comparison with either Precision Plus Protein™ Dual Colour (Panel A) or Spectra™ Multicolour Low Range protein standards (Panel B). Arrows (↓) mark observed variations in banding patterns. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

contrast to Bis-Tris gel profiles, Tris-Tricine gel profiles revealed three distinct bands in *S. horrida* ichthyocrinotoxins from Caloundra (Fig. 1B, Lane 2), corresponding to components with molecular weights of approximately 8, 11, and 18 kDa. These components were also found to be absent in *S. horrida* ichthyocrinotoxins from Cairns (Fig. 1B, Lane 1) and Exmouth (Fig. 1B, Lane 2). *Synanceia horrida* ichthyocrinotoxins from Cairns and Caloundra also shared a band at approximately 28 kDa which was less prominent in samples of *S. horrida* ichthyocrinotoxin from Exmouth. The most abundant shared components, indicated by a greater band intensity, were between approximately 13 to 15 kDa. All profiles also shared 11 kDa and 25 kDa components.

3.2. Size Exclusion Fast Performance Liquid Chromatography of Estuarine Stonefish (*Synanceia horrida*) ichthyocrinotoxin from different Australian locations

Synanceia horrida ichthyocrinotoxin samples from Cairns, Caloundra, and Exmouth shared both similarities and differences in their components when analysed with Size Exclusion Chromatography (SE-FPLC) (Fig. 2). *Synanceia horrida* ichthyocrinotoxin samples from all three locations shared peaks 4 (13.1–14 mL), 5 (14.1–17.9 mL), 7 (19.5–20 mL), 10 (22.6–31 mL), and 11 (31.1–25 mL). However, *S. horrida* ichthyocrinotoxin samples from Caloundra and Exmouth shared peaks 2 (9.1–11 mL), 3 (11.1–13 mL), and 9 (20.6–22.5 mL), which were absent in ichthyocrinotoxin samples from Cairns. Furthermore, ichthyocrinotoxin samples from each location had a peak that was unique to that sample. For instance, peak 1 (7.5–9 mL) was only present in ichthyocrinotoxin samples from Exmouth, peak 6 (18–19.4 mL) was unique to ichthyocrinotoxin samples from Caloundra, and peak 8 (20.1–20.5 mL) was solely observed in ichthyocrinotoxin samples from Cairns.

3.3. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) Profiles of Estuarine Stonefish (*Synanceia horrida*) ichthyocrinotoxin from different Australian locations

Hydrophobicity profiles (RP-HPLC) revealed little difference between *S. horrida* ichthyocrinotoxin from Cairns, Caloundra, and Exmouth (Fig. 3). *Synanceia horrida* ichthyocrinotoxin from Caloundra and Exmouth shared a single peak at approximately 64 min that was absent in the profile of Cairns animals. All other peaks in the ichthyocrinotoxin profiles were shared among all three locations.

4. Discussion

A previous study highlighted similarities and variations in the composition of stonefish ichthyocrinotoxins between two species (*S. horrida* and *S. verrucosa*) from far north Queensland (Cairns), Australia (Lennox-Bulow et al., 2023a,b). The results of this study show that the composition of ichthyocrinotoxins from *S. horrida* also exhibit intraspecific commonalities and variations between geographically isolated Australian populations. A large proportion of the components within *S. horrida* ichthyocrinotoxins that were visible by the applied profiling techniques were shared amongst all three locations. SDS-PAGE revealed that the sizes of most components within *S. horrida* ichthyocrinotoxins ranged between approximately 14 to 25 kDa. However, unique components were identified in *S. horrida* ichthyocrinotoxins from Caloundra and Exmouth populations. In Caloundra populations, the unique components were small (8 and 12 kDa as shown by SDS-PAGE, and given that smaller proteins generally elute later with SE-FPLC, peak 6 in the SE-FPLC chromatograms), and hydrophobic (approximately 62 min retention time as determined by RP-HPLC). In contrast, the unique components in *S. horrida* ichthyocrinotoxins from Exmouth were large (approximately 60 and 150 kDa as shown by SDS-PAGE, and given that larger proteins generally elute earlier with SE-FPLC, peak 1 in the SE-FPLC chromatograms), with no discernible difference in hydrophobicity. To our knowledge, this is the first

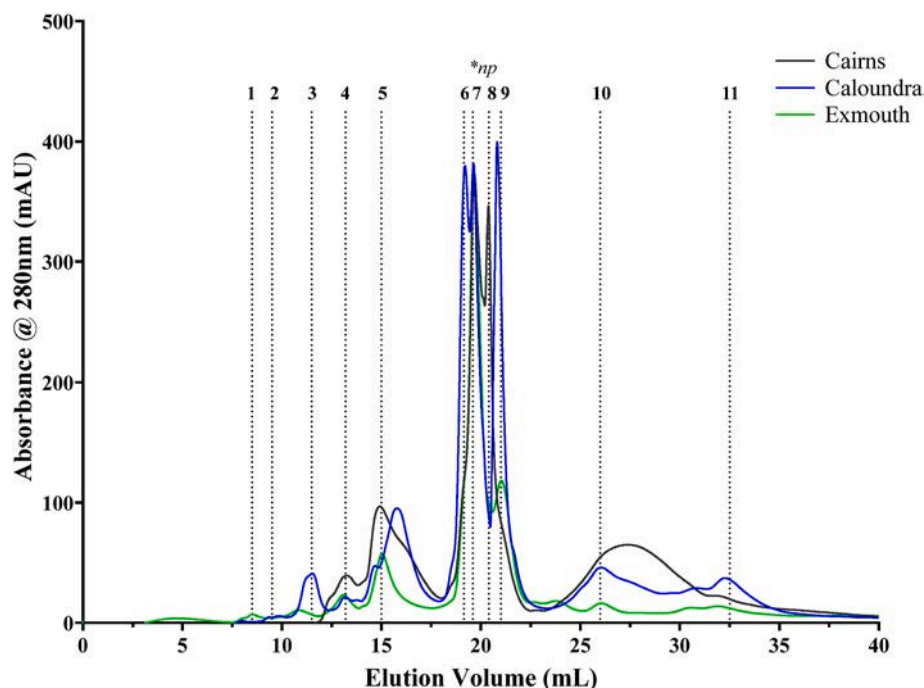


Fig. 2. Size Exclusion Fast Performance Liquid Chromatography (SE-FPLC) Profiles of Estuarine Stonefish (*Synanceia horrida*) Ichthyocrinotoxin from Different Australian Locations. *Synanceia horrida* ichthyocrinotoxin from Cairns (—), Caloundra (—), and Exmouth (—) populations were profiled using an ÄKTA™ Prime system. Absorbance (280 nm) was monitored for 40 mL of the eluant. Chromatograms were normalised for the absorbance intensity of the 19.6 mL peak (marked as 'np'). Numbers 1 to 11 mark identified distinct chromatographic peaks.

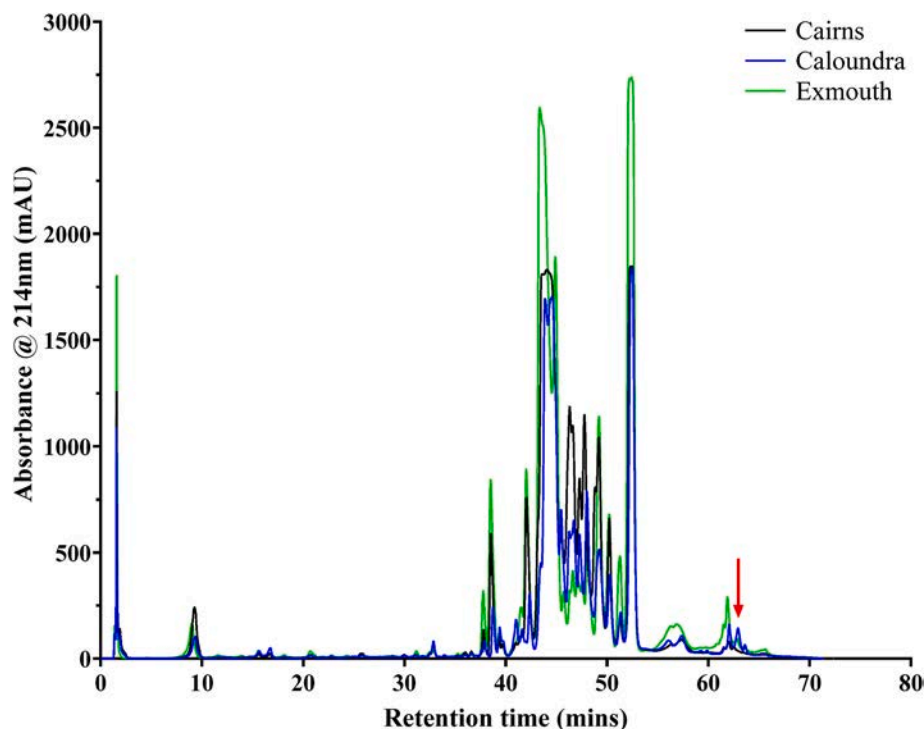


Fig. 3. Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) Profiles of Estuarine Stonefish (*Synanceia horrida*) Ichthyocrinotoxins from Different Australian locations. *Synanceia horrida* ichthyocrinotoxins from Cairns (—), Caloundra (—), and Exmouth (—) populations were profiled using Reverse-Phase High Performance Liquid Chromatography (RP-HPLC). Absorbance was monitored at 214 nm. Arrows (↓) mark observed variations in peak patterns.

documentation of geographic variation in ichthyocrinotoxins produced by fish. These findings further add to the growing body of evidence underscoring the complexity and compositional diversity of ichthyocrinotoxins produced by stonefish.

Geographic variation in the composition of poisons harnessed by animals that secrete toxins acquired from their diet is well-established across numerous taxa, such as poison dart frogs (Albuquerque-Pinna et al., 2024; Daly et al., 1994, 2008), and pufferfish (Ito et al., 2023). While the effect of dietary composition on stonefish toxins has not been directly tested, one study found that the rate at which stonefish replenished their venom after discharge was directly tied to their food intake (Saggiomo et al., 2017). However, the composition of the venom was found to remain unchanged regardless of satiety (Saggiomo et al., 2017). Although diet and/or food intake might also influence the composition of stonefish ichthyocrinotoxins, these factors are unlikely to be the primary drivers of the observed variation. This is because unlike well-studied poisonous taxa that sequester toxins acquired from their diet, stonefish synthesise their ichthyocrinotoxins *de novo* within specialised glands (i.e., tubercles). Additionally, akin to their defensive venom, stonefish ichthyocrinotoxins likely do not aid in prey capture, which suggests that they are not under a selective pressure to adapt to prey dynamics.

Although little is known about compositional variation in poisons or ichthyocrinotoxins that are produced *de novo*, numerous biological and environmental factors have been identified to influence the composition of venoms. These include both biotic factors such as sex (Amorim et al., 2018; Herzig and Hodgson, 2009; Zelanis et al., 2016), ontogeny (da Silva Aguiar et al., 2020; Underwood and Seymour, 2007), diet (Albuquerque-Pinna et al., 2024; Phuong et al., 2016; Weese and Duda, 2019), and community dynamics (Chang and Duda, 2016; Daltry et al., 1996; Smith et al., 2023), as well as abiotic factors such as season (Junior et al., 2010; Tasima et al., 2024), and other physicochemical environmental parameters (O'Hara et al., 2021; Sachkova et al., 2020; Smith et al., 2023). Untangling the ecological drivers of compositional variation in toxins is complex and likely unique to each location.

However, the most compelling evidence of strong positive selection shaping toxin composition is the occurrence of target-specific compounds (Barlow et al., 2009; Smith and Mackessy, 2021). Presently, virtually nothing is known about the ecological function of stonefish ichthyocrinotoxins, however, it has been suggested that they may serve a multifunctional role in mitigating interactions between certain organisms and the animal's integument (Lennox-Bulow et al., 2023a,b). This would suggest that ichthyocrinotoxins secreted by stonefish potentially select for and/or against multiple targets.

Preliminary investigations suggest that one of the roles of stonefish ichthyocrinotoxins may be to facilitate the growth and/or attachment of algae and/or plants on to their integument (Barnett, 2014). As such, one potential driver of variation in the composition of stonefish ichthyocrinotoxins may be differences in benthic flora communities between locations. Directly surveying the benthic flora communities within stonefish collection sites was outside the scope of this study. However, previously published benthic surveys from the generalised areas in which the stonefish used in this study were collected suggest that the dominant algal communities may differ between the locations. For instance, the Trinity Inlet in Cairns is comprised of turbid estuarine waters, a muddy/silty substrate, and abundant in erect macrophyte and filamentous brown (Phaeophyceae) algae (McKenna et al., 2013). Similarly, the arid tropical Exmouth Gulf comprises a mixed substrate (i.e., sand and mud) benthos, with varying turbidity, however, the area also features a fringing coral reef (Ningaloo reef) and numerous turfing and macroalgal species (Cartwright et al., 2023). In contrast, Caloundra's subtropical estuary habitat has generally clearer waters and a sandy substrate that hosts seagrass meadows (Hyland, 1989), and weedy *Sargassum* beds (Phillips and Blackshaw, 2011).

While the epidermis of stonefish is unlikely to host seagrasses and macroalgae, the dominant slime, filamentous, and encrusting algal species also likely differ between these locations. Therefore, it is plausible that the observed intraspecific differences in *S. horrida* ichthyocrinotoxin composition may be influenced by the unique recruitment and/or fertilisation requirements of the dominant algae in each location.

Alternatively, the observed shared compositional traits might suggest that the resources required to recruit flora on to the epidermis, and/or accelerate growth are the same or similar regardless of the species of algae. By facilitating algal attachment and/or growth on the skin, the animal could benefit from enhanced crypsis, which would help to reduce its visibility to both predators and prey. Furthermore, the algal covering could be perceived as a resource by herbivorous fish which, in turn, could draw in potential prey items within striking distance.

Furthermore, although the epidermis of stonefish is reportedly covered in the same colour and type of algae as what is found in their immediate surroundings (Endean, 1961), it does not appear to host fouling fauna. Additionally, despite being highly susceptible to incursion by parasites, particularly species that have a skin-burrowing life stage, an unusually low diversity of endoparasite taxa appear to be associated with stonefish (Miller et al., 2018). As such, it has been suggested that stonefish ichthyocrotinotoxins might also provide a defensive barrier for the skin against the detrimental attachment and/or invasion of fouling or parasitic invertebrates (Lennox-Bulow et al., 2023a,b). While the dynamics of parasitic and fouling invertebrates are likely similar between reef and estuarine environments in a single location within the tropics (Lennox-Bulow et al., 2023a,b), there would likely be some variation between the tropical (i.e., Cairns), arid tropical (i.e., Exmouth) and subtropical (i.e., Caloundra) regions of Australia. We speculate that these differences may also exert some influence on the composition of ichthyocrotinotoxins from *S. horrida*. For instance, variations in the species assemblages or abundance of parasitic or fouling invertebrate taxa between locations may necessitate different components to provide a successful defence for the integument. Alternatively, *S. horrida* ichthyocrotinotoxin may already provide a highly effective defence against numerous invertebrate taxa, which could explain the high degree of conservation observed in *S. horrida* ichthyocrotinotoxin composition between locations.

In conclusion, this study supports to the growing body of evidence suggesting that stonefish ichthyocrotinotoxins are compositionally both complex and diverse concoctions. While many components within the ichthyocrotinotoxins of *S. horrida* were shared across all three Australian populations, unique components were observed. While little is currently known about the ecological function of stonefish ichthyocrotinotoxins, we speculate that the strongest forces acting on its composition are likely benthic flora and invertebrate communities. The task now is to conduct a study that aims to elucidate the role of stonefish ichthyocrotinotoxins in algal growth/recruitment and invertebrate defence to gain a better understanding of the underlying biological and environmental drivers of their compositional diversity.

CRedit authorship contribution statement

Danica Lennox-Bulow: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Robert Courtney:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Jamie Seymour:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Ethical statement for solid state ionics

Hereby, I Danica Lennox-Bulow consciously assure that for the manuscript “Geographic Variation in Ichthyocrotinotoxin from the Estuarine Stonefish (*Synanceia horrida*)” the following is fulfilled.

- 1) This material is the authors’ own original work, which has not been previously published elsewhere.
- 2) The paper is not currently being considered for publication elsewhere.
- 3) The paper reflects the authors’ own research and analysis in a truthful and complete manner.

- 4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
 - 5) The results are appropriately placed in the context of prior and existing research.
 - 6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.
 - 7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.
- The violation of the Ethical Statement rules may result in severe consequences.

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I agree with the above statements and declare that this submission follows the policies of Solid State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Chapter 7

The Palatability of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins to Potential Predators

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D Lennox-Bulow is the principal author of this manuscript and was extensively involved in all aspects of this work under the supervision of J Seymour. R Courtney assisted with animal husbandry, toxin collection, statistical analyses, and in reviewing earlier versions of this manuscript. J Zieth assisted with animal husbandry, data collection, statistical analyses, and in reviewing earlier versions of this manuscript.

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7.1 Abstract

Numerous fish species are known to utilise ichthyocrinotoxins to defend themselves against predators, often by deterring them from feeding. This study investigated whether stonefish ichthyocrinotoxins act as antifeedants by assessing its effect on the feeding behaviours of two potential predator species of stonefish: *Dendrochirus zebra* (Zebra Lionfish) and *Gymnothorax pseudothyrsoides* (Highfin Moray). Food laced with either *Synanceia horrida* (Estuarine Stonefish) or *Synanceia verrucosa* (Reef Stonefish) ichthyocrinotoxin were highly palatable (>85% food acceptance) to both predator species, demonstrating similar acceptance rates as untreated food (>95% food acceptance). In contrast, food coated in *Diploprion bifasciatum* (Barred Soapfish) ichthyocrinotoxin, a known anti-feedant used experimentally as a positive control, prompted high rejection rates (<10% food acceptance). These results suggest that stonefish ichthyocrinotoxins may not play a significant role in antifeedant defence. Stonefish ichthyocrinotoxins also exhibited a greater adhesion to prawn fillets (>45% retention), compared to soapfish ichthyocrinotoxin (27% retention), which may reflect differences in their ecological functions and/or application mechanisms. The findings of this study have expanded our understanding of stonefish toxin ecology.

7.2 Introduction

Stonefish are one of only a few known animals that produce both a venom and a secreted poison (ichthyocrinotoxin). Stonefish venom, which is contained within twin glands associated with each of their 13 dorsal spines, is used exclusively for defence against predators (Endean, 1961). Due to its deleterious nature to people, stonefish venom is also considered a medically significant toxin (Saggiomo *et al.*, 2021). Consequently, most of the research on stonefish toxins is focused on the venom, particularly around improving outcomes in cases of envenomation (Saggiomo *et al.*, 2021). However, very little is known about the ichthyocrinotoxins that these animals secrete on to their skin.

Ichthyocrinotoxins from various fish species, including soapfish, flatfish, and boxfish, are widely evidenced to serve in predator defence, and are often rapidly dispensed when the user is provoked (see review by Lennox-Bulow *et al.*, 2023a). The behavioural responses of predators to the toxin reportedly include avoiding, rejecting or regurgitating the ichthyocrinotoxic fish, jerking away, swimming erratically, mouth gaping, and increased opercular movements (Clark, 1983; Randall *et al.*, 1971). In early studies where brevity-stricken researchers physically tasted ichthyocrinotoxic secretions, it was noted that many, including ichthyocrinotoxins secreted by stonefish, harbour a distinctive bitter taste (Cameron & Endean, 1973; Maretzki & Del Castillo, 1967). As such, it was hypothesised that ichthyocrinotoxins may deter predation by reducing the palatability of the user, thus functioning as an antifeedant.

Later studies determined that the active components within the ichthyocrinotoxins of numerous fish species, such as grammistin from soapfish, pardaxin from flatfish, and pahutoxin from boxfish, are amphipathic surfactants (Shiomi *et al.*, 2000; Primor *et al.*, 1978). Furthermore, the gill membranes of predatory species, such as *Squalus acanthias* (Dogfish Shark), reportedly exhibited increased permeability to solutes following exposure to ichthyocrinotoxin from *Pardachirus marmoratus* (Red Sea Moses Sole) (Primor *et al.*, 1984; Primor *et al.*, 1980). As such, it was hypothesised that ichthyocrinotoxins may also serve a defensive role by disrupting the physiological processes of their target. Specifically, the surfactant components likely function by forming pores in gill membranes and incapacitating the osmoregulatory capabilities of the target (Shiomi *et al.*, 2000; Primor *et al.*, 1980).

Despite limited research, stonefish ichthyocrinotoxins present an intriguing case. Unlike ichthyocrinotoxins secreted by other fish taxa, stonefish ichthyocrinotoxins reportedly exhibit limited toxicity to fish (i.e., blennies and mosquito fish) and vertebrate cell lines (i.e., red blood

cell erythrocytes) (Cameron *et al.*, 1981). Conversely, it immobilised ciliate protozoans and mussel gill cilia, as well as increased the tone of barnacle scutum rostralis muscle (Cameron *et al.*, 1981). This suggests that stonefish ichthyocrinotoxins likely serve a different function than disrupting the physiology of vertebrate predators. However, whether they perform an anti-feedant function is currently unknown. Therefore, the aim of this study was to determine whether stonefish ichthyocrinotoxins play a role in predator defence by reducing the animal's palatability to predators. The natural predators of stonefish reportedly include sharks, stingrays, and eels (Harris *et al.*, 2021), but also likely encompass octopus, sea snakes, and opportunistic predatory fish (i.e., lionfish). This study quantified the effect of ichthyocrinotoxins from *Synanceia horrida* (Estuarine Stonefish) and *Synanceia verrucosa* (Reef Stonefish) on the feeding behaviours of two potential predator species of stonefish, *Dendrochirus zebra* (Zebra Lionfish) and *Gymnothorax pseudothyrsoides* (Highfin Moray).

7.3 Materials and Methods

7.3.1 Animal Husbandry

Thirty specimens, comprising five *S. verrucosa*, five *S. horrida*, and five *Diploprion bifasciatum* (Barred Soapfish), as well as three *D. zebra*, and twelve *G. pseudothyrsoides* were collected from the wild by local commercial fish suppliers, Cairns Marine and Monsoon Aquatics, within the Cairns region (Queensland, Australia) under James Cook University Animal Ethics Committee approval (# A2848). The number of predators used in the study depended on the availability of the animals in the local area at the time of the study. All species used in this study will now be referred to by their common names to improve readability.

Animals were relocated to the eduQuarium at James Cook University (JCU) Ngumbada campus in Cairns, Queensland. All specimen aquariums were connected to a 65,000 L recirculating salt-water system maintained with the following water quality parameters: pH 8.3 ± 0.1 ; salinity $35\text{‰} \pm 0.5\text{‰}$; temperature $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$; with nitrate and nitrite levels between ~ 0 to 0.1 mg/L ; and dissolved oxygen above 90% saturation. Stonefish were housed in small groups (three animals) in 350 L tanks with an exchange rate of 500 L per hour. Soapfish were housed individually in 130 L tanks with an exchange rate of 220 L/hour. Lionfish were housed in groups of three in 150 L tanks with an exchange rate of 500 L/hour. Morays were housed individually in 200 L tanks with an exchange rate of 600 L/hour. The photoperiod within each tank was maintained as a 12:12 day:night cycle, turning on at 06:00 and off at 18:00. Animal welfare was monitored daily, and tank conditions were regularly maintained by aquarium staff. Stonefish and soapfish were acclimated to the aquarium conditions for a minimum of two weeks before undergoing toxin extraction. Lionfish and Morays were acclimated to the aquarium conditions for one month prior to commencing the experiment.

7.3.2 Toxin Extraction

Extracting Stonefish Ichthyocrinotoxin.

Stonefish ichthyocrinotoxins were collected from three to five mature individuals under James Cook University Animal Ethics Committee approval (approval # A2848). The sex of each individual was unable to be determined. The extraction of stonefish ichthyocrinotoxins followed the protocol outlined by Lennox-Bulow *et al.* (2023b). In brief, an individual stonefish was transferred to a saltwater-dampened surface and secured by gently holding either side of the pectoral fins. A saltwater-dampened cotton cloth was placed over the animal's eyes to

minimise stress throughout the process. Ichthyocrinotoxin was then extracted from the skin tubercles using targeted mechanical massage under a controlled vacuum. This vacuum was generated by a purpose-built extraction apparatus comprised of a 100 mm segment of clear vinyl tubing affixed to a 50 mL syringe. To account for potential individual variation in toxin composition and activity, stonefish ichthyocrinotoxin was extracted and pooled from three to five mature individuals of each species. Pooled ichthyocrinotoxin samples were stored in 1.5 mL Eppendorf tubes at -80°C until required.

Extracting Soapfish Ichthyocrinotoxin.

Soapfish ichthyocrinotoxins were collected from two to three mature individuals under James Cook University Animal Ethics Committee approval (approval # A2848). The sex of each individual was unable to be determined. To induce toxin release, each Barred Soapfish was removed from its holding tank and gently massaged unidirectionally along its lateral line from head to caudal fin for a maximum of 30 seconds. The expelled ichthyocrinotoxin was drip-collected in a clean plastic weigh boat (150 × 150 mm) that was placed underneath the animal. To account for potential individual variation in toxin composition and activity, Barred Soapfish ichthyocrinotoxin was extracted and pooled from two to three individuals. Pooled ichthyocrinotoxin samples were stored in 1.5 mL Eppendorf tubes at -80°C until required.

7.3.3 *Monitoring the Feeding Behaviours of Predators*

The palatability of stonefish ichthyocrinotoxins to potential predators was assessed by monitoring the feeding behaviours of Lionfish and Morays following exposure to toxin-laced food. Predators ($n = 3$ Lionfish, and $n = 12$ Morays) were acclimated to a regular feeding regime (daily for Lionfish, and every three days for Morays) of deshelled prawn fillets, which constituted a normal diet, for one month to establish baseline feeding behaviours. Following acclimation, predators were presented with four food types: 1) untreated prawn fillet (i.e., no toxin, used as an indicator for high palatability); 2) prawn fillet laced with Barred Soapfish ichthyocrinotoxin (i.e., a known defensive toxin, used as an indicator for low palatability); 3) prawn fillet laced with Estuarine Stonefish ichthyocrinotoxin; and 4) prawn fillet laced with Reef Stonefish ichthyocrinotoxin. Each food type was offered once per feeding event. Toxin-lacing the food involved patting the prawn fillets dry with a paper towel, then soaking them in the crudest obtainable samples of ichthyocrinotoxin for five minutes. The soaking was carried out in a Ziploc bag containing enough crude ichthyocrinotoxin to completely submerge the prawn fillet. During feeding events, appropriately sized prawn pieces ($5 \times 5 \text{ mm} \pm 2 \text{ mm}$ for

Lionfish, and ~10 mm length pieces $\pm$ 5 mm for Morays) were skewered onto a 60 cm long $\times$ 3 mm diameter dowel rod (i.e., feeding stick) and presented to a predator for 30 seconds.

Predators that completely consumed the prawn fillet within 30 seconds were recorded as 'ate'. Predators that did not attempt to consume the food (i.e., full food refusal), or expelled the food after tasting (i.e., partial food refusal) within 30 seconds were recorded as 'did not eat'. Following toxin-exposure, predators were returned to their regular diet (i.e., untreated prawn fillet) for a least two feedings, or until feeding behaviours returned to baseline. Additionally, predators were monitored closely for 24 hours after each toxin-laced feeding event to ensure there were no adverse physical and/or behavioural effects. This process was repeated until each predator had been offered each food type at least three times.

The proportion of feeding attempts where each individual predator ate the food offering was calculated across all feeding attempts for each food treatment. These proportions were arcsine square root transformed to normalise the data prior to analysis. A two-way Analysis of Variance (ANOVA) was conducted to determine the effect of food treatment (no toxin, or food laced with ichthyocriotoxin from Estuarine Stonefish, Reef Stonefish, and Barred Soapfish) and predator species (Zebra Lionfish, Highfin Moray) on food acceptance rates. Following a significant ANOVA result, a post-hoc least significant difference (LSD) test was used to identify specific food treatments that significantly influenced food acceptance rates. Statistical analyses were performed in IBM SPSS version 27, and figures were generated using Graph Pad Prism version 10.4.0.

7.3.4 Reversed Phase-High Performance Liquid Chromatography Analysis of Ichthyocriotoxin Adhesion to Prawn

To ensure that the predators would be exposed to ichthyocriotoxin during the feeding experiment, toxin adhesion to deshelled prawn fillet following a simulated feeding event was quantified using Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). In brief, deshelled prawn fillets (~5 $\times$ 5 mm) were pat dry with a piece of paper towel and then immersed in the crudest obtainable samples of Estuarine Stonefish, Reef Stonefish, or Barred Soapfish ichthyocriotoxin for five minutes ($n = 1$ prawn fillet per toxin). This was performed in a Ziploc bag containing enough crude ichthyocriotoxin to completely submerge each prawn fillet. To simulate a feeding event (i.e., treatment conditions), each toxin-laced prawn fillet was submerged in an animal-free 250 L saltwater aquarium tank for 30 seconds. After treatment,

fillets were placed in a 1:1 buffer mixture of solvent A (250 μ L H₂O/0.05% trifluoroacetic acid (TFA; Auspep): solvent B (250 μ L 90% ACN (Merck)/10% H₂O/0.045% TFA). An additional prawn fillet was soaked in seawater (i.e., prawn-only control) which was later used for prawn contaminant identification. Furthermore, three ‘native toxin’ controls (i.e., 10 μ L aliquots of each ichthyocrinotoxin in 500 μ L 1:1 A:B buffer solution) were prepared. All samples were then refrigerated (4°C) overnight. The following day, prawn pieces were removed, and samples were centrifuged at 4°C, 15,000 rpm for 10 min to remove debris. The resulting supernatants were then transferred to new 1.5 mL Eppendorf tubes. Sample supernatants (10 μ L) were analysed using a C₁₈ Phenomenex Aeris® PEPTIDE XB liquid chromatography column (250 x 10 mm, 5 μ m, 100 Å). The separation used a gradient of 0 – 60% solvent B in 60 min, 60 – 90% solvent B in 5 min, 90% solvent B for 10 min, and 90 – 0% solvent B in 5 min. The flow rate remained constant at 0.250 mL/min. The absorbance at 214 nm was continuously monitored over 80 mL of the eluant.

Raw absorbance data (collected by OpenLAB CDS ChemStation v1.8.1) was processed to remove prawn component contaminants from the toxin-laced treatment conditions groups. This was achieved by subtracting the absorbance values from the ‘prawn-only control’ at each elution volume from the corresponding values in the ‘treatment conditions’ data. Chromatograms were generated in GraphPad Prism 10. To quantify the retention of ichthyocrinotoxin under the treatment conditions against the native toxin, the area under the curve was calculated for each species. The area under the curve was quantified for each ‘treatment conditions’ and ‘native toxin’ trace by multiplying the difference between each consecutive x-value by the average of the respective y-values and summing the products. The percentage of toxin retained following exposure to the treatment conditions was calculated by dividing the total area of the ‘treatment conditions’ trace for each species by the total area of the corresponding ‘native toxin’ trace and multiplying this by 100.

7.4 Results

7.4.1 *Toxin Present on Prawn Fillet Following a Simulated Feeding Event*

RP-HPLC revealed a high degree of similarity between the absorbance profiles of native toxin (i.e., ichthyocrinotoxin extracted directly from the animal) and treatment conditions (i.e., ichthyocrinotoxin coated on prawn fillets and submerged in an aquarium for 30 seconds) for Barred Soapfish, Estuarine Stonefish, and Reef Stonefish ichthyocrinotoxin (**Figure 7.1**). For Barred Soapfish, the treatment conditions profile showed good consistency with the native toxin for peaks eluting around 1 minute, 45 minutes, and between 55 – 60 minutes retention time (**Figure 7.1A**). However, lower absorbances were observed in the treatment conditions profile for peaks between 40 – 44 minutes, 46 – 54 minutes, and 61 – 70 minutes, potentially suggesting a weaker adherence of these components onto the prawn fillet. Similarly, both Estuarine Stonefish (**Figure 7.1B**) and Reef Stonefish (**Figure 7.1C**), treatment conditions profiles displayed a high fidelity to the native toxin up to approximately 50 minutes retention time. After 50 minutes, peaks present in the native toxin were absent in the treatment conditions profile of Reef Stonefish, and less consistent in the treatment conditions profile for Estuarine Stonefish, indicating that highly hydrophobic components may have a weaker adherence to the prawn fillet. Furthermore, area under the curve calculations revealed that ichthyocrinotoxins from Estuarine Stonefish (65.5% adhesion) and Reef Stonefish (45% adhesion) exhibited a greater adhesion to prawn fillet following exposure to treatment conditions than Barred Soapfish ichthyocrinotoxin (27% adhesion). However, the adhesion of Reef Stonefish ichthyocrinotoxin on prawn fillet following immersion was approximately 20% less than Estuarine Stonefish ichthyocrinotoxin.

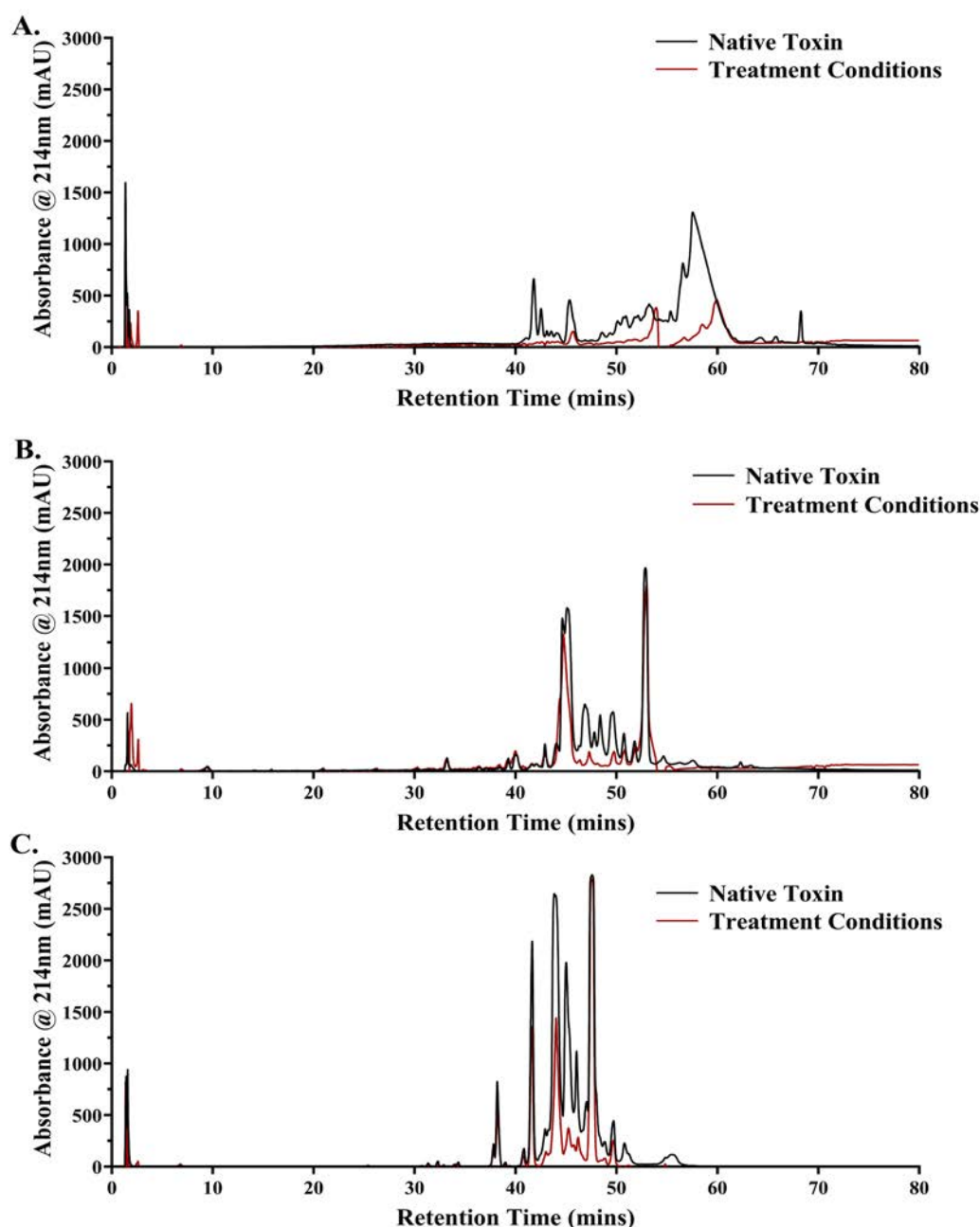


Figure 7.1 The Adhesion of Ichthyocrinotoxins on Prawn Fillet Following a Simulated Feeding Event. RP-HPLC was used to profile ichthyocrinotoxin samples from Barred Soapfish (*Diploprion bifasciatum*, **Panel A**), Estuarine Stonefish (*Synanceia horrida*, **Panel B**), and Reef Stonefish (*Synanceia verrucosa*, **Panel C**). For each species toxin, the absorbance profiles of two sample types were compared: 1) native toxin (—) which was unprocessed ichthyocrinotoxin extracted directly from the animal; and 2) treatment conditions (—) which was ichthyocrinotoxin that had been coated on a prawn fillet and submerged in an aquarium for 30 seconds (i.e., a simulated feeding event). Peaks corresponding to prawn components were removed from the treatment conditions profiles for better visualisation of ichthyocrinotoxin components. Absorbance was monitored at 214 nm.

7.4.2 The Effect of Ichthyocrinotoxins on the Feeding Behaviour of Predators

There was no significant interaction between food treatment (no-toxin, or food laced with ichthyocrinotoxin from Stonefish or Soapfish) and predator species (Highfin Moray, and Zebra Lionfish) on food acceptance rates (**Figure 7.2**, $F_{3, 52} = 1.08$, $p = 0.36$). There was also no significant difference in food acceptance rates between the two predator species ($F_{1, 52} = 1.99$, $p = 0.16$). However, food treatment was found to have a significant effect on the rate of food acceptance ($F_{3, 52} = 65.86$, $p < 0.001$). Both predator species exhibited a strong aversion to food laced with Soapfish ichthyocrinotoxins (less than 10% acceptance). In contrast, predators readily accepted food laced with ichthyocrinotoxins from Reef and Estuarine Stonefish (over 85% acceptance), which was comparable to the no-toxin control group (over 95% acceptance).

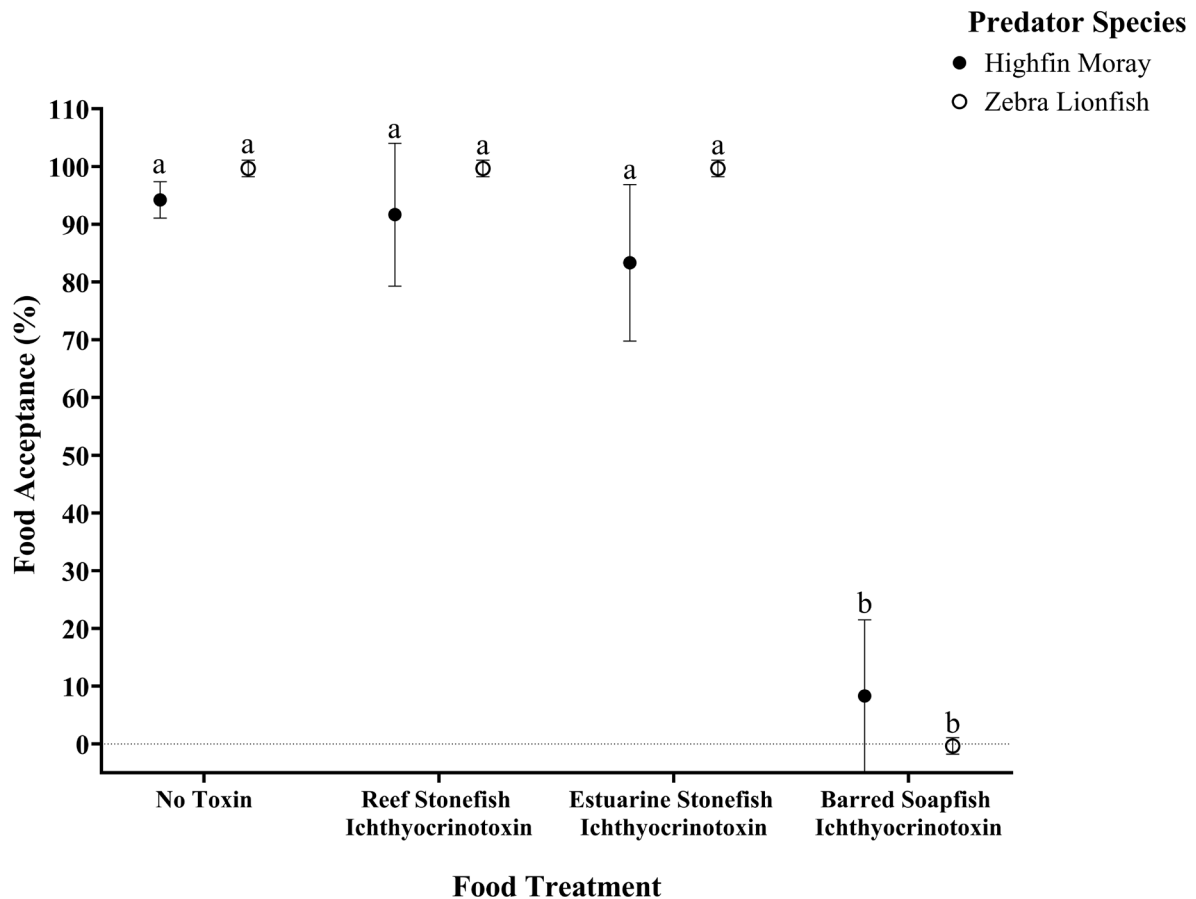


Figure 7.2 The Palatability of Ichthyocrinotoxins to Predators. Highfin Moray (*Gymnothorax pseudothyrsoideus*, ●, $n = 12$) and Zebra Lionfish (*Dendrochirus zebra*, ○, $n = 3$) were offered untreated prawn fillet (i.e., no toxin), or prawn fillet laced with ichthyocrinotoxin from Reef Stonefish (*Synanceia verrucosa*), Estuarine Stonefish (*Synanceia horrida*), *Synanceia verrucosa*, or Barred Soapfish (*Diploprion bifasciatum*). Food acceptance (%) was determined based on proportion of feeding events where predators consumed the food offering within 30 seconds. Letters (a – b) represent statistically significant post-hoc LSD groupings. Error bars represent 95% CI.

7.5 Discussion

Previous studies have shown that ichthyocrinotoxin from *S. horrida* exhibited minimal ichthyotoxic activity against blennies, *Petroscirtes faponicus* (synonym for *Omobranchus punctatus*) and mosquito fish (*Gambusia affinis*) (Cameron *et al.*, 1981). This led to the suggestion that stonefish ichthyocrinotoxins are unlikely to function as a predator deterrent or repellent, unless their bitter taste renders them unpalatable (Cameron *et al.*, 1981). The present study found that the feeding behaviours of Lionfish (*D. zebra*) and Morays (*G. pseudothyrsoideus*) were unchanged by ichthyocrinotoxin from either Reef (*S. verrucosa*) or Estuarine Stonefish (*S. horrida*). Ichthyocrinotoxins from both *S. horrida* and *S. verrucosa* were highly palatable (>85% food acceptance) to both predator species, comparable to untreated food (>94% food acceptance). Additionally, no adverse effects on the predators were observed following exposure to stonefish ichthyocrinotoxins, indicating that they may be non-toxic to these predator species. In contrast, food coated in Barred Soapfish (*D. bifasciatum*) ichthyocrinotoxin, a known anti-feedant, prompted high rejection rates (>90% rejection) from both predators. These findings suggest that stonefish ichthyocrinotoxins do not function as an anti-feedant against these predator species. Moreover, the findings support the hypothesis that stonefish ichthyocrinotoxins likely do not play a major role in predator defence, acting as neither a defensive toxin nor an anti-feedant.

While the primary function of ichthyocrinotoxins secreted by other well-studied fish taxa (i.e., soapfish, boxfish, and flatfish) is predator defence (Clark, 1983; Thomson, 1969; Maretzki & Del Castillo, 1967), stonefish ichthyocrinotoxins appear to be an anomaly. Furthermore, although ichthyocrinotoxins typically exhibit proteolytic and/or haemolytic activity towards vertebrates (Indumathi & Khora, 2013; Abdul-Haqq & Shier, 2008; Maretzki & Del Castillo, 1967), stonefish ichthyocrinotoxins were found to lack proteolytic action, and possess only mild haemolytic properties (Cameron *et al.*, 1981). Another markable distinction between stonefish and other ichthyocrinotoxin-producing taxa is in the equipment of an injectable toxin (i.e., venom). In contrast to their ichthyocrinotoxin, stonefish venom is highly noxious to vertebrates, and undeniably serves in predator defence (Saggiomo *et al.*, 2021; Endean, 1961). Some evolutionary theories suggest that fish venoms may have evolved from the ability to produce a toxic glandular secretion, such as ichthyocrinotoxin (Cameron & Endean, 1973). The rationale being that it is likely a more effective defensive strategy to inject toxin directly into a threat, rather than secrete one into an environment of infinite dilution

(Cameron & Endean, 1973). This might suggest that the evolutionary trajectory of stonefish ichthyocrinotoxins has diverged from that of other fish species, leading to a unique function, and, though unlikely, a vestigial role in predator defence.

Although the results of this study suggest that it is unlikely that stonefish ichthyocrinotoxins serve in predator defence, they may still serve a defensive function. Stonefish are devoid of scales and readily bury themselves into the substratum (Endean, 1961). Consequently, their skin is presumably highly susceptible to a variety of benthic fauna, particularly invertebrate taxa like parasites and fouling animals. Previous studies showed that ichthyocrinotoxin from *S. horrida* immobilised ciliated protozoans and mussel gill cilia, as well as increased the tone of barnacle scutum rostralis muscle (i.e., caused muscle contraction) (Cameron *et al.*, 1981). Additionally, very few internal parasite taxa have currently been identified within members of *Synanceia* (Miller *et al.*, 2018). This suggests that one potential function of stonefish ichthyocrinotoxins may be to provide a defensive barrier against skin incursions from invertebrate taxa. Preliminary investigations suggest that another potential function of stonefish ichthyocrinotoxins may be to facilitate the growth and/or attachment of algae on to their skin (Barnett, 2014). Algal recruitment onto the skin may serve to enhance the animal's camouflage, thereby reducing its likelihood of being discovered by predators and prey, while also potentially luring herbivorous prey into their ambush range. However, further research is required to determine whether stonefish ichthyocrinotoxins play a role in integumentary defence and/or aid in algal recruitment or growth.

Stonefish ichthyocrinotoxins also exhibited a greater adhesion to prawn fillets (65% retention for *S. horrida* and 45% retention for *S. verrucosa*), compared to soapfish ichthyocrinotoxin (27% retention). This differential adhesion may be further indicative of the distinct biological roles of these toxins. Soapfish are known to employ a rapid-release strategy, secreting copious amounts of their ichthyocrinotoxin when threatened (Randall *et al.*, 1971). Previous studies report that the active component within Soapfish (family Grammistidae) ichthyocrinotoxins, called grammistin, is a surfactant peptide (Onuki *et al.*, 1993; Oshima, 1974). Biological surfactants facilitate the mixing of different substances by reducing their interfacial tension (Otzen, 2017). Therefore, the weaker adhesion of Barred Soapfish ichthyocrinotoxin may be attributed to the surfactant properties of one or more of its components. These properties likely support the function of Barred Soapfish ichthyocrinotoxin in predator defence by facilitating its rapid dispersal in seawater. In contrast, stonefish do not

secrete large quantities of ichthyocrinotoxin when threatened. This might suggest that stonefish ichthyocrinotoxins provide localised protection, making it advantageous to secrete a toxin with greater adhesive properties.

Interestingly, the adhesive strength of stonefish ichthyocrinotoxins to prawn fillet also appeared to differ between species. Ichthyocrinotoxins from Estuarine Stonefish seemingly exhibited a greater adhesion to prawn fillet (65% retention) compared to those from Reef Stonefish (45% retention). Recent work elucidated that the composition of stonefish ichthyocrinotoxin varies between these two species (Lennox-Bulow *et al.*, 2023b). As such, it is possible that the different adhesive properties of stonefish ichthyocrinotoxins between species may be due to variations in their chemical composition. However, further research is needed to support these observations, as well as determine whether interspecific differences in the physical properties of stonefish ichthyocrinotoxins might reflect functional and/or mechanistic adaptations.

In conclusion, the findings of this study suggest that stonefish ichthyocrinotoxins likely do not play a major role in predator defence. Furthermore, differences in the adhesive properties of ichthyocrinotoxins between stonefish and soapfish, as well as among stonefish species, potentially reflect distinct ecological functions or application mechanisms. Future research should focus on the potential defensive role of these toxins against invertebrate taxa that are likely to interact with the animal's skin, such as parasites and fouling organisms. The findings of this study have expanded our understanding of the toxin ecology of stonefish and highlight potential avenues for future research that may lead to the discovery of novel therapeutics or industrial solutions.

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7.7 APPENDIX F

The Palatability of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins to Potential Predators

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The palatability of stonefish (*Synanceia* spp.) ichthyocrinotoxins to potential predators

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ABSTRACT

Numerous fish species are known to utilise ichthyocrinotoxins to defend themselves against predators, often by deterring the predator from feeding on them. This study investigated whether stonefish ichthyocrinotoxins act as antifeedants by assessing its effect on the feeding behaviours of two potential predators of stonefish: lionfish and morays. Food laced with either *Synanceia horrida* (Estuarine Stonefish) or *Synanceia verrucosa* (Reef Stonefish) ichthyocrinotoxin were highly palatable (>85% food acceptance) to both predator types, demonstrating similar acceptance rates as untreated food (>95% food acceptance). In contrast, food coated in *Diploprion bifasciatum* (Barred Soapfish) ichthyocrinotoxin, a known antifeedant used experimentally as a positive control, prompted high rejection rates (<10% food acceptance). These results suggest that stonefish ichthyocrinotoxins may not play a significant role in antifeedant defence. Stonefish ichthyocrinotoxins also exhibited a greater adhesion to prawn fillets (>45% retention), compared to soapfish ichthyocrinotoxin (27% retention), which may reflect differences in their ecological functions and/or application mechanisms. The findings of this study have expanded our understanding of stonefish toxin ecology.

Stonefish are one of only a few known animals that produce both a venom and a secreted poison (ichthyocrinotoxin). Stonefish venom, which is contained within twin glands associated with each of their 13 dorsal spines, is used exclusively for defence against predators (Endean 1961). Due to its deleterious nature to people, stonefish venom is also considered a medically significant toxin (Saggiomo et al. 2021). Consequently, most of the research on stonefish toxins is focused on the venom, particularly around improving outcomes in cases of envenomation (Saggiomo et al. 2021). However, very little is known about the ichthyocrinotoxins that these animals secrete onto their skin.

Ichthyocrinotoxins from various fish species, including soapfish, flatfish and boxfish, are widely evidenced to serve in predator defence, and are often rapidly dispensed when the user is provoked (see review by Lennox-Bulow et al. 2023a). The behavioural responses of predators to the toxin reportedly include avoiding, regurgitating or rejecting the ichthyocrinotoxic fish, jerking away, swimming erratically, mouth gaping, and increased opercular movements (Clark 1983, Randall et al. 1971). In early studies where brevity-stricken researchers physically tasted ichthyocrinotoxic secretions, it was noted that many, including ichthyocrinotoxins secreted by stonefish, harbour a distinctive bitter taste (Cameron & Endean 1973, Maretzki & Del Castillo 1967). As such, it was hypothesised that ichthyocrinotoxins may deter predation by reducing the palatability of the user, thus functioning as an antifeedant.

Later studies determined that the active components within the ichthyocrinotoxins of numerous fish species, such as grammistin from soapfish, pardaxin from flatfish, and pahutoxin from boxfish, are amphipathic surfactants (Primor et al. 1978, Shiomi et al. 2000). Furthermore, the gill membranes of predatory species, such as *Squalus acanthias* (Dogfish Shark), reportedly exhibited increased permeability to solutes following exposure to ichthyocrinotoxin from *Pardachirus marmoratus* (Red Sea Moses Sole) (Primor et al. 1980, Primor et al. 1984). As such, it was hypothesised that ichthyocrinotoxins may also serve a defensive role by disrupting the physiological processes of their target. Specifically, the surfactant components likely

function by forming pores in gill membranes and incapacitating the osmoregulatory capabilities of the target (Primor et al. 1980, Shiomi et al. 2000).

Despite limited research, stonefish ichthyocrinotoxins present an intriguing case. Unlike ichthyocrinotoxins secreted by other fish taxa, stonefish ichthyocrinotoxins reportedly exhibit limited toxicity to fish (i.e. blennies and mosquito fish) and vertebrate cell lines (i.e. red blood cell erythrocytes) (Cameron et al. 1981). Conversely, it immobilised ciliated protozoans and mussel gill cilia, as well as increased the tone of barnacle scutorum rostralis muscle (Cameron et al. 1981). This suggests that stonefish ichthyocrinotoxins likely serve a different function than disrupting the physiology of vertebrate predators. However, whether they perform an antifeedant function is currently unknown. Therefore, the aim of this study was to determine whether stonefish ichthyocrinotoxins play a role in predator defence by reducing the animal's palatability to predators. Although limited data exists on the natural predators of stonefish across all life stages, potential predators reportedly include sharks, stingrays and eels (Harris et al. 2021), and likely also encompass octopus, sea snakes, morays and opportunistic predatory fish (such as lionfish). This study quantified the effect of ichthyocrinotoxins from *Synanceia horrida* (Estuarine Stonefish) and *Synanceia verrucosa* (Reef Stonefish) on the feeding behaviours of two potential predators of stonefish: lionfish and morays.

MATERIALS AND METHODS

Animal husbandry

Thirty specimens, comprising five *S. verrucosa*, five *S. horrida*, and five *Diploprion bifasciatum* (Barred Soapfish), as well as three *Dendrochirus zebra* (Zebra Lionfish) and twelve *Gymnothorax pseudothyrsoides* (Highfin Moray) were collected from the wild by local commercial fish suppliers — Cairns Marine and Monsoon Aquatics — within the Cairns region (Queensland, Australia) under James Cook University Animal Ethics Committee approval (#A2848). While limited data on the natural predators of stonefish are currently available, a documented incidence revealed predation by morays (National Geographic 2018). Lionfish are

also well known to predate on a wide range of fish species (Batjakas et al. 2023, Eddy et al. 2016, Morris & Akins 2009). In addition, the predators *D. zebra* and *G. pseudothyroideus* were selected for this study due to their generalist, opportunistic and voracious feeding behaviours, as well as their overlapping distribution with both stonefish species. All species used in this study will now be referred to by their common names to improve readability.

Animals were relocated to the eduQuarium at James Cook University (JCU) Nguma-bada campus in Cairns, Queensland. All specimen aquariums were connected to a 65,000 L recirculating saltwater system maintained with the following water quality parameters: pH 8.3 ± 0.1 ; salinity $35\text{‰} \pm 0.5\text{‰}$; temperature $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$; with nitrate and nitrite levels between ~ 0 to 0.1 mg/L ; and dissolved oxygen above 90% saturation. Stonefish were housed in small groups (three animals) in 350 L tanks with an exchange rate of 500 L/hour. Soapfish were housed individually in 130 L tanks with an exchange rate of 220 L/hour. Lionfish were housed in groups of three in 150 L tanks with an exchange rate of 500 L/hour. Morays were housed individually in 200 L tanks with an exchange rate of 600 L/hour. The photoperiod within each tank was maintained as a 12:12 day:night cycle, turning on at 06:00 and off at 18:00. Animal welfare was monitored daily and tank conditions were regularly maintained by aquarium staff. Stonefish and soapfish were acclimated to the aquarium conditions for a minimum of two weeks before undergoing toxin extraction. Lionfish and morays were acclimated to the aquarium conditions for one month prior to commencing the experiment.

Toxin extraction

Extracting stonefish ichthyocrinotoxin

Stonefish ichthyocrinotoxins were collected from three to five mature individuals under James Cook University Animal Ethics Committee approval (approval #A2848). The sex of each individual was unable to be determined. The extraction of stonefish ichthyocrinotoxins followed the protocol outlined by Lennox-Bulow et al. (2023b). In brief, an individual stonefish was transferred to a saltwater-dampened surface and secured by gently holding either side of the pectoral fins. A saltwater-dampened cotton cloth was placed over the animal's eyes to minimise

stress throughout the process. Ichthyocrinotoxin was then extracted from the skin tubercles using targeted mechanical massage under a controlled vacuum. This vacuum was generated by a purpose-built extraction apparatus comprised of a 100 mm segment of clear vinyl tubing affixed to a 50 mL syringe. To account for potential individual variation in toxin composition and activity, stonefish ichthyocrinotoxin was extracted and pooled from three to five mature individuals of each species. Pooled ichthyocrinotoxin samples were stored in 1.5 mL Eppendorf tubes at -80°C until required.

Extracting soapfish ichthyocrinotoxin

Soapfish ichthyocrinotoxins were collected from two to three mature individuals under James Cook University Animal Ethics Committee approval (approval #A2848). The sex of each individual was unable to be determined. To induce toxin release, each soapfish was removed from its holding tank and gently massaged unidirectionally along its lateral line from head to caudal fin for a maximum of 30 seconds. The expelled ichthyocrinotoxin was drip-collected in a clean plastic weigh boat (150 x 150 mm) that was placed underneath the animal. To account for potential individual variation in toxin composition and activity, soapfish ichthyocrinotoxin was extracted and pooled from two to three individuals. Pooled ichthyocrinotoxin samples were stored in 1.5 mL Eppendorf tubes at -80°C until required.

Reversed Phase-High Performance Liquid Chromatography Analysis of ichthyocrinotoxin adhesion to prawn

To ensure that the predators would be exposed to ichthyocrinotoxin during the feeding experiment, toxin adhesion to deshelled prawn fillet following a simulated feeding event was quantified using Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). In brief, deshelled prawn fillets ($\sim 5 \times 5 \text{ mm}$) were pat dry with a piece of paper towel and then immersed in the crudest obtainable samples of Estuarine Stonefish, Reef Stonefish, or Barred Soapfish ichthyocrinotoxin for five minutes ($n = 1$ prawn fillet per toxin). This was performed in a Ziploc bag containing enough crude ichthyocrinotoxin to completely submerge each prawn fillet. To simulate a feeding event

(treatment conditions), each toxin-laced prawn fillet was submerged in an animal-free 250 L saltwater aquarium tank for 30 seconds. After treatment, fillets were placed in a 1:1 buffer mixture of solvent A (250 μ L H₂O/0.05% trifluoroacetic acid (TFA; Auspep): solvent B (250 μ L 90% ACN (Merck)/10% H₂O/0.045% TFA). An additional prawn fillet was soaked in seawater (prawn-only control) which was later used for prawn contaminant identification. Furthermore, three 'native toxin' controls (10 μ L aliquots of each ichthyocrinotoxin in 500 μ L 1:1 A:B buffer solution) were prepared. All samples were then refrigerated (4°C) overnight. The following day, prawn pieces were removed, and samples were centrifuged at 4°C, 15,000 rpm for 10 min to remove debris. The resulting supernatants were then transferred to new 1.5 mL Eppendorf tubes. Sample supernatants (10 μ L) were analysed using a C18 Phenomenex Aeris® PEPTIDE XB liquid chromatography column (250 x 10 mm, 5 μ m, 100 Å). The separation used a gradient of 0–60% solvent B in 60 min, 60–90% solvent B in 5 min, 90% solvent B for 10 min, and 90–0% solvent B in 5 min. The flow rate remained constant at 0.250 mL/min. The absorbance at 214 nm was continuously monitored over 80 mL of the eluant.

Raw absorbance data (collected by OpenLAB CDS ChemStation v1.8.1) was processed to remove prawn component contaminants from the toxin-laced treatment conditions groups. This was achieved by subtracting the absorbance values from the 'prawn-only control' at each elution volume from the corresponding values in the 'treatment conditions' data. Chromatograms were generated in GraphPad Prism 10. To quantify the retention of ichthyocrinotoxin under the treatment conditions against the native toxin, the area under the curve was calculated for each species. The area under the curve was quantified for each 'treatment conditions' and 'native toxin' trace by multiplying the difference between each consecutive x-value by the average of the respective y-values and summing the products. The percentage of toxin retained following exposure to the treatment conditions was calculated by dividing the total area of the 'treatment conditions' trace for each species by the total area of the corresponding 'native toxin' trace and multiplying this by 100.

Monitoring the feeding behaviour of predators

The palatability of stonefish ichthyocrinotoxins to potential predators was assessed by monitoring the feeding behaviours of lionfish and morays following exposure to toxin-laced food. Predators ($n = 3$ lionfish and $n = 12$ morays) were acclimated to a regular feeding regime (daily for lionfish and every three days for morays) of deshelled prawn fillets, which constituted a normal diet, for one month to establish baseline feeding behaviours. Following acclimation, predators were presented with four food types: 1) untreated prawn fillet (no toxin, used as an indicator for high palatability); 2) prawn fillet laced with Barred Soapfish ichthyocrinotoxin (a known defensive toxin, used as an indicator for low palatability); 3) prawn fillet laced with Estuarine Stonefish ichthyocrinotoxin; and 4) prawn fillet laced with Reef Stonefish ichthyocrinotoxin. Each food type was offered once per feeding event. Toxin-lacing the food involved patting the prawn fillets dry with a paper towel, then soaking them in the crudest obtainable samples of ichthyocrinotoxin for five minutes. The soaking was carried out in a Ziploc bag containing enough crude ichthyocrinotoxin to completely submerge the prawn fillet. During feeding events, appropriately sized prawn pieces (5 x 5 mm $\pm$ 2 mm for lionfish and ~10 mm length pieces $\pm$ 5 mm for morays) were skewered onto a 60 cm long $\pm$ 3 mm diameter dowel rod (feeding stick) and presented to a predator for 30 seconds.

Predators that completely consumed the prawn fillet within 30 seconds were recorded as 'ate'. Predators that did not attempt to consume the food (full food refusal) or expelled the food after tasting (partial food refusal) within 30 seconds were recorded as 'did not eat'. Following toxin exposure, predators were returned to their regular diet (untreated prawn fillet) for at least two feedings, or until feeding behaviours returned to baseline. Additionally, predators were monitored closely for 24 hours after each toxin-laced feeding event to ensure there were no adverse physical and/or behavioural effects. This process was repeated until each predator had been offered each food type at least three times.

The proportion of feeding attempts where each individual predator ate the food offering was calculated across all feeding attempts for each food treatment. These proportions were arcsine square root transformed to normalise the data prior to analysis. A two-way Analysis of Variance (ANOVA) was conducted to determine the effect of food treatment (no toxin, or food laced with ichthyocrinotoxin from Estuarine Stonefish, Reef Stonefish and Barred Soapfish) and predator species (Zebra Lionfish, Highfin Moray) on food acceptance rates. Following a significant ANOVA result, a post-hoc least significant difference (LSD) test was used to identify specific food treatments that significantly influenced food acceptance rates. Statistical analyses were performed in IBM SPSS version 27, and figures were generated using Graph Pad Prism version 10.4.0.

RESULTS

Toxin present on prawn fillet following a simulated feeding event

RP-HPLC revealed a high degree of similarity between the absorbance profiles of native toxin (ichthyocrinotoxin extracted directly from the animal) and treatment conditions (ichthyocrinotoxin coated on prawn fillets and submerged in an aquarium for 30 seconds) for Barred Soapfish, Estuarine Stonefish, and Reef Stonefish ichthyocrinotoxin (Figure 1). For Barred Soapfish, the treatment conditions profile showed good consistency with the native toxin for peaks eluting around 1 minute, 45 minutes, and between 55–60 minutes retention time (Figure 1A). However, lower absorbances were observed in the treatment conditions profile for peaks between 40–44 minutes, 46–54 minutes, and 61–70 minutes, potentially suggesting a weaker adherence of these components onto the prawn fillet. Similarly, both Estuarine Stonefish (Figure 1B) and Reef Stonefish (Figure 1C) treatment conditions profiles displayed a high fidelity to the native toxin up to approximately 50 minutes retention time. After 50 minutes, peaks present in the native toxin were absent in the treatment conditions profile of Reef Stonefish, and less consistent in the treatment conditions profile for Estuarine Stonefish, indicating that highly hydrophobic components

may have a weaker adherence to the prawn fillet. Furthermore, area under the curve calculations revealed that ichthyocrinotoxins from Estuarine Stonefish (65.5% adhesion) and Reef Stonefish (45% adhesion) exhibited a greater adhesion to prawn fillet following exposure to treatment conditions than Barred Soapfish ichthyocrinotoxin (27% adhesion). However, the adhesion of Reef Stonefish ichthyocrinotoxin on prawn fillet following immersion was approximately 20% less than Estuarine Stonefish ichthyocrinotoxin.

The effect of ichthyocrinotoxin on the feeding behaviours of predators

There was no significant interaction between food treatment (no toxin, or food laced with ichthyocrinotoxin from stonefish or soapfish) and predator species (Highfin Moray and Zebra Lionfish) on food acceptance rates (Figure 2, $F_{3,52} = 1.08$, $p = 0.36$). There was also no significant difference in food acceptance rates between the two predator species ($F_{1,52} = 1.99$, $p = 0.16$). However, food treatment was found to have a significant effect on the rate of food acceptance ($F_{3,52} = 65.86$, $p < 0.001$). Both predator species exhibited a strong aversion to food laced with Barred Soapfish ichthyocrinotoxins (less than 10% acceptance). In contrast, predators readily accepted food laced with ichthyocrinotoxins from Reef and Estuarine Stonefish (over 85% acceptance), which was comparable to the no toxin control group (over 95% acceptance).

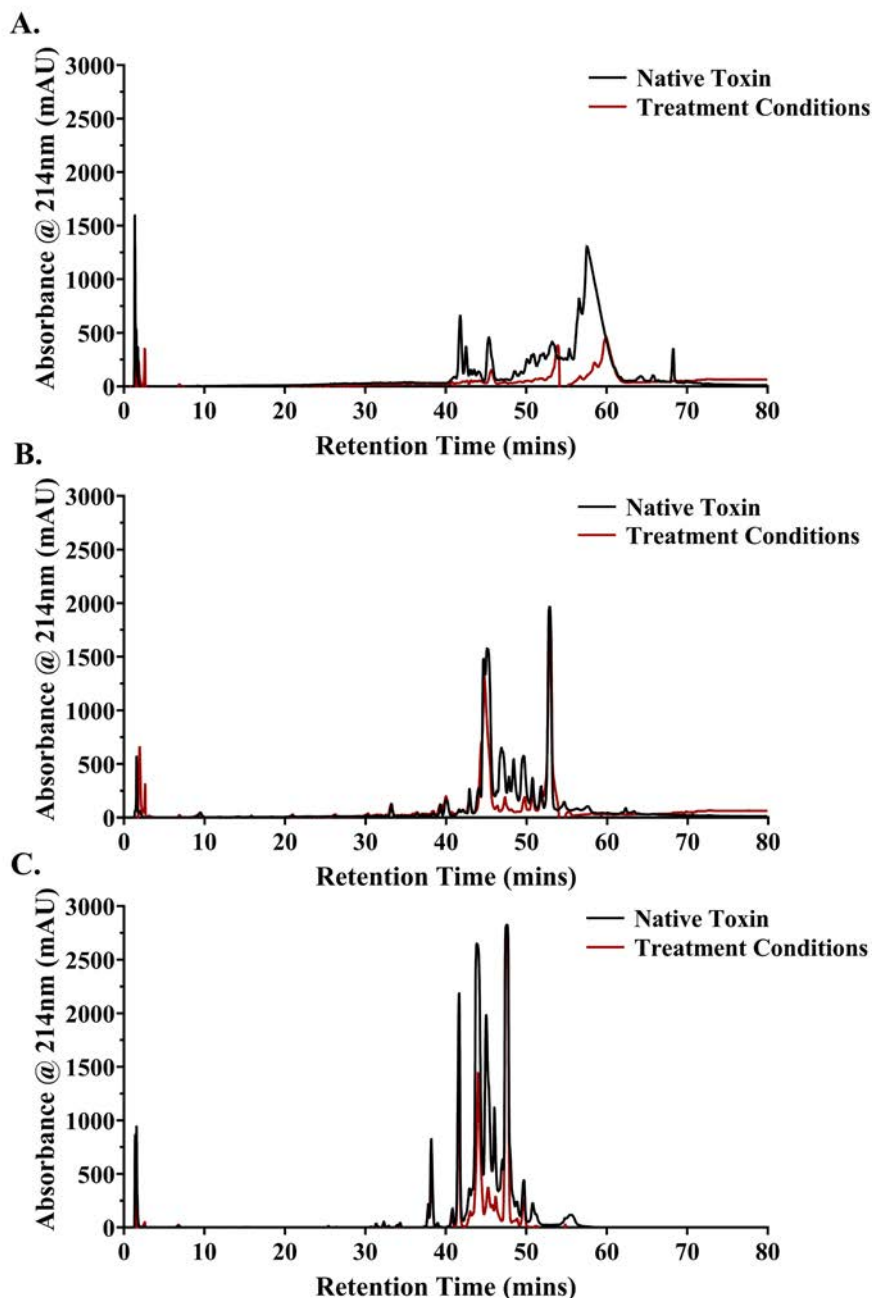


Figure 1. The adhesion of ichthyocrinotoxins on prawn fillet following a simulated feeding event. RP-HPLC was used to profile ichthyocrinotoxin samples from Barred Soapfish (*Diploprion bifasciatum*, Panel A), Estuarine Stonefish (*Synanceia horrida*, Panel B), and Reef Stonefish (*Synanceia verrucosa*, Panel C). For each species toxin, the absorbance profiles of two sample types were compared: 1) native toxin (—), unprocessed ichthyocrinotoxin extracted directly from the animal; and 2) treatment conditions (—), ichthyocrinotoxin that had been coated on a prawn fillet and submerged in an aquarium for 30 seconds (a simulated feeding event). Peaks corresponding to prawn components were removed from the treatment conditions profiles for better visualisation of ichthyocrinotoxin components. Absorbance was monitored at 214 nm.

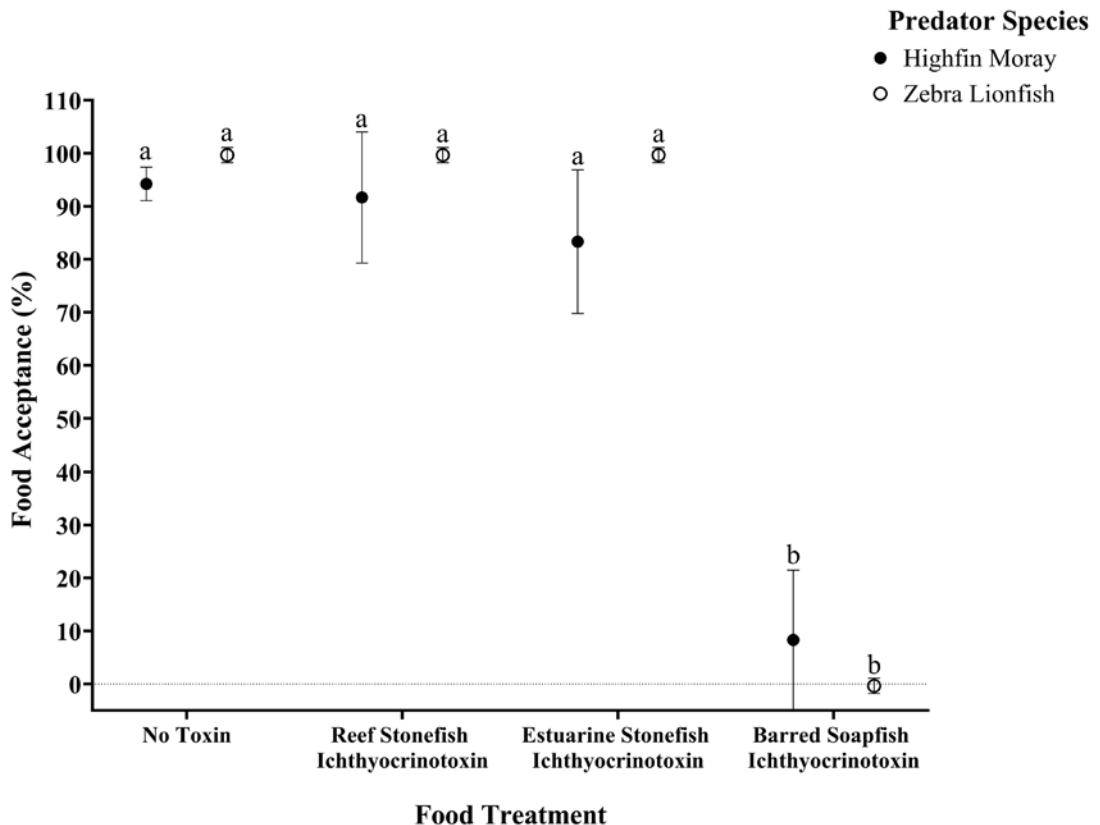


Figure 2. The palatability of ichthyocrinotoxins to predators. Highfin Morays (*Gymnothorax pseudothyrsoides*, ●, $n = 12$) and Zebra Lionfish (*Dendrochirus zebra*, ○, $n = 3$) were offered untreated prawn fillet (no toxin), or prawn fillet laced with ichthyocrinotoxin from Reef Stonefish (*Synanceia verrucosa*), Estuarine Stonefish (*Synanceia horrida*), or Barred Soapfish (*Diploprion bifasciatum*). Food acceptance (%) was determined based on proportion of feeding events where predators consumed the food offering within 30 seconds. Letters (a–b) represent statistically significant post-hoc LSD groupings. Error bars represent 95% CI.

DISCUSSION

Previous studies have shown that ichthyocrinotoxin from *S. horrida* exhibited minimal ichthyotoxic activity against blennies, *Petroscirtes japonicus* (synonym for *Omobranchus punctatus*) and mosquito fish (*Gambusia affinis*) (Cameron et al. 1981). This led to the suggestion that stonefish ichthyocrinotoxins are unlikely to function as a predator deterrent or repellent, unless their bitter taste renders them unpalatable (Cameron et al. 1981). The present study found that the feeding behaviours of Zebra Lionfish (*D. zebra*) and Highfin Moray (*G. pseudothyrsoides*) were unchanged by ichthyocrinotoxin from either Reef (*S. verrucosa*) or

Estuarine Stonefish (*S. horrida*). Ichthyocrinotoxins from both *S. horrida* and *S. verrucosa* were highly palatable (>85% food acceptance) to both predator species, comparable to untreated food (>94% food acceptance). Additionally, no adverse effects on the predators were observed following exposure to stonefish ichthyocrinotoxins, indicating that they may be non-toxic to these predator species. In contrast, food coated in Barred Soapfish (*D. bifasciatum*) ichthyocrinotoxin, a known antifeedant, prompted high rejection rates (>90% rejection) from both predators. These findings demonstrate that stonefish ichthyocrinotoxins do not function as an antifeedant against these

predator species. Moreover, the findings support the hypothesis that stonefish ichthyocrinotoxins likely do not play a major role in predator defence, acting as neither a defensive toxin nor an antifeedant.

While the primary function of ichthyocrinotoxins secreted by other well-studied fish taxa (soapfish, boxfish and flatfish) is predator defence (Clark 1983, Maretzki & Del Castillo 1967, Thomson 1969), stonefish ichthyocrinotoxins appear to be an anomaly. Furthermore, although ichthyocrinotoxins typically exhibit proteolytic and/or haemolytic activity towards vertebrates (Abdul-Haqq & Shier 2008, Indumathi & Khora 2013, Maretzki & Del Castillo 1967), stonefish ichthyocrinotoxins were found to lack proteolytic action and possess only mild haemolytic properties (Cameron et al. 1981). Another markable distinction between stonefish and other ichthyocrinotoxin-producing taxa is stonefish being equipped with an injectable toxin (i.e. venom). In contrast to their ichthyocrinotoxin, stonefish venom is highly noxious to vertebrates and undeniably serves in predator defence (Edean 1961, Saggiomo et al. 2021). Some evolutionary theories suggest that fish venoms may have evolved from the ability to produce a toxic glandular secretion, such as ichthyocrinotoxin (Cameron & Edean 1973). The rationale is that it is likely a more effective defensive strategy to inject toxin directly into a threat, rather than secrete one into an environment of infinite dilution (Cameron & Edean 1973). This might suggest that the evolutionary trajectory of stonefish ichthyocrinotoxins has diverged from that of other fish species, leading to a unique function, and, though unlikely, a vestigial role in predator defence.

Although the results of this study suggest that it is unlikely that stonefish ichthyocrinotoxins serve in predator defence, they may still serve a defensive function. Stonefish are devoid of scales and readily bury themselves into the substratum (Edean 1961). Consequently, their skin is presumably highly susceptible to a variety of benthic fauna, particularly invertebrate taxa like parasites and fouling animals. Previous studies showed that ichthyocrinotoxin from *S. horrida* immobilised ciliated protozoans and mussel gill cilia, as well as increased the tone of barnacle scutorum rostralis muscle (i.e. caused muscle contraction) (Cameron et

al. 1981). Additionally, very few internal parasite taxa have currently been identified within members of *Synanceia* (Miller et al. 2018). This suggests that one potential function of stonefish ichthyocrinotoxins may be to provide a defensive barrier against skin incursions from invertebrate taxa. Preliminary investigations suggest that another potential function of stonefish ichthyocrinotoxins may be to facilitate the growth and/or attachment of algae on to their skin (Barnett 2014). Algal recruitment onto the skin may serve to enhance the animal's camouflage, thereby reducing its likelihood of being discovered by predators and prey, while also potentially luring herbivorous prey into their ambush range. However, further research is required to determine whether stonefish ichthyocrinotoxins play a role in integumentary defence and/or aid in algal recruitment or growth.

Stonefish ichthyocrinotoxins also exhibited a greater adhesion to prawn fillets (65% retention for *S. horrida* and 45% retention for *S. verrucosa*), compared to soapfish ichthyocrinotoxin (27% retention). This differential adhesion may be further indicative of the distinct biological roles of these toxins. Soapfish are known to employ a rapid-release strategy, secreting copious amounts of their ichthyocrinotoxin when threatened (Randall et al. 1971). Previous studies report that the active component within soapfish (family Grammistidae) ichthyocrinotoxins, called grammistin, is a surfactant peptide (Onuki et al. 1993, Oshima 1974). Biological surfactants facilitate the mixing of different substances by reducing their interfacial tension (Otzen 2017). Therefore, the weaker adhesion of soapfish ichthyocrinotoxin may be attributed to the surfactant properties of one or more of its components. These properties likely support the function of soapfish ichthyocrinotoxin in predator defence by facilitating its rapid dispersal in seawater (and specifically within the buccal cavity of aquatic vertebrate predators). In contrast, stonefish do not secrete large quantities of ichthyocrinotoxin when threatened. This might suggest that stonefish ichthyocrinotoxins provide localised protection, making it advantageous to secrete a toxin with greater adhesive properties.

Interestingly, the adhesive strength of stonefish ichthyocrinotoxins to prawn fillet also appeared

to differ between species. Ichthyocrinotoxins from Estuarine Stonefish seemingly exhibited a greater adhesion to prawn fillet (65% retention) compared to those from Reef Stonefish (45% retention). Recent work elucidated that the composition of stonefish ichthyocrinotoxin varies between these two species (Lennox-Bulow et al. 2023b). As such, it is possible that the different adhesive properties of stonefish ichthyocrinotoxins between species may be due to variations in their chemical composition. However, further research is needed to support these observations, as well as determine whether interspecific differences in the physical properties of stonefish ichthyocrinotoxins might reflect functional and/or mechanistic adaptations.

In conclusion, the findings of this study suggest that stonefish ichthyocrinotoxins likely do not play a major role in predator defence. Furthermore, differences in the adhesive properties of ichthyocrinotoxins between stonefish and soapfish, as well as among stonefish species, potentially reflect distinct ecological functions or application mechanisms. Future research should focus on the potential defensive role of these toxins against invertebrate taxa that are likely to interact with the animal's skin, such as parasites and fouling animals. The findings of this study have expanded our understanding of the toxin ecology of stonefish and highlight potential avenues for future research that may lead to the discovery of novel therapeutics or industrial solutions for utilitarian purpose.

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Chapter 8

Optimising the xWORM Assay for Monitoring Hookworm Larvae Motility

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D Lennox-Bulow is the principal author of this manuscript and was extensively involved in all aspects of this work under the supervision of J Seymour. M Smout assisted with data collection, statistical analyses, and in reviewing earlier versions of this manuscript. L Becker assisted with parasite cultivation and in the editing and review of this manuscript. A Loukas assisted with reviewing earlier versions of this manuscript and provided the parasites used in this work.

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8.1 Abstract

Parasitic worms (helminths) infect almost all taxa across the animal kingdom, and pose significant challenges to public health and economies, particularly in developing countries. To address this problem, researchers have developed various tools to measure the motility and viability of helminths. However, the conditions used in anthelmintic screening assays are often not optimised, and they can vary considerably between research teams. These un-optimised conditions can impact new drug screens as little is known about the effect of different conditions on the health of the target parasites. To improve future research this study determined the effect of key assay parameters such as media type, media concentration, in-well parasite density, and assay duration on the infective third-stage larva (L3) of two types of hookworms, *Nippostrongylus brasiliensis* in rodents and *Necator americanus* in humans. Conditions were screened over several days using the xWORM assay, a real time impedance-based helminth motility assay using the xCELLigence system with 96-well microplates. While results varied depending on the species and media used, the study found that 500-1000 L3/200µL well volume and a media concentration of 3.13 – 25% generally produced good to excellent assay conditions. This study's findings can guide future selection of xWORM assay parameters for novel drug trials involving these parasite species and serve as a suggested model for optimising trial conditions for alternate parasite targets and assays.

8.2 Introduction

One of the major drawbacks of conducting an experiment on any living organism outside of its natural habitat is the potential for the artificial conditions to confound results (Akhtar, 2015; Morgan & Tromborg, 2007). As such, selecting the abiotic factors that make up the artificial conditions requires careful consideration of the ecology and biology of the target organism, whilst in some cases, balancing the requirements of the equipment being used (i.e., the xCELLigence RTCA system). This becomes particularly important when the experimental design involves measuring a physiological response, (i.e., parasite motility), to a treatment, such as novel drug targets with a pre-determined or desired effect. Recently, a number of screening techniques with reduced subjectivity and higher-throughput capabilities have emerged to measure the physiological responses (i.e., motility) of parasitic worms to novel drug targets including; video assays (Paveley *et al.*, 2012), enzymatic assays (Mansour & Bickle, 2010), colorimetric assays (Tritten *et al.*, 2012), fluorescence (Peak *et al.*, 2010) and other mechanisms (Howe *et al.*, 2015; Lalli *et al.*, 2015). Amongst these was the development of the xCELLigence worm real-time motility assay, i.e., xWORM (Rinaldi *et al.*, 2015; Smout *et al.*, 2010).

The xWORM assay uses the xCELLigence real time cell analyser system, which is principally used to monitor cellular events in real time, to measure parasite motility through fluctuations in electrical impedance (Smout *et al.*, 2010). To measure electrical impedance, the xCELLigence system applies an electrical potential across gold interdigitated micro-electrodes fused to the bottom surface of each well of a 96-well E-plate. When cells, or helminths make contact with these electrodes it increases the resistance inside the well, which relays to the analysing software as an increase in the Cell Index (CI) value. Conversely, where there is no contact there is less resistance, and thus there is a decrease in the CI value. As described by Smout *et al.* (2010), live parasites writhe in culture, as they do *in vivo*, and constantly vary their contact with the electrodes on the bottom surface of the E-Plate. This induces a variable resistance that results in an irregular CI value with an amplitude proportional to parasite motility. When the helminths become weakened or die, the resistance across the electrodes stabilises as the helminths are stationary on the base of the E-plate. Since its conception, the xWORM assay has had numerous applications across helminth species and life stages including screening novel compounds for anthelmintic activity, the determination of the 50% inhibitory concentration of numerous pre-existing anthelmintic treatment options, as well as for the

detection of phenotypic resistance to gold standard anthelmintics (Wangchuk *et al.*, 2016; Rinaldi *et al.*, 2015; Tritten *et al.*, 2012; Silbereisen *et al.*, 2011; Smout *et al.*, 2010).

Interestingly, some of the baseline assay conditions which the parasites are exposed to, i.e., media type, media concentration, and in-well parasite density vary considerably between published works including Smout *et al.* (2010), Tritten *et al.* (2012), and Rinaldi *et al.* (2015). This is particularly true for assays involving the larval stages (i.e., L2 – L4) of helminths. In some cases, these conditions have been shown to underpin the success of the assay (Tritten *et al.*, 2012). However, to date very little is known about their effect on both the measurement system and the target parasites. Therefore, the aim of this study was to measure the effect of key assay parameters, i.e., media type (phosphate-buffered saline and Dulbecco's Modified Eagle Medium), media concentration (3.13 – 100%), and in-well parasite density (67 – 2000 L3/200 μ L well volume) on the motility of infective third-stage hookworm larvae (L3), specifically *Nippostrongylus brasiliensis* (infective rodent hookworm larvae) and *Necator americanus* (infective human hookworm larvae). The goal of this was to identify and select the combination of xWORM assay parameters suitable for future studies aiming to screen novel compounds for anthelmintic activity against these species.

8.3 Materials and Methods

8.3.1 *Nippostrongylus brasiliensis* Cultivation

The cultivation of *N. brasiliensis* L3 followed a protocol outlined by Camberis *et al.* (2003). Using a 21G needle, 250 μ L of 1 $\times$ PBS solution containing approximately 2500 to 3000 *N. brasiliensis* L3 was injected subcutaneously between the shoulder blades of female Sprague-Dawley rats (ethics approval number A2626). Rats were monitored daily for physical and/or behavioural abnormalities. Rats were transferred to a new cage on the fifth day post-infection. Infected faecal pellets were collected on days six and seven post-infection. To culture the parasite, faeces were first hydrated in reverse-osmosis (R/O) H₂O and mixed thoroughly to form a smooth slurry. Granulated and fine particulate activated charcoal was added to the slurry at a ratio of 2:1 or until the mixture resembled natural soil. The charcoal mixture was distributed onto R/O H₂O dampened filter paper (80 mm rounded) that had been placed within 90 mm sterile plastic petri dishes. Plates were then set to incubate in the dark at 26°C. The larvae were left undisturbed for five days to allow for migration to the periphery of the plate. Collecting L3 *N. brasiliensis* involved gently washing the parasites off the sides and lid of the plate with a Pasteur pipette and deionised (R/O) H₂O. Collected *N. brasiliensis* L3 were transferred to a 50 mL falcon tube. To calculate the number of *N. brasiliensis* L3 harvested, the falcon tube was topped up with R/O H₂O to the nearest 10 mL gradation. Next, 100 μ L of the original solution was aliquoted into a 1.5 mL Eppendorf tube containing 900 μ L of R/O H₂O. The number of *N. brasiliensis* L3 in a 100 μ L subsample of the diluted solution was then counted using a top light microscope and extrapolated to yield the total number of parasite larvae harvested.

8.3.2 *Necator americanus* Cultivation

Faecal material for culture was obtained from a chronically infected donor from James Cook University who was carrying an *N. americanus* strain originating from Madang, Papua New Guinea. The donor was a 50-year-old male, inoculated with 20 *N. americanus* L3 in May 2020 with human research and ethics approvals provided by James Cook University (ethics approval number H5936). The donor was confirmed to be negative for transmissible blood-borne viruses (HIV, HBV, HCV) and for infection with significant bacterial enteropathogens (*Salmonella*, *Shigella*, *Campylobacter*). Faecal cultures were established within 48 hours of defecation following the same procedure outlined above for *N. brasiliensis*.

8.3.3 Measuring Parasite Motility Using xCELLigence RTCA

In previous studies, the xCELLigence Real Time Cell Analyser (RTCA, Agilent) has been used to differentiate between live and dead helminths across several species, and developmental stages (Zeraik *et al.*, 2014; You *et al.*, 2013; Tritten *et al.*, 2012; Silbereisen *et al.*, 2011; Smout *et al.*, 2010). While this was chiefly used to assess the efficacy of novel anthelmintics, our aim was to measure the effect of baseline assay parameters, i.e., media type, media concentration, and in-well parasite density, on the motility of *N. brasiliensis* L3 (rodent hookworm) and *N. americanus* L3 (human hookworm) for the purpose of optimising xCELLigence measuring conditions for future novel drug screening trials (Supplementary data 1).

To achieve this, we used an adjusted version of the xWORM assay developed by Rinaldi *et al.* (2015). In brief, Phosphate-Buffered Saline (PBS) and Dulbecco's Modified Eagle Medium (DMEM) media was prepared at six specified concentrations: 100, 50, 25, 12.5, 6.25, or 3.13%. These concentrations were achieved by diluting the media with deionised (MilliQ) H₂O. Subsequently, 150 µL of the prepared media and 2% Antibiotic-Antimycotic (AA) were added to each well of a 96-well E-plate. Plates were placed into an xCELLigence RTCA single plate (SP) instrument to undergo an initial background read. Alive (i.e., treatment group) and heat-killed (i.e., positive control group) *N. brasiliensis* or *N. americanus* L3 at densities of 2000, 1000, 500, 250, 125, and 67 L3/200µL well volume were then added to all wells in triplicate, apart from three that were reserved as a media-only control group. Parasites were added in 50 µL aliquots of solution made up with chosen media and 2% AA. Media-only control wells were devoid of parasites and consisted of 200 µL of chosen media and 2% AA. As hookworms are less active in an environment lacking the presence of a mammalian host, plates and RTCA SP instruments were incubated at 37°C in a humidified atmosphere containing 5% CO₂ throughout the duration of the experiment to simulate mammalian body temperature, and thus stimulate parasite motility. Parasite motility was registered at 25 kHz in regular user defined reads at 15 second intervals over 72 hours. Parasite motility was measured for all media type, media concentration and in-well parasite density combinations for both *N. brasiliensis* and *N. americanus* L3. Cell index (CI) figures were produced in GraphPad Prism 9.

Given that CI represents the real-time electrical impedance within all wells of a 96-well E-plate, CI scatter curves are a great way to screen for data corruptions (i.e., faulty wells) prior to proceeding with further data conversions and analyses. However, while changes to parasite

motility can be roughly identified in the raw CI scatter, with multiple wells and conditions, comparisons can become difficult. Therefore, as a more intuitive way to visualise and compare parasite motility, CI data was converted to a motility index (MI).

8.3.4 Motility Index Transformation

Motility Index (MI) is a measure of the amplitude of the CI curve scatter over time. As described by Smout *et al.* (2010), MI is calculated as the standard deviation over 800 data points of the CI difference from the rolling average over 30 data points. To calculate MI, raw CI readings from RTCA Software Pro were exported to Excel. To account for background noise, MI data was blank adjusted by subtracting each data point for treatment and positive control groups from the corresponding data point of the media-only control. Hypermotility was observed in all MI traces over the first 24 hours of the assay, and thus was designated as an equilibration period and excluded from further analyses. As such, 24-hours after assay commencement will hereafter be referred to as treatment time 0.

In contrast to CI scatter plots, MI curves are an intuitive way of visualising parasite motility over time (Supplementary data 2). Another benefit of converting CI data to MI is that it is suitable for use in a wide range of statistical analyses, the most common for drug trials being dose response. In this case, MI data is subsequently converted to a percentage of maximum motility which can be used to generate dose response curves for traditional IC₅₀ calculations. However, MI can also be used in a wide range of hit selection models, such as Strictly Standardised Mean Difference Prime (SSMD'). Therefore, to determine the optimal media type, media concentration, and in-well parasite density for xWORM assays with *N. brasiliensis* and *N. americanus* L3 we used SSMD'.

8.3.5 Assessing Assay Quality using Strictly Standardised Mean Difference Prime

Strictly standardised mean difference prime (SSMD') is a hit selection model developed by Zhang (2011). SSMD' is a way of measuring the size of an effect from the control group, relative to its variation, and is particularly useful for comparisons across groups measuring the same effect but operating under different physical parameters. For xWORM data, SSMD' scores represent the mean difference in parasite motility between treatment groups, and corresponding heat-killed parasite controls, relative to the variation within each group. Hence,

treatment groups with the greatest magnitude of difference from the heat-killed parasite control group coupled with low variability between replicates yield high SSMD' scores. Previous studies have used SSMD' to determine the optimal measurement frequency, and in-well density for *Schistosoma mansoni* egg hatching and cercariae motility (Rinaldi *et al.*, 2015). For future xWORM trials aimed at screening novel compounds for anthelmintic activity against *N. brasiliensis* and *N. americanus* L3, we targeted conditions that yielded a stable motility with the greatest magnitude of difference from the positive control group, and low variance between replicates. To identify these conditions quantitatively, SSMD' values were generated for each condition combination and parasite species using the following protocol

Raw MI data was first blank-adjusted by subtracting all data points from the average MI of the corresponding media-only control to account for background signal. The standard deviation of each treatment and heat-killed parasites control group was then calculated across the triplicates for each time point. SSMD' scores were then calculated for each hookworm species (i.e., *N. brasiliensis* L3 or *N. americanus* L3) and assay condition combination (i.e., media type, media concentration, and in-well parasite density) from treatment time 0 (i.e., 24 hours after assay commencement) up to 48 hours as:

$$SSMD' = \left| \frac{\overline{AC} - \overline{HK}}{\sqrt{s_{AC}^2 + s_{HK}^2}} \right|$$

$\overline{AC}$ - is the average MI value of hookworm larvae in y 'assay condition' (i.e., media type, media concentration and in-well parasite density combination)

$\overline{HK}$ – is the average MI value of 'heat-killed' hookworm larvae

s_{AC} – is the standard deviation of the MI of hookworm larvae in y 'assay condition'

s_{HK} – is the standard deviation of the MI of 'heat-killed' hookworm larvae

SSMD' is calculated as the mean of the differences divided by the standard deviation of the differences between the treatment group (parasites in various conditions) and a negative reference (heat-killed parasites in the same conditions). Where the selected assay conditions (i.e., the media type, media concentration, and in-well parasite density combination) and their matching positive control (i.e., heat-killed parasites) have mean values designated as AC and HK with standard deviations of S_{AC} and S_{HK} respectively.

Identifying the optimal conditions (i.e., hit selection) for xWORM assays with *N. brasiliensis* and *N. americanus* L3 was based on the classification system outlined by Zhang (2011) (**Table 8.1**). Whereby assay conditions (i.e., media type, media concentration, and parasite in-well density combinations) scoring between zero to three, and three to five, were considered poor to inferior, respectively. However, assay conditions that scored between five to seven were considered good, and those scoring greater than seven were considered excellent.

Table 8.1 Classification of Assay Quality using SSMD' Values Based on Zhang (2011).

Assay Quality	Absolute SSMD'
Excellent	>7
Good	5 – 7
Inferior	3 – 5
Poor	0 – 3

Isopleths were generated in MATLAB[®] (MathWorks R2022a) and used to visualise the interaction between parasite motility (as determined by the xWORM assay) and baseline assay condition combinations on mean SSMD' scores. Optimal condition ranges were further compared between parasite species (*N. brasiliensis* and *N. americanus* L3) and across two time points (24- and 48-hours treatment time). Conditions that produced highly variable MI traces were deemed as outliers. The mean SSMD' values for the outliers were replaced with the average SSMD' of the adjacent scores.

8.4 Results

8.4.1 SSMD' Assay Quality Assessment for Nippostrongylus brasiliensis L3

The quality of xWORM assays with *N. brasiliensis* L3 were affected by both media concentration and parasite density (**Figure 8.1**). These trends also varied between media types (PBS/DMEM) and over time (24- and 48- hours). PBS conditions that produced good to excellent assay scores over 24 hours ranged between approximately 3.13 – 25% media concentration with 67 – 1000 L3/200 μ L well volume (**Figure 8.1A**). This range was slightly more restricted when measuring over 48 hours, where approximately 3.13 – 25% media concentration and 125 – 800 L3/200 μ L well volume yielded good to excellent assay scores (**Figure 8.1B**). However, PBS conditions that had the highest assay quality (> 7 excellent assay) over time were with a media concentration roughly between 3.13 – 6.25% coupled with a parasite density between 250 – 500 L3/200 μ L well volume. DMEM conditions that produced good to excellent assay scores over 24 hours had a slightly broader range inclusive of 3.13 – 100 % media concentrations and parasite densities between 250 – 2,000 L3/200 μ L well volume (**Figure 8.1C**). These conditions maintained mostly good to excellent assay scores over 48 hours, although some exhibited a slight drop in quality over time (**Figure 8.1D**). The DMEM conditions that maintained the strongest assay scores (> 7) between the 24- and 48-hour time points were 50 – 100 % media with 1000 – 1500 L3/200 μ L well volume, 12.5 – 25% media with 500 – 1000 L3/200 μ L well volume, and 6.25 – 12.5% media with 1,000 – 2,000 L3/200 μ L well volume.

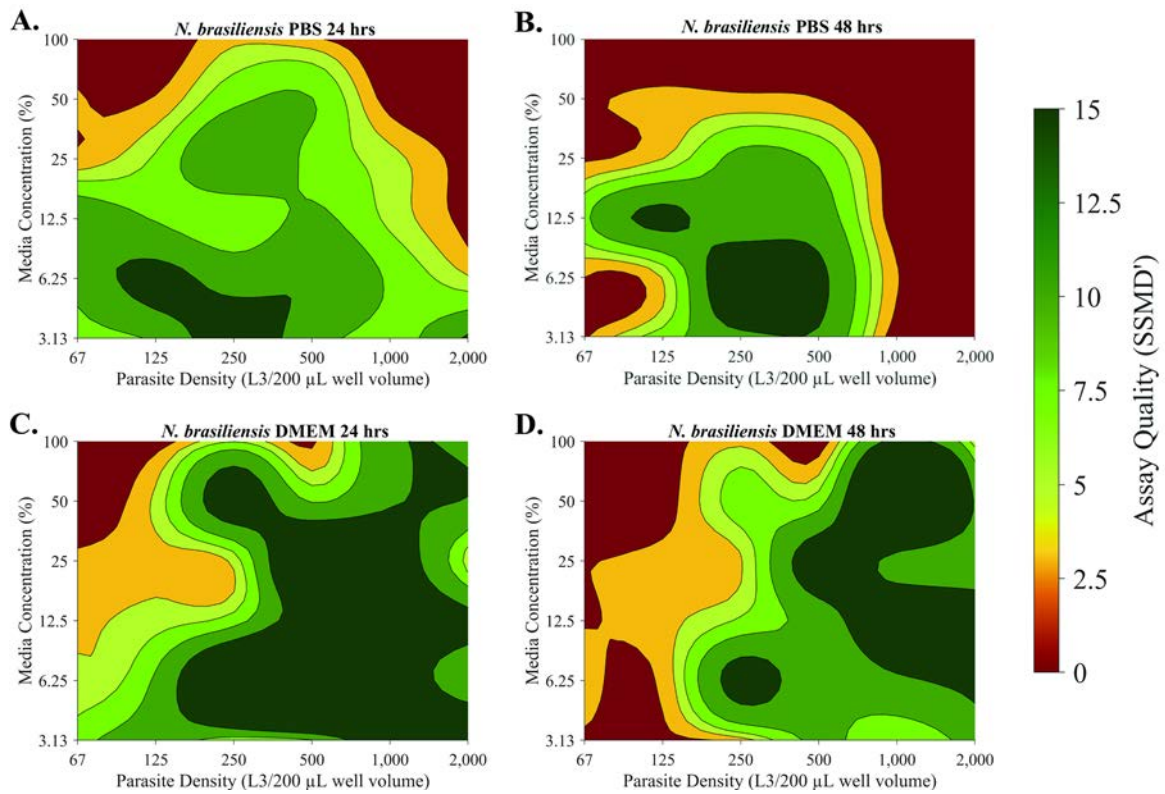


Figure 8.1 The Effect of Baseline xWORM Assay Conditions on Rodent Hookworm (*Nippostrongylus brasiliensis*) L3 Vitality. Strictly Standardised Mean Difference Prime (SSMD') was used to assess xWORM assay quality for the infective larval (L3) stage of *N. brasiliensis*. SSMD' scores were calculated for each baseline condition combination of media type (PBS and DMEM), media concentration (3.13 – 100%), and in-well parasite density (67 – 2000 L3/200 μ L well volume) at 24-hour and 48-hour time points (**Panels A – D**). Isopleths are defined as the mean SSMD' score where < 3 indicates a poor assay, 3 – 5 an inferior assay, and > 7 an excellent assay.

8.4.2 SSMD' Assay Quality Assessment for Necator americanus L3

The quality of xWORM assays with *N. americanus* L3 were affected by media concentration and parasite density (**Figure 8.2**). These trends also varied between the two media types as well as over time. PBS conditions yielding good to excellent assay scores over 24 hours were ~3.13 – 30% media concentration with a parasite density of ~250 – 2,000 L3/200 μ L well volume (**Figure 8.2A**). These conditions maintained mostly high SSMD' scores with only slight variance over 48 hours (**Figure 8.2B**). The PBS conditions with the highest assay quality over time were ~3.13 – 12.5 % media with an in-well parasite density between ~1,500 – 2,000 L3/200 μ L well volume. DMEM conditions with *N. americanus* exhibited a similar pattern to what was observed for PBS conditions (**Figure 8.2C,D**). Good to excellent assay scores were recorded for all DMEM media concentrations (3.13 – 100%) with parasite densities between 500 – 2,000 L3/200 μ L well volume over both 24- and 48- hour time periods. However, assay scores falling within the excellent category were much less abundant at 48 hours than 24 hours.

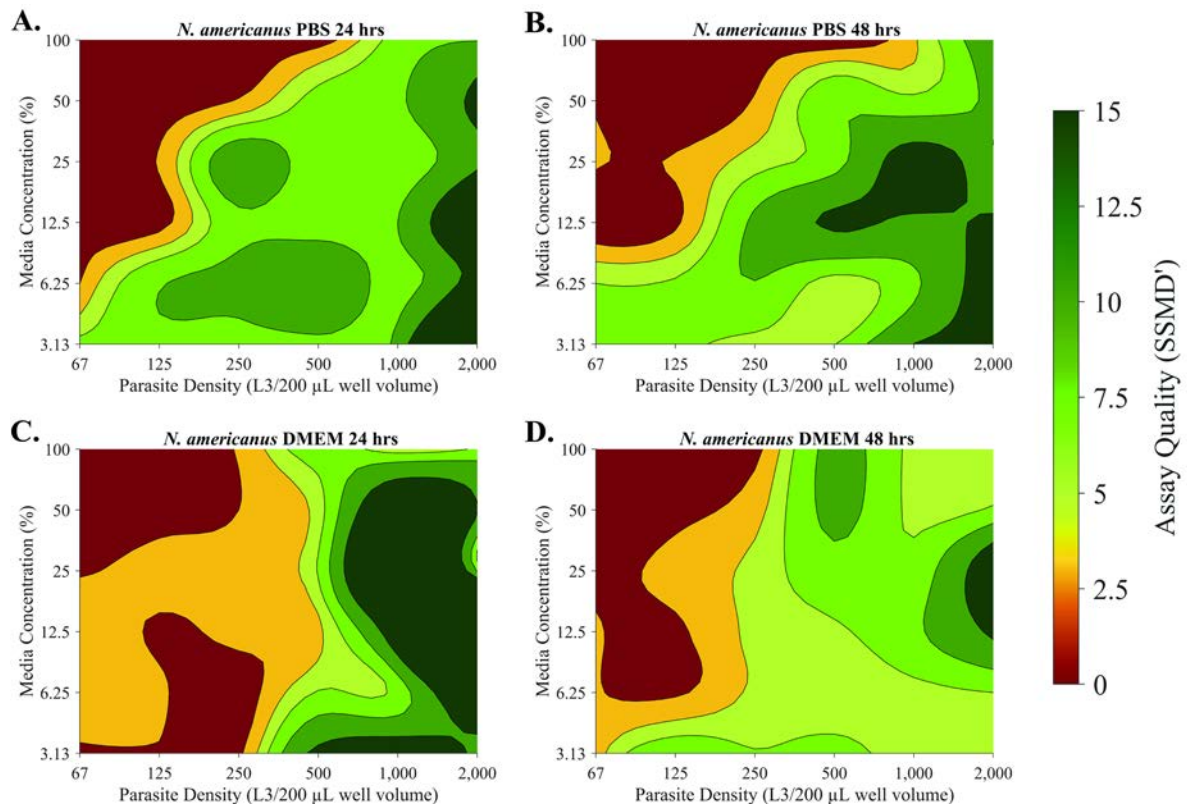


Figure 8.2 The Effect of Baseline xWORM Assay Conditions on Human Hookworm (*Necator americanus*) L3 Vitality. Strictly Standardised Mean Difference Prime (SSMD') was used to assess xWORM assay quality for the infective larval (L3) stage of the human hookworm (*N. americanus*). SSMD' scores were calculated for each baseline condition combination of media type (PBS and DMEM), media concentration (3.13 – 100%), and in-well parasite density (67 – 2000 L3/200 μ L well volume) at 24-hour and 48-hour time points (**Panels A – D**). Isopleths are defined as the mean SSMD' score where < 3 indicates a poor assay, 3 – 5 an inferior assay, 5 – 7 a good assay, and > 7 an excellent assay.

8.5 Discussion

The results of this study demonstrate that assay quality is affected by the combination of media type, media concentration, and in-well parasite density selected, as well as assay duration. We further show that the effect these parameters have on assay quality differs between two closely related hookworm species of the same life stage, *N. brasiliensis* and *N. americanus* L3. For instance, *N. americanus* performed better in PBS media over longer time periods than *N. brasiliensis*. However, *N. brasiliensis* performed better overall in DMEM media. Additionally, in PBS media, higher densities of *N. americanus* resulted in a stronger assay in most cases. Conversely, this resulted in a weak assay for *N. brasiliensis*, particularly with high concentrations of PBS media, and assays conducted over longer periods of time.

Since its conception, the xWORM assay has been used to measure the motility of numerous species and life stages of helminths. However, one of the most striking elements within the methodology of the published literature is that selected assay conditions including media type, media concentration, and in-well parasite density, vary considerably. Across several studies, the type of media used in xWORM assays has been selected to mimic the natural environment of the parasite at that life stage (Rinaldi *et al.*, 2015; Smout *et al.*, 2010). For instance, as adult and some later larval stages are known to occur *in vivo*, cell culture medium (i.e., DMEM or RPMI) was selected in attempt to replicate conditions experienced inside a host animal. Conversely, as eggs and early larval stages commonly occur in the environment, such as in soil or bodies of freshwater, saline solutions (i.e., PBS) are often selected. Nonetheless, the distinct preferences of human and rodent hookworm larvae for DMEM and PBS media suggests that choosing the appropriate medium may be more complex than previously assumed. This study highlights that factors such as media concentration and in-well parasite density should be considered in conjunction with media type for a more comprehensive approach. However, in previous xWORM studies these conditions have frequently been selected based on subjective choice or constrained by resource availability, rather than systematic evaluation. In some instances, resource constraints, possibly combined with an unsuitable pairing of media type and concentration, have resulted in the failure of the assay. According to a study by Tritten *et al.* (2012), the third larval stage (L3) of the hookworm *Ancylostoma ceylanicum* were immeasurable at densities of 100 L3/well, which was presumed to be related to the activity level of the species. On the other hand, previously successful assays, particularly those involving larval life stages, have utilised high numbers of parasites. One study, for example, measured

Haemonchus contortus L3 at a density of 3,000 L3/well, and *Strongyloids ratti* L3 at a density of 300 L3/well (Smout et al., 2010). Similarly, using SSMD' as an indicator for assay quality, another study determined that 4,500 *Schistosoma mansoni* cercariae/well yielded the strongest assay for the selected media (10% PBS) and duration (16 – 20 hours; Rinaldi et al., 2015). This has resulted in the notion that xWORM assays require large numbers of parasites to be successful (Tritten *et al.*, 2012). However, obtaining high numbers of parasites can be challenging due to the complex life cycle of most parasite species, which involves multiple morphological stages, and sometimes, several host animals (Ahmed, 2014). This can render artificially simulating the parasites natural conditions difficult from both a cultivation and experimental perspective (Ahmed, 2014).

In experimental settings, ensuring suitable conditions for monitoring parasite health *in vitro* becomes increasingly challenging when researchers must simultaneously accommodate the specific requirements of the measuring system in use. For example, to assess parasite motility through changes in electrical impedance using the xCELLigence system, an ionic solution is required in the measuring environment. The ionic strength of the media needed for the system to accurately monitor parasite motility is likely influenced by various ecological and physiological factors related to the study organism, particularly physical size and activity level. However, the tolerance of the study organism to these conditions is also anticipated to differ significantly among parasite species and life stages. Hence, when selecting assay conditions, it is crucial to strike a balance between meeting the system's requirements and preserving the study organism's viability to ensure the accuracy and success of the research. It is crucial to recognise that the influence of artificial conditions on parasite behaviour and sensitivity, especially in relation to treatments, is currently unknown. Nevertheless, striving to simulate the natural environment of the parasite as closely as possible is likely the most productive set of conditions for xWORM assays.

Previous studies have shown that the sensitivity of impedance measurements captured by xCELLigence can also be improved by modifying the system's frequency settings (Rinaldi *et al.*, 2015). This proved to be particularly useful when measuring the hatching frequency of *S. mansoni* eggs, which require near freshwater conditions to maintain their viability (Rinaldi *et al.*, 2015). When this is combined with the appropriate combination of media type, media concentration and in-well parasite density tailored to the study organism and intended assay duration, we showed that lower parasite numbers can yield a strong assay. Although the overall

strongest assay conditions for both *N. brasiliensis* and *N. americanus* were determined to involve high parasite numbers (>125 L3/200 µL well volume), as little as 67 L3/200 µL well volume yielded high scores under certain conditions, specifically with a low media concentration, and shorter assay duration. However, while frequency was not considered by this study due to the promising results obtained at 25 MHz (Rinaldi *et al.*, 2015), parasites or specific conditions may benefit from additional frequency optimisation. Similarly, while high numbers of *S. mansoni* (4,500 cercariae/well) in select conditions reportedly produced the strongest assay, a good assay could still be achieved with 562 cercariae/well (Rinaldi *et al.*, 2015). Our results suggest that adjusting media type and/or concentration may potentially further allow for lower cercariae densities whilst maintaining a high assay quality.

While this study offers a preliminary framework for optimising the xWORM assay, the effect of several other critical assay parameters, including pH, temperature, and the availability of nutrients and oxygen, requires further investigation. For instance, our findings illustrated that heightened larval densities could sometimes impair assay quality, as evidenced in the case of *N. brasiliensis* L3 in PBS media. A plausible explanation for the observed detrimental effects associated with increased larval densities could be fluctuations in critical media parameters, such as pH, over time. This phenomenon is known to be associated with decreased viability of mammalian cells maintained *in vitro*. As cells generate waste products (for instance, lactate), they can exceed the buffering capacity of the culture media, especially when maintained at high densities and/or over prolonged durations, leading to pH fluctuations (Gaze *et al.*, 2014). Similarly, hookworm adults, including *N. americanus*, excrete and secrete proteinaceous material which reportedly enables the parasite to avoid detection by the hosts immune system (Logan *et al.*, 2020; Hewitson *et al.*, 2009). Following manual extraction from the host and subsequent *in vitro* cultivation, viable adult hookworms continue to generate excretory and secretory (ES) products, as well as reproductive materials (Logan *et al.*, 2020), which over time likely affects parasite vitality. Although it is currently uncertain, it is plausible that L3 larvae also generate potentially toxic by-products, the effects of which could be exacerbated by high in-well larval densities.

Conversely, as exemplified by *N. americanus* L3 in both PBS and DMEM media, high parasite densities were sometimes required for preserving parasite vitality. Higher in-well parasite densities may be advantageous when monitoring the motility of certain species like *N. americanus*, which could exhibit lower activity levels. In this scenario, higher numbers would

be required to amplify motility signals. Alternatively, these species might experience stimulation through body contact. For instance, L3 larvae are known to detect hosts in nature through positive thigmotaxis (Gaze *et al.*, 2014; Haas *et al.*, 2005). Moreover, upon hatching in culture, L3 hookworm larvae rapidly disperse in all directions, moving towards the periphery of the plate (Camberis *et al.*, 2016; Camberis *et al.*, 2003). This behaviour could represent an evolutionary adaptation aimed at enhancing the success of transmission to a host. Specifically, close proximity with other larvae might trigger a response in the larvae to move away from each other to expand their spatial footprint, thereby increasing the likelihood of encountering a viable host. However, this is purely speculative and requires a deeper understanding of parasite behaviour.

Another factor to consider when selecting xWORM assay conditions is oxygen and nutrient availability. A recent study by Hass *et al.* (2023) reported that in-well media volume significantly impacts the metabolic activity of retinal pigment epithelium (RPE) cells by affecting oxygen and nutrient availability. Researchers may initially overlook this as a potential factor when measuring the motility of L3 as it is widely believed that they are in a state of arrested development and survive solely on the energy accumulated during previous larval stages (Gaze *et al.*, 2014). However, studies have shown that various environmental stimuli can trigger larval metamorphosis and the resumption of feeding, including warmer temperatures and the presence of serum or host-specific secretions like sweat (Pasuralertsakul & Ngrenngarm, 2006; Haas *et al.*, 2005; Kumar & Pritchard, 1994; Hawdon & Schad, 1990). The incubation temperature commonly used in xWORM trials (37°C) to stimulate worm motility could thus trigger an artificial metamorphosis in hookworm parasites from a free-living to a parasitic lifestyle and/or a resumption of feeding. This could potentially account for the observed decline in assay quality for *N. brasiliensis* L3 in PBS media over a 48-hour period. Therefore, future studies should aim to explore the effect of serum supplementation and oxygen availability on xWORM assay quality, particularly for studies conducted over longer timeframes.

It is also worth considering that extended exposure to elevated temperatures may be detrimental to en-sheathed hookworm larvae. Since the sheath acts as a thermal buffer in a dynamic environment, prolonged heat exposure could trap heat and potentially decrease larval vitality. The success rate at which larvae shed their protective sheath in response to artificially induced environmental stimuli, such as temperature or the presence of serum, is currently

unknown and should be a focus of future research. However, this may explain the broad spectrum of optimal conditions identified in this study, potentially reflecting variations in the ability of L3 larvae to undergo temperature-induced ex-sheathing and/or metamorphosis in the absence of a host.

In summary, the quality of xWORM assays is influenced by numerous interrelated parameters, including media type, media concentration, in-well parasite density, and assay duration. Furthermore, the effect of these parameters was found to vary between closely related helminth species at the same larval stage. This reveals species-specific enviro-physiological differences in hookworms that can impact the conditions necessary for achieving high-quality xWORM assays. We anticipate that these effects are likely relevant to other helminth species and life stages as well. Moreover, we have emphasised the importance of several other critical assay parameters, including pH, temperature, and nutrient and oxygen availability, which should be considered in future works for their potential impact on larval viability and assay quality. This study highlights the need for a dedicated optimisation phase and a deeper understanding of the conditions under which xWORM assays are conducted, specifically their effect on parasite physiology and behaviour. By addressing these factors, researchers can ensure the generation of accurate and reliable results, ultimately contributing to more effective research in the future.

8.6 References

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8.7 APPENDIX G

Optimising the xWORM Assay for Monitoring Hookworm Larvae Motility

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Optimizing the xWORM assay for monitoring hookworm larvae motility

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Parasitic worms (helminths) infect almost all taxa across the animal kingdom, and pose significant challenges to public health and economies, particularly in developing countries. To address this problem, researchers have developed various tools to measure the motility and viability of helminths. However, the conditions used in anthelmintic screening assays are often not optimized, and can vary considerably between research teams. These unoptimized conditions may impact novel drug screens, as little is known about the effects of different conditions on the health of the target parasites. To improve future research, this study determined the effects of key assay parameters including, media type, media concentration, in-well parasite density, and assay duration on the infective third-stage larva (L3) of two types of hookworms, namely *Nippostrongylus brasiliensis* in rodents, and *Necator americanus* in humans. Conditions were screened over several days using the xCELLigence worm real-time motility assay (xWORM); a real-time impedance-based helminth motility assay using the xCELLigence system with 96-well microplates. While results varied depending on the species and media used, the study found that 500–1,000 L3/200-μL and a media concentration of 3.13–25% generally produced good to excellent assay conditions. The findings of this study can guide the future selection of xWORM assay parameters for novel drug trials involving these parasite species and serve as a suggested model for optimizing trial conditions for alternative parasite targets and assays.

KEYWORDS

xWORM, drug screening and discovery, hookworm larvae, helminth culture, assay conditions, method optimization

1 Introduction

One of the major drawbacks of conducting an experiment on any living organism outside of its natural habitat is the potential for the artificial conditions to confound results (Morgan and Tromborg, 2007; Akhtar, 2015). As such, selecting the abiotic factors that make up the artificial conditions requires careful consideration of the ecology and biology

of the target organism, whilst, in some cases, balancing the requirements of the equipment being used [i.e., the xCELLigence® Real Time Cell Analyzer (RTCA; ACEA Biosciences) system]. This becomes particularly important when the experimental design involves measuring a physiological response, (i.e., parasite motility) to a treatment, such as novel drug targets with a predetermined or desired effect. Recently, a number of screening techniques with reduced subjectivity and higher-throughput capabilities have emerged to measure the physiological responses (i.e., motility) of parasitic worms to novel drug targets, including; video assays (Paveley et al., 2012), enzymatic assays (Mansour and Bickle, 2010), colorimetric assays (Tritten et al., 2012), fluorescence (Peak et al., 2010), and other mechanisms (Howe et al., 2015; Lalli et al., 2015). Among these was the development of the xCELLigence worm real-time motility (xWORM) assay, (Smout et al., 2010; Rinaldi et al., 2015).

The xWORM assay uses the xCELLigence real-time cell analyzer system, which is principally used to monitor cellular events in real time, to measure parasite motility through fluctuations in electrical impedance (Smout et al., 2010). To measure electrical impedance, the xCELLigence system applies an electrical potential across gold interdigitated microelectrodes fused to the bottom surface of each well of a 96-well E-plate. When cells or helminths make contact with these electrodes, it increases the resistance inside the well, which is relayed to the analyzing software as an increase in the cell index (CI) value. Conversely, where there is no contact, there is less resistance, resulting in a decrease in the CI value. As described by Smout et al. (2010), live parasites writhe in culture, as they do *in vivo*, and constantly vary their contact with the electrodes on the bottom surface of the E-plate. This induces a variable resistance that results in an irregular CI value with an amplitude that is proportional to parasite motility. When the helminths become weakened or die, the resistance across the electrodes stabilizes as the helminths are stationary on the base of the E-plate. Since its conception, the xWORM assay has had numerous applications across helminth species and life stages, including screening novel compounds for anthelmintic activity, the determination of the 50% inhibitory concentration of numerous pre-existing anthelmintic treatment options, and also the detection of phenotypic resistance to gold standard anthelmintics (Smout et al., 2010; Silbereisen et al., 2011; Tritten et al., 2012; Rinaldi et al., 2015; Wangchuk et al., 2016).

Interestingly, some of the baseline assay conditions that the parasites are exposed to, i.e., media type, media concentration, and in-well parasite density, vary considerably between published works, including those by Smout et al. (2010); Tritten et al. (2012), and Rinaldi et al. (2015). This is particularly true for assays involving the larval stages of helminths [i.e., infective second- to fourth-stage larvae (L2–L4)]. In some cases, these conditions have been shown to underpin the success of the assay (Tritten et al., 2012). However, to date, very little is known about their effects on both the measurement system and the target parasites. Therefore, the aim of this study was to measure the effect of key assay parameters, including, media type [phosphate-

buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM)], media concentration (3.13–100%), and in-well parasite density (67–2000 L3/200-μL well volume) on the motility of infective third-stage (L3) hookworm larvae, specifically *Nippostrongylus brasiliensis* (infective rodent hookworm larvae) and *Necator americanus* (infective human hookworm larvae). The goal of this was to identify and select the combination of xWORM assay parameters suitable for future studies aiming to screen novel compounds for anthelmintic activity against these species.

2 Materials and methods

2.1 *N. brasiliensis* cultivation

The cultivation of *N. brasiliensis* L3 followed a protocol outlined by Camberis et al. (2003). Using a 21-gauge needle, 250-μL of 1 × PBS solution containing ≈ 2500 – 3000 *N. brasiliensis* L3 was injected subcutaneously between the shoulder blades of female Sprague Dawley® rats (ethics approval number A2626). Rats were monitored daily for physical and/or behavioral abnormalities. Rats were transferred to a new cage on the fifth day post infection. Infected fecal pellets were collected on days six and seven post infection. To culture the parasite, feces were first hydrated in reverse-osmosis (R/O) H₂O and mixed thoroughly to form a smooth slurry. Granulated and fine particulate activated charcoal was then added to the slurry at a ratio of 2 : 1 or until the mixture resembled natural soil. The charcoal mixture was distributed onto R/O H₂O dampened filter paper (80-mm rounded), which had been placed within 90-mm sterile plastic Petri dishes. Plates were then set to incubate in the dark at 26°C. The larvae were left undisturbed for five days to allow for migration to the periphery of the plate. Collecting *N. brasiliensis* L3 involved gently washing the parasites off the sides and lid of the plate with a Pasteur pipette and R/O H₂O. Collected *N. brasiliensis* L3 were transferred to a 50-mL Falcon™ tube. To calculate the number of *N. brasiliensis* L3 harvested, the Falcon tube was topped up with R/O H₂O to the nearest 10-mL gradation. Subsequently, 100-μL of the original solution was aliquoted into a 1.5-mL Eppendorf tube containing 900-μL of R/O H₂O. The number of *N. brasiliensis* L3 in a 100-μL subsample of the diluted solution was then counted using a top light microscope and extrapolated to yield the total number of parasite larvae harvested.

2.2 *N. americanus* cultivation

Fecal material for culture was obtained from a chronically infected donor from James Cook University who was carrying an *N. americanus* strain originating from Madang, Papua New Guinea. The donor was a 50-year-old male who had been inoculated with 20 *N. americanus* L3 in May 2020, with human research and ethics approvals provided by James Cook University (ethics approval number H5936). The donor was confirmed to be negative for

transmissible blood-borne viruses [i.e., human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV)] and for infection with significant bacterial enteropathogens (*Salmonella*, *Shigella*, and *Campylobacter*). Fecal cultures were established within 48 hours of defecation following the procedure outlined in section 2.1.

2.3 Measuring parasite motility using xCELLigence RTCA

In previous studies, the xCELLigence RTCA has been used to differentiate between live and dead helminths across several species and developmental stages (Smout et al., 2010; Silbereisen et al., 2011; Tritten et al., 2012; You et al., 2013; Zeraik et al., 2014). Although this was chiefly used to assess the efficacy of novel anthelmintics, our aim was to measure the effect of baseline assay parameters, i.e., media type, media concentration, and in-well parasite density, on the motility of *N. brasiliensis* L3 (rodent hookworm) and *N. americanus* L3 (human hookworm) for the purpose of optimizing xCELLigence measuring conditions for future novel drug screening trials (Supplementary Data 1).

To achieve this, we used an adjusted version of the xWORM assay developed by Rinaldi et al. (2015). In brief, PBS and DMEM media were prepared at six specified concentrations: 100%, 50%, 25%, 12.5%, 6.25%, and 3.13%. These concentrations were achieved by diluting the media with deionized (Milli-Q®) H₂O. Subsequently, 150-μL of the prepared media and 2% antibiotic-antimycotic (AA) were added to each well of a 96-well E-plate. Plates were placed into an xCELLigence RTCA single-plate (SP) instrument to undergo an initial background read. Live (i.e., treatment group) and heat-killed (i.e., positive control group) *N. brasiliensis* or *N. americanus* L3 at densities of 2000, 1000, 500, 250, 125, and 67 L3/200-μL well volume were then added to all wells in triplicate, apart from three that were reserved as a media-only control group. Parasites were added in 50-μL aliquots of solution made up with chosen media and 2% AA. Media-only control wells were devoid of parasites and consisted of 200-μL of the chosen media and 2% AA. As hookworms are less active in an environment lacking the presence of a mammalian host, plates and RTCA SP instruments were incubated at 37°C in a humidified atmosphere containing 5% CO₂ throughout the duration of the experiment to simulate mammalian body temperature, and thus stimulate parasite motility. Parasite motility was registered at 25 kHz in regular user-defined reads at 15 second intervals over 72 hours. Parasite motility was measured for all media type, media concentration, and in-well parasite density combinations for both *N. brasiliensis* and *N. americanus* L3. CI figures were produced in GraphPad Prism 9 (GraphPad Software Inc., CA, USA).

Given that CI represents the real-time electrical impedance within all wells of a 96-well E-plate, CI scatter curves are a great way to screen for data corruptions (i.e., faulty wells) prior to proceeding with further data conversions and analyses. However, although changes to parasite motility can be roughly identified in the raw CI scatter, with multiple

wells and conditions, comparisons can become difficult. Therefore, as a more intuitive way to visualize and compare parasite motility, CI data were converted to a motility index (MI).

2.4 MI transformation

MI is a measure of the amplitude of the CI curve scatter over time. As described by Smout et al. (2010), MI is calculated as the standard deviation over 800 data points of the CI difference from the rolling average over 30 data points. To calculate the MI, raw CI readings from the RTCA Software Pro were exported to Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA). To account for background noise, MI data was blank adjusted by subtracting each data point for treatment and positive control groups from the corresponding data point of the media-only control. Hypermotility was observed in all MI traces over the first 24 hours of the assay, and thus was designated as an equilibration period and excluded from further analyses. As such, the 24 hours after assay commencement will hereafter be referred to as treatment time 0.

In contrast to CI scatter plots, MI curves are an intuitive way of visualizing parasite motility over time (Supplementary Data 2). Another benefit of this conversion is that MI is suitable for use in a wide range of statistical analyses, the most common for drug trials being dose response. In this case, MI data are subsequently converted to a percentage of maximum motility, which can be used to generate dose-response curves for traditional IC₅₀ calculations. However, MI can also be used in a wide range of hit selection models, such as strictly standardized mean difference prime (SSMD'). Therefore, to determine the optimal media type, media concentration, and in-well parasite density for xWORM assays with *N. brasiliensis* and *N. americanus* L3 we used SSMD'.

2.5 Assessing assay quality using SSMD'

SSMD' is a hit selection model developed by Zhang (2011). SSMD' is a way of measuring the size of an effect from the control group, relative to its variation, and is particularly useful for comparisons across groups that measure the same effect but operate under different physical parameters. For xWORM data, SSMD' scores represent the mean difference in parasite motility between treatment groups, and corresponding heat-killed parasite controls, relative to the variation within each group. Hence, treatment groups with the greatest magnitude of difference from the heat-killed parasite control group coupled with low variability between replicates, yield high SSMD' scores. Previous studies have used SSMD' to determine the optimal measurement frequency, and in-well density for *Schistosoma mansoni* egg hatching and cercariae motility (Rinaldi et al., 2015). For future xWORM trials aimed at screening novel compounds for anthelmintic activity against *N. brasiliensis* and *N. americanus* L3, we targeted conditions that yielded a stable motility with the greatest magnitude of difference from the positive control group, and low variance between

replicates. To identify these conditions quantitatively, SSMD' values were generated for each condition combination and parasite species using the following protocol:

First, raw MI data were blank adjusted by subtracting all data points from the average MI of the corresponding media-only control to account for background signal. The standard deviation of each treatment and heat-killed parasites control group was then calculated across the triplicates for each time point. SSMD' scores were then calculated for each hookworm species (i.e., *N. brasiliensis* L3 or *N. americanus* L3) and assay condition combination (i.e., media type, media concentration, and in-well parasite density) from treatment time 0 (i.e., 24 hours after assay commencement) up to 48 hours as:

$$SSMD' = \frac{|\overline{AC} - \overline{HK}|}{\sqrt{s_{AC}^2 + s_{HK}^2}} \quad (1)$$

$\overline{AC}$ is the average MI value of hookworm larvae in y "assay condition" (i.e., media type, media concentration, and in-well parasite density combination). $\overline{HK}$ is the average MI value of "heat-killed" hookworm larvae. s_{AC} is the standard deviation of the MI of hookworm larvae in y "assay condition". s_{HK} is the standard deviation of the MI of 'heat-killed' hookworm larvae.

SSMD' is calculated as the mean of the differences divided by the standard deviation of the differences between the treatment group (parasites in various conditions) and a negative reference (heat-killed parasites in the same conditions), where the selected assay conditions (i.e., the media type, media concentration, and in-well parasite density combination) and their matching positive control (i.e., heat-killed parasites) have mean values designated as $\overline{AC}$ and $\overline{HK}$ with standard deviations of s_{AC} and s_{HK} , respectively.

Identifying the optimal conditions (i.e., hit selection) for xWORM assays with *N. brasiliensis* and *N. americanus* L3 was based on the classification system outlined by Zhang (2011, Table 1), whereby assay conditions (i.e., media type, media concentration, and parasite in-well density combinations) with scores between zero and three, and between three and five, were considered to be poor and inferior, respectively. However, assay conditions that scored between five and seven were considered good, and those scoring greater than seven were considered excellent.

Isopleths were generated in MATLAB[®] (MathWorks R2022a) and used to visualize the interaction between parasite motility (as determined by the xWORM assay) and baseline assay condition combinations on mean SSMD' scores. Optimal condition ranges were further compared between parasite species (*N. brasiliensis* and *N.*

americanus L3) and across two time points (24- and 48-hours treatment time). Conditions that produced highly variable MI traces were deemed as outliers. The mean SSMD' values for the outliers were replaced with the average SSMD' of the adjacent scores.

3 Results

3.1 SSMD' assay quality assessment for *N. brasiliensis* L3

The quality of xWORM assays with *N. brasiliensis* L3 were affected by both media concentration and parasite density (Figure 1). These trends also varied between media types (PBS/DMEM) and over time (24- and 48-hour treatment time points). PBS conditions that produced good to excellent assay scores over 24 hours ranged between ≈ 3.13 –25% media concentration with 67–1000 L3/200- μ L well volume (Figure 1A). This range was slightly more restricted when measuring over 48 hours, where ≈ 3.13 –25% media concentration and 125–800 L3/200- μ L well volume yielded good to excellent assay scores (Figure 1B). However, PBS conditions that had the highest assay quality (> 7 , excellent assay) over time were with a media concentration of ≈ 3.13 –6.25%, coupled with a parasite density of 250–500 L3/200 μ L well volume. DMEM conditions that produced good to excellent assay scores over 24 hours had a slightly broader range inclusive of 3.13–100% media concentrations and parasite densities between 250–2000 L3/200- μ L well volume (Figure 1C). These conditions maintained mostly good to excellent assay scores over 48 hours, although some exhibited a slight drop in quality over time (Figure 1D). The DMEM conditions that maintained the strongest assay scores (> 7) between the 24- and 48-hour time points were 50–100% media with 1000–1500 L3/200- μ L well volume, 12.5–25% media with 500–1000 L3/200- μ L well volume, and 6.25–12.5% media with 1000–2000 L3/200- μ L well volume.

3.2 SSMD' assay quality assessment for *N. americanus* L3

The quality of xWORM assays with *N. americanus* L3 were affected by media concentration and parasite density (Figure 2). These trends also varied between the two media types as well as over time. PBS conditions yielding good to excellent assay scores over 24 hours were ≈ 3.13 –30% media concentration with a parasite density of ≈ 250 –2000 L3/200- μ L well volume (Figure 2A). These conditions maintained mostly high SSMD' scores, with only slight variance over 48 hours (Figure 2B). The PBS conditions with the highest assay quality over time were ≈ 3.13 –12.5% media with an in-well parasite density between ≈ 1500 –2000 L3/200- μ L well volume. DMEM conditions with *N. americanus* L3 exhibited a similar pattern to what was observed for PBS conditions (Figures 2C, D). Good to excellent assay scores were recorded for all DMEM media concentrations (3.13–100%) with parasite densities between 500–2000 L3/200- μ L well volume over both the 24- and 48-hour time periods. However, assay scores falling within the excellent category

TABLE 1 Classification of assay quality using SSMD' values based on Zhang (2011).

Assay quality	Absolute SSMD'
Excellent	> 7
Good	5–7
Inferior	3–5
Poor	0–3

SSMD', strictly standardized mean difference prime.

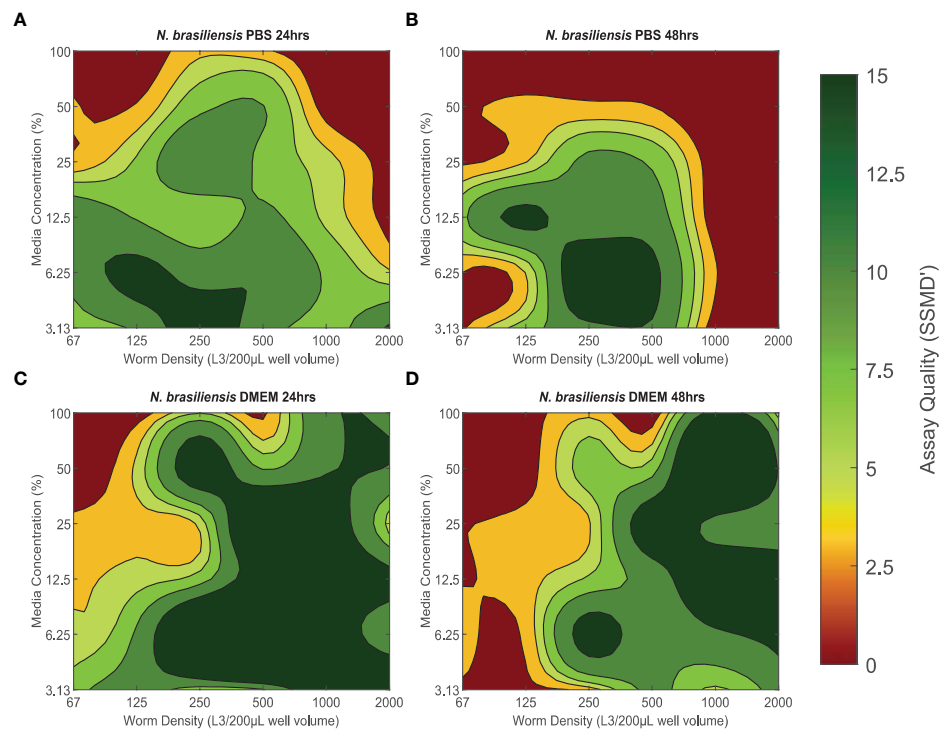


FIGURE 1

(A–D) The effect of baseline conditions on *Nippostrongylus brasiliensis* L3 xWORM [xCELLigence Worm Real-Time Motility] assay quality over time. SSMD' [Strictly standardized mean difference prime] was used to assess xWORM assay quality for the infective larval (L3) stage of the rodent hookworm (*N. brasiliensis*). SSMD' scores were calculated for each baseline condition combination of media type PBS [Phosphate-Buffered Saline] or DMEM [Dulbeccos Modified Eagle's Medium], media concentration (3.13–100%), and in-well parasite density (67–2000 L3/200 µL well volume) at two time points (24 and 48 hours). Isoleths are defined as the mean SSMD' score, where a score of < 3 indicates a poor assay; 3–5, an inferior assay; 5–7, a good assay; and > 7, an excellent assay.

were much less abundant at the 48-hour time point than at the 24-hour time point.

4 Discussion

The results of this study demonstrate that assay quality is affected by the combination of media type, media concentration, and in-well parasite density selected, as well as assay duration. We further show that the effect these parameters have on assay quality differs between two closely related hookworm species of the same life stage, *N. brasiliensis* and *N. americanus* L3. For instance, *N. americanus* performed better in PBS media over longer time periods than *N. brasiliensis*. However, *N. brasiliensis* performed better overall in DMEM media. Additionally, in PBS media, higher densities of *N. americanus* resulted in a stronger assay in most cases. Conversely, this resulted in a weak assay for *N. brasiliensis*, particularly with high concentrations of PBS media, and assays conducted over longer periods of time.

Since its conception, the xWORM assay has been used to measure the motility of numerous species and life stages of helminths. However, one of the most striking elements within the methodology of the published literature is that selected assay conditions including media type, media concentration, and in-well parasite density, vary considerably. Across several studies, the

type of media used in xWORM assays has been selected to mimic the natural environment of the parasite at that life stage (Smout et al., 2010; Rinaldi et al., 2015). For instance, as adult and some later larval stages are known to occur *in vivo*, cell culture medium [i.e., DMEM or Roswell Park Memorial Institute (RPMI)] was selected in attempt to replicate the conditions experienced inside a host animal. Conversely, as eggs and early larval stages commonly occur in the environment, such as in soil or bodies of freshwater, saline solutions (i.e., PBS) are often selected. Nonetheless, the distinct preferences of human and rodent hookworm larvae for DMEM and PBS media suggest that choosing the appropriate medium may be more complex than previously assumed. This study highlights that factors such as media concentration and in-well parasite density should be considered in conjunction with media type for a more comprehensive approach. However, in previous xWORM studies, these conditions have frequently been selected based on subjective choice or constrained by resource availability, rather than systematic evaluation. In some instances resource constraints, possibly combined with an unsuitable pairing of media type and concentration, have resulted in the failure of the assay. According to a study by Tritten et al. (2012), the third-larval stage of the hookworm *Ancylostoma ceylanicum* was immeasurable at densities of 100 L3/well which was presumed to be related to the activity level of the species. On the other hand, previously successful assays, particularly those involving larval life stages, have utilized

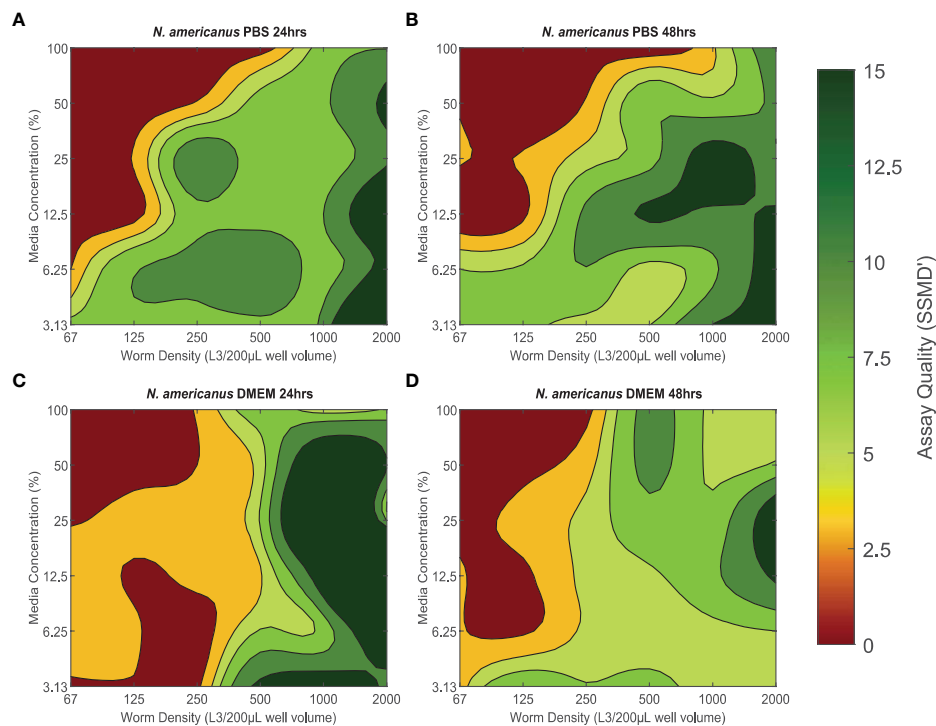


FIGURE 2

(A–D) The effect of baseline conditions on *Necator americanus* L3 xWORM [xCELLigence Worm Real-Time Motility] assay assay quality over time. SSMD' [Strictly standardized mean difference prime] was used to assess xWORM assay quality for the infective larval (L3) stage of the human hookworm (*N. americanus*). SSMD' scores were calculated for each baseline condition combination of media type (PBS [Phosphate-Buffered Saline] or DMEM [Dulbeccos Modified Eagle's Medium]), media concentration (3.13–100%), and in-well parasite density (67–2000 L3/200 µL well volume) at two time points (24 hours and 48 hours). Isopleths are defined as the mean SSMD' score where < 3 indicates a poor assay; 3–5, an inferior assay; 5–7, a good assay; and > 7, an excellent assay.

high numbers of parasites. One study, for example, measured *Haemonchus contortus* L3 at a density of 3000 L3/well, and *Strongyloides ratti* L3 at a density of 300 L3/well (Smout et al., 2010). Similarly, using SSMD' as an indicator for assay quality, another study determined that 4500 *Schistosoma mansoni* cercariae/well yielded the strongest assay for the selected media (10% PBS) and duration (16–20 hours; Rinaldi et al., 2015). This has resulted in the belief that xWORM assays require large numbers of parasites to be successful (Tritten et al., 2012). However, obtaining high numbers of parasites can be challenging due to the complex life cycle of most parasite species, which involves multiple morphological stages, and sometimes, several host animals (Ahmed, 2014). This can render artificially simulating the parasites natural conditions difficult from both a cultivation and experimental perspective (Ahmed, 2014).

In experimental settings, ensuring suitable conditions for monitoring parasite health *in vitro* becomes increasingly challenging when researchers must simultaneously accommodate the specific requirements of the measuring system in use. For example, to assess parasite motility through changes in electrical impedance using the xCELLigence system, an ionic solution is required in the measuring environment. The ionic strength of the media needed for the system to accurately monitor parasite motility is likely influenced by various ecological and physiological factors related to the study organism, particularly physical size and activity

level. However, the tolerance of the study organism to these conditions is also anticipated to differ significantly among parasite species and life stages. Hence, when selecting assay conditions, it is crucial to strike a balance between meeting the system's requirements and preserving the study organism's viability to ensure the accuracy and success of the research. It is crucial to recognize that the influence of artificial conditions on parasite behavior and sensitivity, especially in relation to treatments, is currently unknown. Nevertheless, striving to simulate the natural environment of the parasite as closely as possible is likely the most productive set of conditions for xWORM assays.

Previous studies have shown that the sensitivity of impedance measurements captured by xCELLigence can also be improved by modifying the system's frequency settings (Rinaldi et al., 2015). This proved to be particularly useful when measuring the hatching frequency of *S. mansoni* eggs, which require near-freshwater conditions to maintain their viability (Rinaldi et al., 2015). When this was combined with the appropriate combination of media type, media concentration, and in-well parasite density tailored to the study organism and intended assay duration, we showed that smaller parasite numbers can yield a strong assay. Although the overall strongest assay conditions for both *N. brasiliensis* and *N. americanus* were determined to involve high parasite numbers (> 125 L3/200-µL well volume), as little as 67 L3/200-µL well volume yielded high scores under certain conditions, specifically

with a low media concentration, and shorter assay duration. However, although frequency was not considered by this study due to the promising results obtained at 25 MHz (Rinaldi et al., 2015), parasites or specific conditions may benefit from additional frequency optimization. Similarly, while high numbers of *S. mansoni* (4500 cercariae/well) in select conditions reportedly produced the strongest assay, a good assay could still be achieved with 562 cercariae/well (Rinaldi et al., 2015). Our results suggest that adjusting media type and/or concentration may potentially further allow for lower cercariae densities while maintaining a high assay quality.

While this study offers a preliminary framework for optimizing the xWORM assay, the effect of several other critical assay parameters, including pH, temperature, and the availability of nutrients and oxygen, requires further investigation. For instance, our findings illustrated that increased larval densities could sometimes impair assay quality, as evidenced in the case of *N. brasiliensis* L3 in PBS media. A plausible explanation for the observed detrimental effects associated with increased larval densities could be fluctuations in critical media parameters, such as pH, over time. This phenomenon is known to be associated with decreased viability of mammalian cells maintained *in vitro*. As cells generate waste products (for instance, lactate), they can exceed the buffering capacity of the culture media, especially when maintained at high densities and/or over prolonged durations, leading to pH fluctuations (Gaze et al., 2014). Similarly, hookworm adults, including *N. americanus*, excrete and secrete proteinaceous material which that reportedly enables the parasite to avoid detection by the host's immune system (Hewitson et al., 2009; Logan et al., 2020). Following manual extraction from the host and subsequent *in vitro* cultivation, viable adult hookworms continue to generate excretory and secretory (ES) products and reproductive materials (Logan et al., 2020), which over time likely affects parasite vitality. Although it is currently uncertain, it is plausible that third-stage larvae also generate potentially toxic by-products, the effects of which could be exacerbated by high in-well larval densities.

Conversely, as exemplified by *N. americanus* L3 in both PBS and DMEM media, high parasite densities were sometimes required for preserving parasite vitality. Higher in-well parasite densities may be advantageous when monitoring the motilities of certain species such as *N. americanus*, which could exhibit lower activity levels. In this scenario, higher numbers would be required to amplify motility signals. Alternatively, these species might experience stimulation through body contact. For instance, third-stage larvae are known to detect hosts in nature through positive thigmotaxis (Haas et al., 2005; Gaze et al., 2014). Moreover, upon hatching in culture, hookworm larvae rapidly disperse in all directions, moving toward the periphery of the plate (Camberis et al., 2003; Camberis et al., 2016). This behavior could represent an evolutionary adaptation aimed at enhancing the success of transmission to a host. Specifically, close proximity with other larvae might trigger a response in the larvae to move away from each other to expand their spatial footprint, thereby increasing the likelihood of encountering a viable host. However, this is purely speculative and requires a deeper understanding of parasite behavior.

Another factor to consider when selecting xWORM assay conditions is oxygen and nutrient availability. A recent study by Hass et al. (2023) reported that in-well media volume significantly impacts the metabolic activity of retinal pigment epithelium (RPE) cells by affecting oxygen and nutrient availability. Researchers may initially overlook this as a potential factor when measuring the motility of L3, as it is widely believed that they are in a state of arrested development and survive solely on the energy accumulated during previous larval stages (Gaze et al., 2014). However, studies have shown that various environmental stimuli can trigger larval metamorphosis and the resumption of feeding, including warmer temperatures and the presence of serum or host-specific secretions such as sweat (Hawdon and Schad, 1990; Kumar and Pritchard, 1994; Haas et al., 2005; Pasuralertsakul and Ngrenngarmert, 2006). The incubation temperature commonly used in xWORM trials (37°C) to stimulate worm motility could thus trigger an artificial metamorphosis in hookworm parasites from a free-living to a parasitic lifestyle and/or a resumption of feeding. This could potentially account for the observed decline in assay quality for *N. brasiliensis* L3 in PBS media over a 48-hour period. Therefore, future studies should aim to explore the effect of serum supplementation and oxygen availability on xWORM assay quality, particularly for studies conducted over longer time frames.

It is also worth considering that extended exposure to elevated temperatures may be detrimental to ensheathed hookworm larvae. As the sheath acts as a thermal buffer in a dynamic environment, prolonged heat exposure could trap heat and potentially decrease larval vitality. The rate at which larvae successfully shed their protective sheaths in response to artificially induced environmental stimuli, such as temperature or the presence of serum, is currently unknown and should be a focus of future research. However, this phenomena may explain the broad spectrum of optimal conditions identified in this study, potentially reflecting variations in the ability of third-stage larvae to undergo temperature-induced exsheathing and/or metamorphosis in the absence of a host.

In summary, the quality of xWORM assays is influenced by numerous interrelated parameters, including media type, media concentration, in-well parasite density, and assay duration. Furthermore, the effect of these parameters was found to vary between closely related helminth species at the same larval stage. This reveals that there are species-specific enviro-physiological differences in hookworms that can impact the conditions necessary for achieving high-quality xWORM assays. We anticipate that these effects are likely relevant to other helminth species and life stages as well. Moreover, we have emphasised the importance of several other critical assay parameters, including pH, temperature, and nutrient and oxygen availability, which should be considered in future works for their potential impact on larval viability and assay quality. This study highlights the need for a dedicated optimization phase and a deeper understanding of the conditions under which xWORM assays are conducted, specifically their effect on parasite physiology and behavior. By addressing these factors, researchers can ensure the generation of accurate and reliable results, ultimately contributing to more effective research in the future.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The robust collection of Motility Index (MI) and Strictly Standardised Mean Difference Prime (SSMD') figures generated for all conditions investigated in this study is publicly accessible through the following link: <https://doi.org/10.5281/zenodo.7927398>.

Ethics statement

The studies involving human participants were reviewed and approved by James Cook University Human Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by James Cook University Animal Research Ethics Committee.

Author contributions

DL-B, MS, and JS contributed to the conception and design of this study. DL-B, MS, and LB were involved in data collection. DL-B, JS, MS, and AL aided in data analysis and presentation. DL-B wrote the original draft. All authors contributed to the final article and approved the submitted version.

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Conflict of interest

The author AL declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpara.2023.1189872/full#supplementary-material>

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Chapter 9

The Anthelmintic Activity of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins and their Potential as Novel Therapeutics

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D Lennox-Bulow is the principal author of this manuscript and was extensively involved in all aspects of this work under the supervision of J Seymour. M Smout assisted with data collection, statistical analyses, and in reviewing earlier versions of this manuscript. A Loukas assisted with reviewing earlier versions of this manuscript and provided the parasites used in this work.

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9.1 Abstract

Parasitic gastrointestinal worms (i.e., helminths) remain a significant global health and economic burden. The increasing inefficacy of current anthelmintic drugs against parasitic diseases necessitates the discovery of novel therapeutic options. This study investigated the anthelmintic properties and therapeutic potential of stonefish ichthyocrinotoxins (i.e., secreted skin toxins). xWORM (xCELLigence Worm Real-Time Motility Assay) was used to evaluate the anthelmintic activity of ichthyocrinotoxins from two stonefish species, *Synanceia horrida* (Estuarine Stonefish) and *Synanceia verrucosa* (Reef Stonefish), against the infective third-stage larvae of *Nippostrongylus brasiliensis* (Rodent Hookworm). Both toxins demonstrated potent anthelmintic effects, with *S. horrida* ichthyocrinotoxin exhibiting greater potency ($IC_{50} = 196.0 \mu\text{g/mL}$) compared to ichthyocrinotoxin from *S. verrucosa* ($IC_{50} = 329.7 \mu\text{g/mL}$). Fractionation revealed that the anthelmintic activity of *S. verrucosa* is likely driven by synergistic interactions between the large ($>3 \text{ kDa}$) and small ($<3 \text{ kDa}$) components. In contrast, the small components isolated from *S. horrida* ichthyocrinotoxin were responsible for the majority of the observed activity, making them a more attractive therapeutic candidate. Furthermore, despite the cytotoxicity of crude *S. horrida* ichthyocrinotoxin against human skin and bile duct cell lines, the isolated small components exhibited potent anthelmintic effects ($IC_{50} = 70.5 \mu\text{g/mL}$) with negligible cytotoxicity ($<10\%$ decrease in survival at $100 \mu\text{g/mL}$). While further research is necessary to fully characterise these compounds and assess their clinical suitability, this study highlights the potential of stonefish ichthyocrinotoxins as a novel source of anthelmintic therapeutics.

9.2 Introduction

Parasitic gastrointestinal worms (or helminths) infect almost all taxa across the animal kingdom (Carlson *et al.*, 2020). It is estimated that over two billion people, or a little over 24% of the global population, are currently infected with helminths (W.H.O, 2023; Riaz *et al.*, 2020). The highest prevalence of helminth infections in humans is found in developing countries, particularly in places with poor sanitation and limited access to healthcare (W.H.O, 2023; Riaz *et al.*, 2020). Different helminths cause distinct pathophysiological conditions, depending on the niches they occupy, including intestinal issues (i.e., diarrhoea, abdominal pain), organ damage (i.e., blindness, kidney disease), dermatological conditions (i.e., skin irritation, skin rashes), malnutrition, and impaired growth and physical development (Piazzesi & Putignani, 2023; Segoviano-Lorenzo *et al.*, 2022). Beyond their toll on health and wellbeing, helminths also impose substantial economic losses, particularly in agricultural and aquacultural sectors, by compromising the productivity of livestock and the market value of animal products (Menconi *et al.*, 2023; Rinaldo *et al.*, 2021; Charlier *et al.*, 2020). Such production losses can also affect consumers, as reduced supply can strain the market, which in turn, drives up retail prices (Menconi *et al.*, 2023; Begley *et al.*, 2019). The devastating impact of helminths on health and the economy is further compounded by the emergence of singular or multi-drug resistance across numerous parasite species. It is now well-established that resistance has developed against all classes of anthelmintic drugs used against helminth species that infect livestock (Fissiha & Kinde, 2021). Moreover, within the last decade, cases of drug resistance have also emerged for helminth species that infect companion animals (Prichard, 2021; Bourguinat *et al.*, 2015) and, concerningly, humans (Orr *et al.*, 2019; Crellen *et al.*, 2016). Novel anthelmintic treatment options with distinct mechanisms of action are thus urgently needed to address this issue (Jayawardene *et al.*, 2021; Nixon *et al.*, 2020; Zajickova *et al.*, 2020). Nature has already provided a profusion of modern therapeutics, including analgesics (i.e., penicillin, opium, and aspirin), decongestants (i.e., pseudoephedrine/Sudafed), cholesterol lowering medications (i.e., statins), anthelmintics (i.e., ivermectin), and much more (Jayawardene *et al.*, 2021; McCurdy & Scully, 2005). Yet, a vast reservoir of unexplored compounds remains, some of which are likely to have therapeutic potential. Animal toxins, which include venoms and poisons, are particularly promising for biodiscovery due to their highly diverse, dynamic, and often complex composition (Luddecke & Blank, 2024).

Stonefish (*Synanceia* spp.) stand out as being one of few organisms known to possess two distinct types of toxins: a potent defensive venom associated with each of their dorsal spines, and a secreted skin toxin called ichthyocrinotoxin (Halstead, 1988; Cameron *et al.*, 1981). While stonefish are widely distributed throughout the shallow, coastal waters of the Indo-Pacific, two species inhabit Queensland waters; *Synanceia horrida* (Estuarine Stonefish) which are primarily found inshore amongst rocky intertidal areas and estuaries, and *Synanceia verrucosa* (Reef Stonefish), which are commonly associated with coral reefs (Endean, 1961). Stonefish generally adopt a cryptic, benthic, and predominantly sedentary lifestyle; a strategy that likely assists with prey capture and predator avoidance (Lennox-Bulow *et al.*, 2024; Endean, 1961). However, the intimate association that stonefish have with the benthos, combined with their lack of squamation (i.e., scales) (Cameron *et al.*, 1981; Endean, 1961), should render these animals highly susceptible to parasites, especially those, like some helminths, that burrow through the skin.

Parasites are ubiquitous in the aquatic realm, and as such, their occurrence in fish is common and widely reported, particularly for species targeted or reared for consumption (Timi & Buchmann, 2023; Suthar & Shamsi, 2021). While investigations into the abundance and diversity of parasites in members of the genus *Synanceia* (including stonefish) are limited, they appear to harbour a surprisingly low diversity of internal parasite taxa (Miller *et al.*, 2018). One hypothesis for the paucity of internal parasites in stonefish may be that their ichthyocrinotoxins afford the skin some protection (Cameron & Endean, 1973). Although the role of stonefish ichthyocrinotoxins in parasite defence is currently unknown (Lennox-Bulow *et al.*, 2023b), a previous study on ichthyocrinotoxic *Gobiodon* species supports the hypothesis that some ichthyocrinotoxins may aid in preventing parasitism (Munday *et al.*, 2003). This study observed that *Gobiodon* ichthyocrinotoxins influenced the attachment sites of external parasites (gnathiid isopods), with parasites demonstrating a preference for areas, such as the fins, where the toxin glands were less abundant (Munday *et al.*, 2003).

Furthermore, a previous study demonstrated the broad-spectrum toxicity of stonefish ichthyocrinotoxins against invertebrates. Specifically, *S. horrida* ichthyocrinotoxin was found to inhibit the cilia of invertebrates, including those associated with mussel gills, and protozoan locomotory appendages (Cameron *et al.*, 1981). The toxin was also found to increase the tone of barnacle depressor scutorum rostralis muscles (i.e., the muscle that controls the scuta lips) for up to one hour after immersion (Cameron *et al.*, 1981). Additionally, while

ichthyocrinotoxins secreted by other fish commonly exhibit both ichthyotoxic and hemotoxic properties (Lennox-Bulow *et al.*, 2023b), stonefish ichthyocrinotoxins do not appear to follow suit. Ichthyocrinotoxins from *S. horrida* were found to demonstrate negligible protease, haemolytic and haemagglutinating activity, and were only mildly toxic to mosquito fishes and blennies (Cameron *et al.*, 1981). However, *S. horrida* ichthyocrinotoxin was found to inhibit contractions in guinea pig ileum and vas deferens (Hoy & Cameron, 1986; Cameron *et al.*, 1981). This suggests that while the toxin may have some effect on vertebrates, it appears to be more potent against invertebrates, which potentially include helminths. Moreover, the apparent limited toxicity of stonefish ichthyocrinotoxins to vertebrates warrants further investigation into its therapeutic potential.

Therefore, the aim of this study was to investigate the anthelmintic properties of stonefish (*Synanceia* spp.) ichthyocrinotoxins as a possible explanation for the paucity of endoparasites observed in these animals. Furthermore, this study evaluated the therapeutic potential of stonefish ichthyocrinotoxins by 1) identifying the components responsible for the toxin's anthelmintic activity against *Nippostrongylus brasiliensis* L3 (infective Rodent Hookworm larvae) and, 2) determining the cytotoxicity of these components against various mammalian cell lines (human skin cells, human bile duct cells, as well as human and rat red blood cells) *in vitro*.

9.3 Materials and Methods

9.3.1 *Ichthyocrinotoxin Collection and Parasite Cultivation*

Ichthyocrinotoxin Collection.

Ichthyocrinotoxin from *S. verrucosa* and *S. horrida* were collected following the protocol outlined by Lennox-Bulow *et al.* (2023c). In brief, a single specimen (*S. horrida* or *S. verrucosa*, 100 to 400 mm body length) was transferred to a damp surface and secured by gently holding either side of the pectoral fins. A saltwater-dampened cloth was placed over the animal's eyes to minimise stress throughout the process. Ichthyocrinotoxin was then extracted from the skin tubercles using targeted mechanical massage under a controlled vacuum. This vacuum was generated by a purpose-built extraction apparatus consisting of a 100 mm segment of clear vinyl tubing affixed to a 50 mL syringe. To account for potential individual variation in toxin composition, ichthyocrinotoxin was extracted and pooled from three to five individuals of each species. Pooled ichthyocrinotoxin samples were stored in 1.5 mL Eppendorf tubes at -80°C until required. The concentration of crude ichthyocrinotoxin was determined for both species using a standard PierceTM Bicinchoninic Acid (BCA) Protein Assay kit, following the manufacturer's instructions.

Parasite Cultivation.

Nippostrongylus brasiliensis L3 were cultivated following a protocol adapted from Camberis *et al.* (2003). Briefly, *N. brasiliensis* L3 were suspended in 1x Phosphate-Buffered Saline (PBS) at a density of 2,500 to 3,000 L3/250 μL . Two female Sprague-Dawley rats were then subcutaneously injected between the shoulder blades with 250 μL of the larval suspension in accordance with James Cook University Animal Ethics approval (A2626). The rats were then monitored daily for any physical or behavioural abnormalities, and on the fifth day post-infection, they were transferred to new cages with clean bedding. Infected faecal pellets were collected on the sixth- and seventh-days post-infection. The faecal pellets were saturated in reverse-osmosis (R/O) water and blended into a slurry. The slurry was then combined with granulated and fine particulate activated charcoal at a ratio of roughly 1-part faecal slurry:2-parts granulated charcoal:1-part fine particulate charcoal or until the mixture resembled natural soil. This mixture was evenly spread across sterile 90 mm plastic petri dishes lined with R/O water-dampened filter paper (Whatman[®] Buckinghamshire, United Kingdom 80 mm rounded, Grade 4, 20-25 μm pore size). The petri-dishes containing the faecal culture were then set to incubate undisturbed in the dark at 26°C for five days to allow the larvae to hatch and migrate

to the periphery of the plates. *Nippostrongylus brasiliensis* L3 were gently washed from the plate sides and lid with a Pasteur pipette and R/O water. The suspension was transferred to a 50 mL Falcon tube and brought to the nearest 10 mL gradation with R/O water. To quantify the number of *N. brasiliensis* L3 harvested, 100 μ L of larval suspension was diluted 1:10 with R/O water. Then, 100 μ L of diluted larval suspension was aliquoted on to a clean 90 mm plastic petri dish and larvae were counted under a top light microscope. The total number of larvae harvested was extrapolated by multiplying counts by the dilution factor (DF = 100).

9.3.2 Measuring Hookworm Larvae Motility with xWORM

Measuring Hookworm Larvae Motility.

The effect of stonefish ichthyocrotoxins on the motility of *N. brasiliensis* L3 was assessed using a modified version of xWORM (xCELLigence Worm Real Time Motility Assay) adapted from Rinaldi *et al.* (2015). Assay parameters including media type, media concentration, and in-well parasite density were selected for *N. brasiliensis* L3 based on the findings from Lennox-Bulow *et al.* (2023a). Briefly, two 96-well E-plates were pre-loaded with 100 μ L of 0.25x PBS and 2% Antibiotic-Antimycotic (AA) solution in each well. The E-plates were then placed on an xCELLigence Real-Time-Cell-Analyser (RTCA) single plate (SP) instrument to undergo an initial background measurement. The xCELLigence SP instrument was incubated in a temperature-controlled cabinet maintained at 37°C and 5% CO₂ for the duration of the experiment to simulate the internal conditions of a mammalian host, and thus, stimulate L3 motility. For each E-plate, a total of 48 wells were loaded with 50 μ L of live *N. brasiliensis* L3 suspension (300 L3/50 μ L of 0.25x PBS larval suspension), and a further 24 wells received 50 μ L of heat-killed (95°C for 5 min) L3 suspension at the same density. To mitigate the effect of media evaporation during incubation, parasites were only added to the inner wells of the E-plates. Wells along the outer perimeter of the plates received 50 μ L 0.25x PBS with 2% AA and were excluded from subsequent analysis. The motility of *N. brasiliensis* L3, which was used as a proxy for hookworm survival, was continuously monitored at 25 kHz with regular user-defined readings taken every 15 seconds over a 72-hour period. The larvae were acclimated to the experimental conditions for 20-hours prior to the administration of treatment.

Data Transformation to Motility Index/Percent Parasite Survival.

Raw Cell Index readings from RTCA Software Pro version 2.0 were exported for processing into motility index, a measure of the standard deviation of the difference from the rolling Cell Index mean over time, as described by Smout *et al.* (2010). To account for background noise, motility index data were blank-adjusted by subtracting each data point of each treatment group from the mean of its corresponding positive control group (i.e., heat-killed L3 that had received the same dosage of *S. horrida* or *S. verrucosa* ichthyocrinotoxin). Variations in baseline motility between live *N. brasiliensis* L3 wells was compensated for by identifying the highest motility value across all wells at a single pre-treatment time point (19 hours post assay commencement) and dividing this by the motility index recorded for each individual well at that time point. This yielded an adjustment factor for each well, which was then applied by multiplying this factor by all proceeding motility index values (post-treatment). Adjusted motility index values were then converted to percent motility, herein referred to as hookworm survival, by calculating the relative motility index of each treatment group compared to the negative control (wells containing live L3 that received 0.25x PBS only) and multiplying by 100.

9.3.3 Assessing the Anthelmintic Activity of Stonefish Ichthyocrinotoxins and Identifying Hookworm-Toxic Components

xWORM and data transformation procedures detailed in Section 9.3.2 were employed for all experiments outlined in this section. To evaluate the effect of ichthyocrinotoxin samples on the survival of *N. brasiliensis* L3, we exposed the parasites to toxins for one hour prior to all subsequent analyses. This exposure duration was chosen to mimic a plausible infection scenario, as the parasite is expected to penetrate a host's skin within this timeframe.

Determining the Effect of Crude Stonefish Ichthyocrinotoxins on Hookworm Larvae Survival.

To determine the effect of stonefish ichthyocrinotoxins on the survival of *N. brasiliensis* L3, crude samples of ichthyocrinotoxin from *S. horrida* and *S. verrucosa* were serially diluted (1-part toxin:2-parts 0.25x PBS) to eleven concentrations that were four times more concentrated than the final in-well treatment concentration. Treatment involved the addition of 50 μ L of crude 4x concentrated *S. horrida* or *S. verrucosa* ichthyocrinotoxin samples to 150 μ L of parasite/0.25x PBS mixture (total well volume 200 μ L). The final in-well ichthyocrinotoxin treatment concentrations applied to *N. brasiliensis* L3 ranged from 6 to 7,200 μ g/mL for both

species' toxins. Each condition (i.e., toxin concentration and stonefish species combination) was replicated four times on live parasite wells which were assigned as the treatment group, and two times on heat-killed parasite wells which served as a positive control group (i.e., 0% survival). An additional four wells containing live parasites served as the negative control group (i.e., 100% survival) and received 50 μ L of 0.25x PBS with 2% AA in lieu of ichthyocrinotoxin.

A four-parameter logistic regression model (log-inhibitor vs normalised response, variable slope) was used to analyse the relationship between ichthyocrinotoxin concentration and *N. brasiliensis* L3 survival. Hookworm survival (%) one hour after receiving treatment was used as the dependent variable, $\log_{10}$ -transformed ichthyocrinotoxin concentration was assigned as the independent variable, and stonefish species (*S. horrida* and *S. verrucosa*) that the ichthyocrinotoxin was sourced from was the covariate. The concentration of ichthyocrinotoxin required to inhibit parasite survival by 50% (IC_{50}) was calculated for each stonefish species using the fitted relationship. A two-tailed t-test was then used to compare IC_{50} values between *S. horrida* and *S. verrucosa* ichthyocrinotoxin treatments following a method outlined by Motulsky (1998).

Isolating the Large (> 3kDa) and Small (< 3kDa) Components from Crude Toxin.

To begin to identify the components responsible for the anthelmintic activity, crude ichthyocrinotoxin samples from *S. horrida* and *S. verrucosa* were each fractionated into two component groups: large (> 3kDa) components and small (< 3kDa) components. In brief, crude ichthyocrinotoxin (400 μ L) was diluted in 4 mL of Milli-Q water and placed in the filter compartment of a centrifugal filter device (Amplicon Millipore Ultra) with a 3 kDa molecular weight cutoff. The device was then centrifuged at 4000 $\times g$ at 4°C in 10 min intervals until the sample volume in the filter compartment was reduced to 100 μ L. Both the retained sample (large components) and the filtrate (small components) were collected. The filtrate was subsequently lyophilized to remove excess water. Both large and small component samples were then diluted/rehydrated to a final volume of 500 μ L each. Protein concentrations were quantified using a standard Pierce™ BCA Protein Assay kit, following the manufacturer's instructions. The protein concentrations of the isolated small and large component groups were used to estimate their relative abundance within the original crude toxin mixture. The abundance of each component group within the crude toxin was determined to be approximately equal.

Measuring the Dose-Dependent Effect of Isolated Toxin Components Isolated and Comparing their Activity Against Crude Toxin.

xWORM was used to determine whether the large and small components isolated from stonefish ichthyocrotoxins exhibited a dose-dependent effect on the survival of *N. brasiliensis* L3. The small (< 3 kDa) components isolated from stonefish ichthyocrotoxins were serially diluted (1-part toxin:2-parts 0.25x PBS) to yield nine treatment concentrations ranging from 3 to 600 µg/mL for *S. horrida*, and eight treatment concentrations ranging from 3 to 345 µg/mL for *S. verrucosa*. The large (> 3 kDa) components isolated from stonefish ichthyocrotoxins were also serially diluted (1-part toxin:2-parts 0.25x PBS) to three treatment concentrations ranging from 341 to 1,368 µg/mL for *S. horrida*, and four treatment concentrations ranging from 51 to 843 µg/mL for *S. verrucosa*. Treatment was administered to *N. brasiliensis* L3 as described in section 9.3.3.

Separate four-parameter logistic regression models (log-inhibitor vs normalised response, variable slope) for each species toxin (*S. horrida* and *S. verrucosa*) were performed to analyse the relationship between ichthyocrotoxin concentration and *N. brasiliensis* L3 survival. Parasite survival (%) one hour after receiving treatment was used as the dependent variable, log₁₀-transformed ichthyocrotoxin concentration was assigned as the independent variable, and toxin type (isolated small components, isolated large components, crude toxin) was the covariate. The concentration of ichthyocrotoxin required to inhibit parasite survival by 50% (IC₅₀) was calculated for each toxin type and stonefish species using the fitted relationship.

9.3.4 Determining the Cytotoxicity of Stonefish Ichthyocrinotoxins to Mammalian Cell Lines In Vitro

Culturing Mammalian Cell Lines *In Vitro*.

Human normal dermal fibroblast (1BR.3.GN) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC, product code 90020509). The cholangiocyte cell line (H69), which is a SV40-transformed bile duct epithelial cell line derived from a non-cancerous human liver, was obtained in 2010 from Dr. Gregory J. Gores, Mayo Clinic, Rochester, Minnesota (Smout *et al.*, 2015). These cell lines will herein be referred to as human skin cells for 1BR.3.GN; and human bile duct cells for H69. Both cell lines were cultured according to manufacturer protocols. In brief, cells were seeded in 75 cm² filter top flasks with 15 mL of an appropriate complete growth medium and incubated at 37°C with 5% CO₂. The complete growth medium for human skin cells consisted of DMEM/Nutrient Mixture F12 with 10% Foetal Bovine Serum (FBS) and 1% Antibiotic-Antimycotic (AA). The complete growth medium for human bile duct cells comprised high glucose DMEM/Nutrient Mixture F12 with 10% FBS, 1% AA, Adenine (25 µg/mL), Insulin (5 µg/mL), Epinephrine (1 µg/mL), Holo-transferrin (8.3 µg/mL), Hydrocortisone (0.62 µg/mL), T3 enzyme mix (13.6 ng/mL), and Epidermal Growth Factor (10 ng/mL).

Cell lines were left to form a monolayer until reaching approximately 80% confluence before being passaged. Cells were then gently washed in 10 mL 1× PBS to remove debris, followed by detachment using 3 mL of 1× Tryple reagent (Gibco). Harvested cells were combined with 12 mL of their respective complete growth medium and centrifuged at 150× g for 5 minutes (Hettich ROTINA 420R Centrifuge). The resulting supernatant was discarded, and the remaining cell pellet was resuspended in fresh complete growth medium. The concentration of all cell lines was determined using a haemocytometer.

xCELLigence Cell Viability Assay.

The cytotoxicity of *S. horrida* and *S. verrucosa* ichthyocrinotoxin samples (crude and isolated small components) to human skin cells and human bile duct cells *in vitro* was assessed using a cell adhesion assay. Cell adhesion, which was used as a proxy for cell survival, was monitored in real time using an xCELLigence RTCA (Agilent) system. E-Plates were loaded with 50 µL of the appropriate complete growth medium and then placed on an xCELLigence RTCA single plate (SP) instrument to undergo an initial background measurement. Then, either 100 µL of human skin cell suspension or 100 µL of human bile duct cell suspension was

aliquoted in each well of a 96-well E-plate at a density of 5,000 cells/100 μ L. Eight E-plates were set up with one cell type (human skin cells or human bile duct cells), stonefish species ichthyocrinotoxin (*S. horrida* or *S. verrucosa* ichthyocrinotoxin), and sample type (crude sample or isolated small components) combination allocated per plate. All E-plates were then incubated at 37°C and 5% CO₂ throughout the duration of the experiment to mimic the internal thermal conditions of a mammal. Cells were left to form a monolayer on the bottom of the wells for 19 hours prior to the addition of treatment.

Samples of crude *S. horrida* and *S. verrucosa* ichthyocrinotoxin and small (< 3kDa) components isolated from *S. horrida* ichthyocrinotoxin were prepared by serial dilution (1-part toxin:2-parts complete growth medium). Treatment involved the addition of 50 μ L of 4x concentrated samples of crude *S. horrida* or *S. verrucosa* ichthyocrinotoxins or isolated small components from *S. horrida* ichthyocrinotoxin to wells containing 150 μ L of human skin or bile duct cells in their relevant complete growth medium. The final in-well treatment conditions were as follows: eleven concentrations of crude samples from *S. horrida* or *S. verrucosa* ichthyocrinotoxin ranging from 0.9 to 725 μ g/mL were applied to human skin cells; ten concentrations of crude samples of *S. horrida* or *S. verrucosa* ichthyocrinotoxin ranging from 0.5 to 275 μ g/mL were applied to human bile duct cells; eight concentrations of isolated small components samples from *S. horrida* ichthyocrinotoxin ranging from 5 to 600 μ g/mL were applied to human skin cells; and, nine concentrations of isolated small components samples from *S. horrida* ichthyocrinotoxin ranging from 3 to 700 μ g/mL were applied to human bile duct cells. Each treatment condition was conducted in triplicate. To establish a reference for 100% cell survival, three wells on each E-plate were designated as a negative control group and received 50 μ L of the relevant complete growth medium in lieu of ichthyocrinotoxin. Following treatment, cell progression was continuously monitored over 72 hours using xCELLigence RTCA (Agilent). The system performed 50 sweeps of the E-plate at intervals of 15 seconds for the first hour, 2 minutes for the second hour, 15 minutes for six hours, and 60 minutes thereafter, generating a continuous Cell Index trace (RTCA Software Pro).

Pre-treatment (19 hours post assay commencement) normalised Cell Index values were used to quantify the percent change in cell adhesion in response to treatment. Percent cell survival was calculated by dividing the normalised Cell Index values of each treatment condition by the average normalised Cell Index value of the negative control group and

multiplying by 100. Percent cell survival data were then exported to GraphPad Prism version 10 for dose-response analysis.

Haemotoxicity Assay.

The haemotoxicity of *S. horrida* and *S. verrucosa* ichthyocrinotoxin to human and rat red blood cell erythrocytes was assessed using an absorbance-based lysis assay outlined by Brinkman *et al.* (2014). Rat blood was collected via cardiac puncture from two female Sprague-Dawley rats that had been recently euthanised (James Cook University Animal Ethics Approval #A2626). Human blood was drawn from the vein (venipuncture) of a healthy donor with human research and ethics approvals provided by James Cook University (James Cook University Human Ethics Approval H8523). To isolate red blood cell erythrocytes, whole blood samples were centrifuged at 3000x g for 10 min at 4°C. Following centrifugation, the resulting supernatant was discarded, and the sedimented erythrocytes were resuspended in sterile 1x PBS. This process was repeated five times to thoroughly wash the erythrocytes and ensure the removal of any remanent plasma. A 1% erythrocyte suspension (approximately 42×10^6 cells/450 μ L), one each for human and rat erythrocytes, was prepared in 1x PBS. Human and rat erythrocyte suspensions (450 μ L) were each aliquoted into 1.5mL Eppendorf tubes.

Treatment involved the addition of 150 μ L of crude *S. horrida* or *S. verrucosa* ichthyocrinotoxin, or isolated small (< 3kDa) components from *S. horrida* ichthyocrinotoxin at various concentrations to individual 1.5 mL Eppendorf tubes containing 450 μ L of human or rat red blood cell erythrocytes ($n = 51$ tubes). Samples of crude *S. horrida* and *S. verrucosa* ichthyocrinotoxin and isolated small components from *S. horrida* ichthyocrinotoxin were first prepared by serial dilution (1-part toxin:2-parts 1x PBS) to a series of samples that were 4 times more concentrated than the final in-well treatment conditions. The following final in-well treatment concentrations were tested for haemolytic activity to human and rat red blood cell erythrocytes: ten concentrations of crude *S. horrida* or *S. verrucosa* ichthyocrinotoxin ranging from 1 to 1,243 μ g/mL were administered to rat red blood cells; eight concentrations of crude *S. horrida* or *S. verrucosa* ichthyocrinotoxin ranging from 10 to 4,400 μ g/mL were administered to human red blood cells; ten concentrations of isolated small component samples from *S. horrida* ichthyocrinotoxin ranging from 0.6 to 180 μ g/mL were administered to human blood cells; and, seven concentrations of isolated small components samples from *S. horrida* ichthyocrinotoxin ranging from 7.5 to 5000 μ g/mL were administered to rat red blood cells. Additionally, one tube per cell type (i.e., human/rat cells) and treatment group (i.e., crude *S.*

horrida ichthyocrinotoxin, isolated small components from *S. horrida* ichthyocrinotoxin, etc.) served as a positive control group (i.e., 100% cell lysis), which received 150 μ L of lysis solution (1% Triton X-100 in 1x PBS) in lieu of toxin ($n = 8$ tubes). Another tube per cell type and treatment group was designated as a negative control group (i.e., 100% cell survival), which received 150 μ L of 1x PBS ($n = 8$ tubes). All samples were then incubated for one hour at 37°C with gentle agitation.

After the incubation period, samples were chilled on ice for 5 mins, then centrifuged at 3000x g for 10 mins at room temperature. Then, 200 μ L of supernatant from each tube was transferred in triplicate to flat-bottomed 96-well plates. To account for background interference in absorbance readings, 200 μ L of 1x PBS (media only wells) was aliquoted into three empty wells. Liberated haemoglobin was quantified by measuring its absorbance at 540 nm using a POLARstar Omega microplate reader. Raw absorbance data were blank adjusted by subtracting the average absorbance of the media-only wells. Percent lysis was determined by dividing each blank-adjusted absorbance value of the treatment group by the average of the positive control group and multiplying by 100. Percent survival was calculated by subtracting percent lysis from 100.

Dose Response Analysis – Mammalian Cell Lines.

Separate four-parameter logistic regression models (log-inhibitor vs response, variable slope) for each species toxin (*S. horrida* and *S. verrucosa*) were performed to analyse the relationship between crude ichthyocrinotoxin concentration and cell survival one hour after treatment administration. The same regression model was used to analyse the relationship between the concentration of the small (< 3 kDa) components from *S. horrida* ichthyocrinotoxins administered and cell survival one hour post treatment. Cell survival (%) one-hour post-treatment was used as the dependent variable, $\log_{10}$ -transformed ichthyocrinotoxin concentration was used as the independent variable, and cell type (i.e., human skin cells, human bile duct cells, human red blood cells, and rat red blood cells) was assigned as the co-variate. Where possible, the half maximal inhibitory concentration (IC_{50}) of ichthyocrinotoxin for cell survival was calculated using the fitted relationship. In cases where only mild cytotoxicity was observed (i.e., where cell survival remained above 50%), the treatment concentration that resulted in a 20% inhibition (IC_{20}) in cell survival was determined using a nonlinear regression model (log agonist vs response – Find ECanything) with the following variables as constants: HillSlope = -1.0 , F = 80, Bottom = 0, Top = 100.

9.3.5 Comparing the Activity of Crude and Small Component Samples from S. horrida Ichthyocrinotoxin Across Targets

The effects of crude toxin and isolated small (<3 kDa) components from *S. horrida* ichthyocrinotoxin on survival rates were compared between parasite and cellular targets using a two-way ANOVA (IBM SPSS version 27). Percent target survival after one hour of exposure to treatment served as the dependent variable, while target (human skin cells, human bile duct cells, human red blood cells, rat red blood cells, and *N. brasiliensis* L3), and toxin type (crude toxin, isolated small components) were assigned as the independent variables. For the isolated small components, the highest achievable treatment concentration shared by all targets (100 µg/mL) was selected for comparison. For the crude toxin, a treatment concentration of 200 µg/mL was chosen for comparison to account for the estimated 50% composition of small components. Prior to analysis, the assumptions of normality and homoscedasticity were confirmed using Kolmogorov-Smirnov and Levene's Statistic, respectively. Following a significant ANOVA result, the post-hoc least significant difference (LSD) was used to identify specific target (cells/parasites) and toxin type (crude/small component) groups that exhibited statistically significant differences in survival. Analyses were conducted in IBM SPSS version 10.

9.4 Results

9.4.1 Assessing the Toxicity of Crude Stonefish Ichthyocrinotoxins to Infective Rodent Hookworm Larvae

A four-parameter logistic regression model (log inhibitor vs. normalised response, variable slope) was used to investigate the dose-dependent effect of crude stonefish ichthyocrinotoxins on the survival of *N. brasiliensis* L3 after one hour of exposure to treatment (**Figure 9.1**). A strong inhibitory dose-response relationship was observed for both *S. horrida* ($R^2 = 0.952$, $AICc = 199.5$, $HillSlope = -2.60$) and *S. verrucosa* ($R^2 = 0.893$, $AICc = 214.1$, $HillSlope = -3.11$) ichthyocrinotoxin treatments. A two-tailed *t*-test revealed a significant difference in the IC_{50} values between *S. horrida* and *S. verrucosa* ichthyocrinotoxins ($t_{0.05(2),36} = 4.32$, $p = 0.0001$), indicating that the anthelmintic activity of these toxins varies between the species. Specifically, ichthyocrinotoxin from *S. horrida* was significantly more toxic to *N. brasiliensis* L3 ($IC_{50} = 196.0 \mu\text{g/mL}$, 95% CI: 175.1 – 221.4 $\mu\text{g/mL}$) compared to ichthyocrinotoxin from *S. verrucosa* ($IC_{50} = 329.7 \mu\text{g/mL}$, 95% CI: 279.9 – 391.5 $\mu\text{g/mL}$).

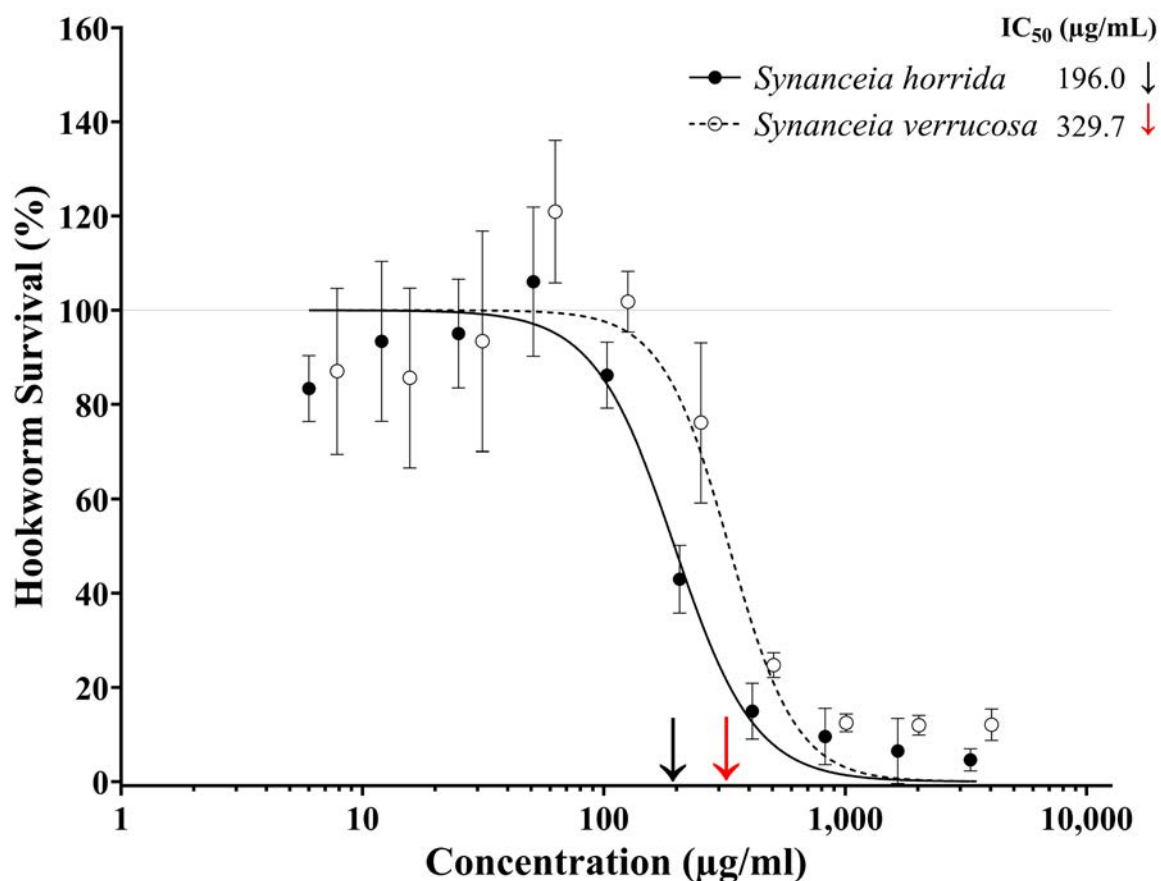


Figure 9.1 Dose-Dependent Effect of Crude Stonefish (*Synanceia* spp.) Ichthyocrinotoxins on Hookworm Larvae Survival. The motility of *N. brasiliensis* L3 (infective larval stage 3 Rodent Hookworm) relative to the negative (i.e., no toxin) control group has been used as an indicator for the percentage of hookworm larvae that survived following one hour of exposure to varying concentrations of *Synanceia horrida* (Estuarine Stonefish, —●—) or *Synanceia verrucosa* (Reef Stonefish, ---○---) ichthyocrinotoxins. The IC_{50} values for each species toxin were calculated from the fitted curve and marked on the x-axis with ↓ for *S. horrida*, and ↓ for *S. verrucosa*. Error bars represent 95% confidence intervals.

9.4.2 Identifying Components in Stonefish Ichthyocrinotoxins with Anthelmintic Activity

A four-parameter logistic regression model (log inhibitor vs. normalised response, variable slope) was used to investigate the dose-dependent effect of the large (>3 kDa) and small (<3 kDa) components isolated from stonefish ichthyocrinotoxins on the survival of *N. brasiliensis* L3 after one hour of exposure to treatment (**Figure 9.2**). A strong inhibitory dose-response relationship was observed for the small (>3 kDa) components isolated from *S. horrida* ichthyocrinotoxin (**Figure 9.2A**, $R_2 = 0.78$, $AICc = 238.6$, $HillSlope = -3.83$). Given that these small components constitute approximately 50% of the crude *S. horrida* ichthyocrinotoxin mixture, their potency to *N. brasiliensis* L3 ($IC_{50} = 70.5 \mu\text{g/mL}$, 95% CI: 55.9 – 92.6 $\mu\text{g/mL}$) was comparable to that of crude toxin ($IC_{50} = 196.0 \mu\text{g/mL}$, 95% CI: 173.9 – 222.1 $\mu\text{g/mL}$). In contrast, much higher concentrations (ten times greater) of the large components isolated from *S. horrida* ichthyocrinotoxin were required to induce a modest 20% inhibitory effect on *N. brasiliensis* L3 survival ($IC_{20} = 990.0 \mu\text{g/mL}$, 95% CI: 806.2 – 1,270.0 $\mu\text{g/mL}$, $R_2 = 0.74$, $AICc = 45.7$, $HillSlope = -1.85$) compared to crude toxin. While crude *S. verrucosa* ichthyocrinotoxin elicited a strong dose-dependent inhibitory effect on hookworm survival ($IC_{50} = 329.7 \mu\text{g/mL}$, 95% CI: 279.9 – 391.5 $\mu\text{g/mL}$), both the isolated large and small components had minimal effect across all tested treatment dosages (**Figure 9.2B**). Only high concentrations of the large components (>500 $\mu\text{g/mL}$) resulted in a slight decrease (~10%) in hookworm survival.

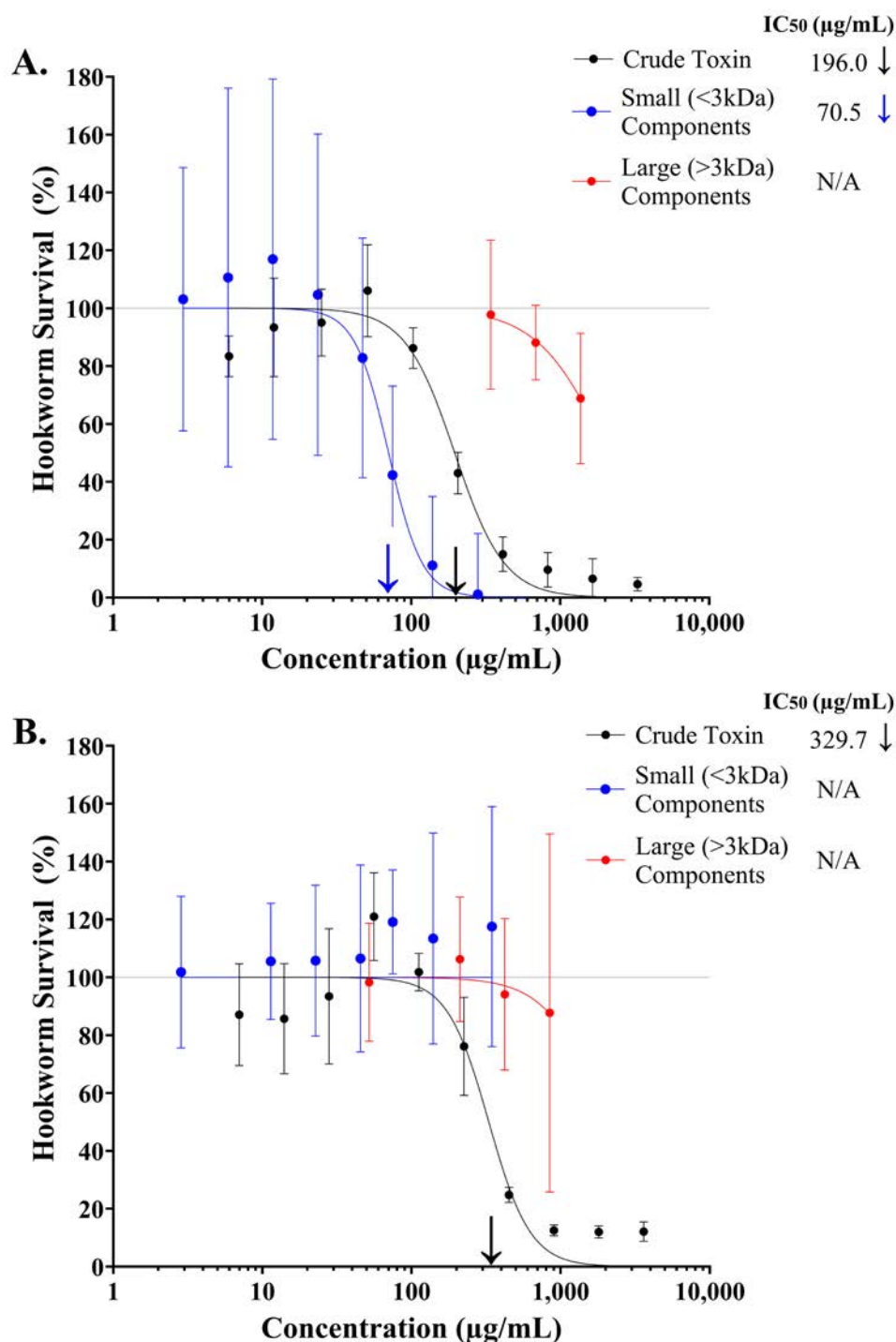


Figure 9.2 The Anthelmintic Activity of the Isolated Components from Stonefish (*Synanceia* spp.) Ichthyocrinotoxins. Dose-response analysis was used to determine the anthelmintic activity of the small (<3 kDa, —●—) and large (>3 kDa, —●—) components isolated from *Synanceia horrida* (Estuarine Stonefish, Panel A), and *S. verrucosa* (Reef Stonefish, Panel B) ichthyocrinotoxins against crude toxin (i.e., unprocessed, —●—). Parasite motility relative to the negative (i.e., no toxin) control group has been used as an indicator for the percentage of *N. brasiliensis* L3 that survived following one hour of exposure to each treatment. Error bars represent 95% confidence intervals. Arrows mark the IC₅₀ values for crude (↓) and isolated small components (↓) from stonefish ichthyocrinotoxins on *N. brasiliensis* L3.

9.4.3 Assessing the Cytotoxicity of Crude Stonefish Ichthyocrinotoxin to Mammalian Cell Lines In Vitro

A four-parameter logistic regression model (log inhibitor vs. response, variable slope) was used to investigate the dose-dependent effect of crude stonefish ichthyocrinotoxins on the survival of several mammalian cell lines *in vitro*, following one-hour of exposure to treatment (**Figure 9.3**). Both *S. horrida* (**Figure 9.3A**) and *S. verrucosa* (**Figure 9.3B**) ichthyocrinotoxin had minimal effect on the survival of human red blood cells across all tested concentrations (*S. horrida*: $R_2 = 0.32$, $AICc = 2.35$, $HillSlope = -0.23$, and *S. verrucosa*: $R_2 = 0.88$, $AICc = -42.25$, $HillSlope = -1.570$). Similarly, both species toxins also had minimal effect on rodent red blood cells (*S. horrida*: $R_2 = 0.90$, $AICc = 6.12$, $HillSlope = -0.41$, and *S. verrucosa*: $R_2 = 0.80$, $AICc = 29.37$, $HillSlope = -0.399$), however, high treatment concentrations ($>250 \mu\text{g/mL}$) elicited a non-significant decrease in survival (up to 10%). Additionally, crude *S. verrucosa* ichthyocrinotoxin exhibited no inhibitory effect on the survival of human skin cells ($R_2 = 0.66$, $AICc = 141.6$, $HillSlope = 0.068$) or human bile duct cells ($R_2 = 0.65$, $AICc = 94.03$, $HillSlope = 0.22$) across all tested concentrations. Instead, the toxin appeared to stimulate the proliferation of both cell types, with a maximum effect of approximately 40% and 20% for skin and bile duct cells, respectively. In contrast, crude *S. horrida* ichthyocrinotoxin exhibited a strong dose-dependent inhibitory effect on the survival of human skin cells ($R_2 = 0.75$, $AICc = 135.9$, $HillSlope = -3.479$), with an IC_{50} of $146.3 \mu\text{g/mL}$ (95% CI: $128.7 - 170.7 \mu\text{g/mL}$). While crude *S. horrida* ichthyocrinotoxin also exerted an inhibitory effect on human bile duct cell survival ($R^2 = 0.43$, $AICc = 129.5$, $HillSlope = -3.361$), its potency against these cells was notably lower, requiring a concentration of $274.6 \mu\text{g/mL}$ (95% CI: $242.5 - 324.9 \mu\text{g/mL}$) to achieve only a 20% reduction in survival. Furthermore, at a concentration that resulted in 50% parasite mortality (IC_{50}), crude *S. verrucosa* ichthyocrinotoxin had no negative effect on the survival of human bile duct and skin cells and had minimal effect on human and rat red blood cells. Similarly, crude *S. horrida* ichthyocrinotoxin exerted minimal effect ($>10\%$ reduction in survival), on the survival of human or rat red blood cells, or human bile duct cells at the concentration that resulted in 50% hookworm death. In contrast, *S. horrida* ichthyocrinotoxin exerted a similar toxic effect on human skin cells, as it did on *N. brasiliensis* L3.

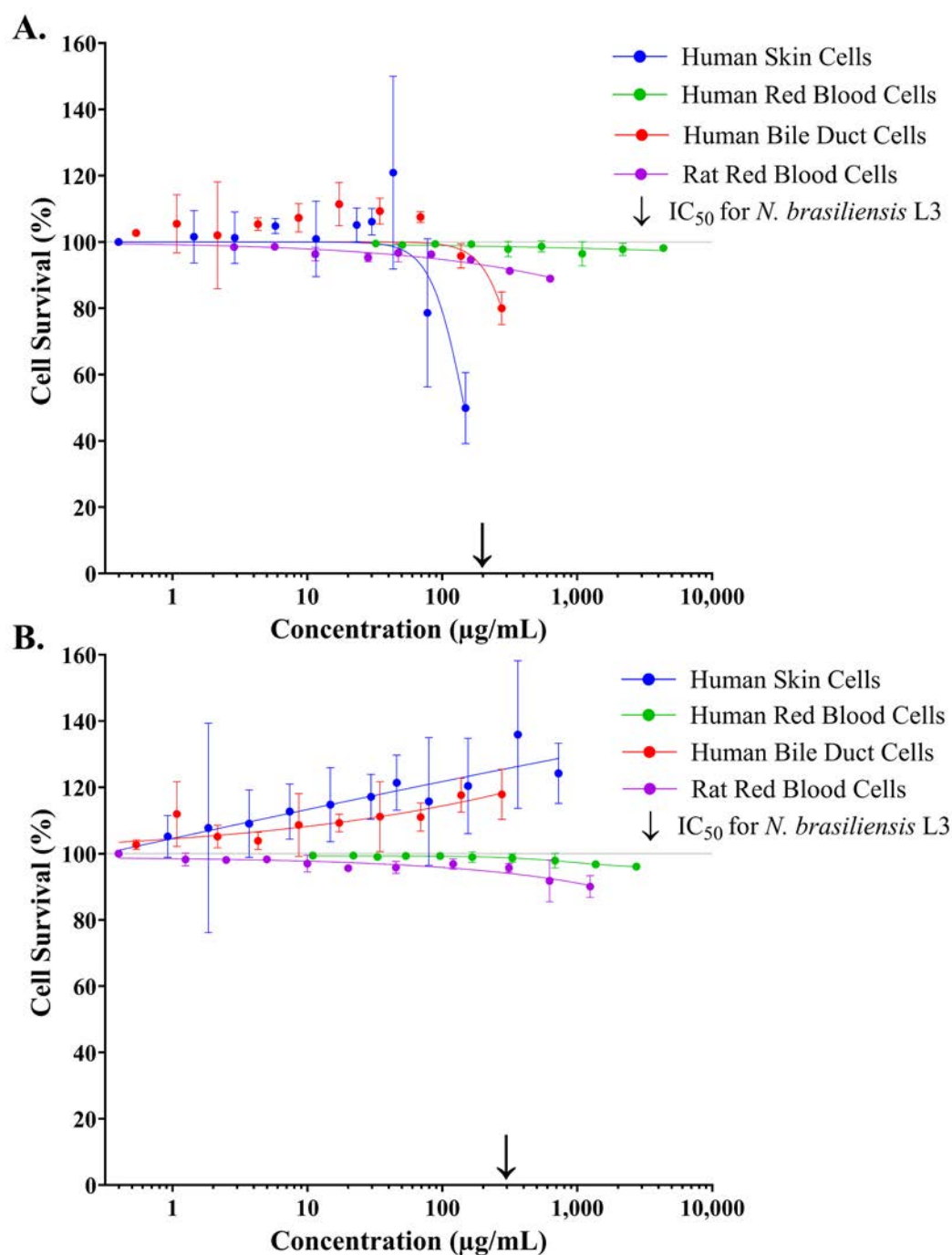


Figure 9.3 The Effect of Crude Stonefish (*Synanceia* spp.) Ichthyocrinotoxins on the Survival of Several Mammalian Cell Lines maintained *In Vitro*. Percent survival (%) of Human Normal Dermal Fibroblast Cells (—●—), Human Bile Duct Epithelial Cells (—●—), Human Red Blood Cell Erythrocytes (—●—) and Rodent Red Blood Cell Erythrocytes (—●—) was assessed one hour after the administration of crude *Synanceia horrida* (Estuarine Stonefish, Panel A), or *Synanceia verrucosa* (Reef Stonefish, Panel B) ichthyocrinotoxin at varying concentrations. Cell adhesion relative to the negative (i.e., no toxin) control group has been used as an indicator for the percentage of cells that survived following one hour of exposure to treatment. Arrows (↓) mark the IC₅₀ values for crude stonefish ichthyocrinotoxins on *N. brasiliensis* L3. Error bars represent 95% confidence intervals.

9.4.4 Assessing the Cytotoxicity of Isolated Small Components from S. horrida Ichthyocrinotoxin to Mammalian Cell Lines In Vitro

A four-parameter logistic regression model (log inhibitor vs. normalised response, variable slope) was used to determine the dose-dependent effect of the small components isolated from *S. horrida* ichthyocrinotoxin on the survival of several mammalian cell lines *in vitro* following one-hour of exposure to treatment (**Figure 9.4**). Like the crude toxin, the isolated small components (< 3kDa) from *S. horrida* ichthyocrinotoxin had minimal effect on the survival of human or rat red blood cell erythrocytes, with ~99% cell survival observed across all tested concentrations (**Figure 9.4C, D**). Conversely, a strong inhibitory dose-response relationship was observed for the survival of human skin cells (**Figure 9.4A**, $R_2 = 0.82$, $AICc = 96.4$, $HillSlope = -1.25$), and human bile duct cells (**Figure 9.4B**, $R_2 = 0.86$, $AICc = 51.25$, $HillSlope = -1.44$). However, the small components demonstrated a weaker inhibitory effect on the survival of both cell lines compared to the crude toxin. While accurate IC_{50} values for the effect of the small components on mammalian cell lines could not be calculated from the available data, the IC_{20} was determined to be 250.4 $\mu\text{g/mL}$ (95% CI: 204.6–314.0 $\mu\text{g/mL}$) for human skin cells, and 706.9 $\mu\text{g/mL}$ (95% CI: 628.9–807.3 $\mu\text{g/mL}$) for bile duct cells. Furthermore, at a concentration that resulted in 50% mortality of *N. brasiliensis* L3, the isolated small components from *S. horrida* ichthyocrinotoxin had minimal effect on the survival (>10% reduction in survival) of all tested cell lines.

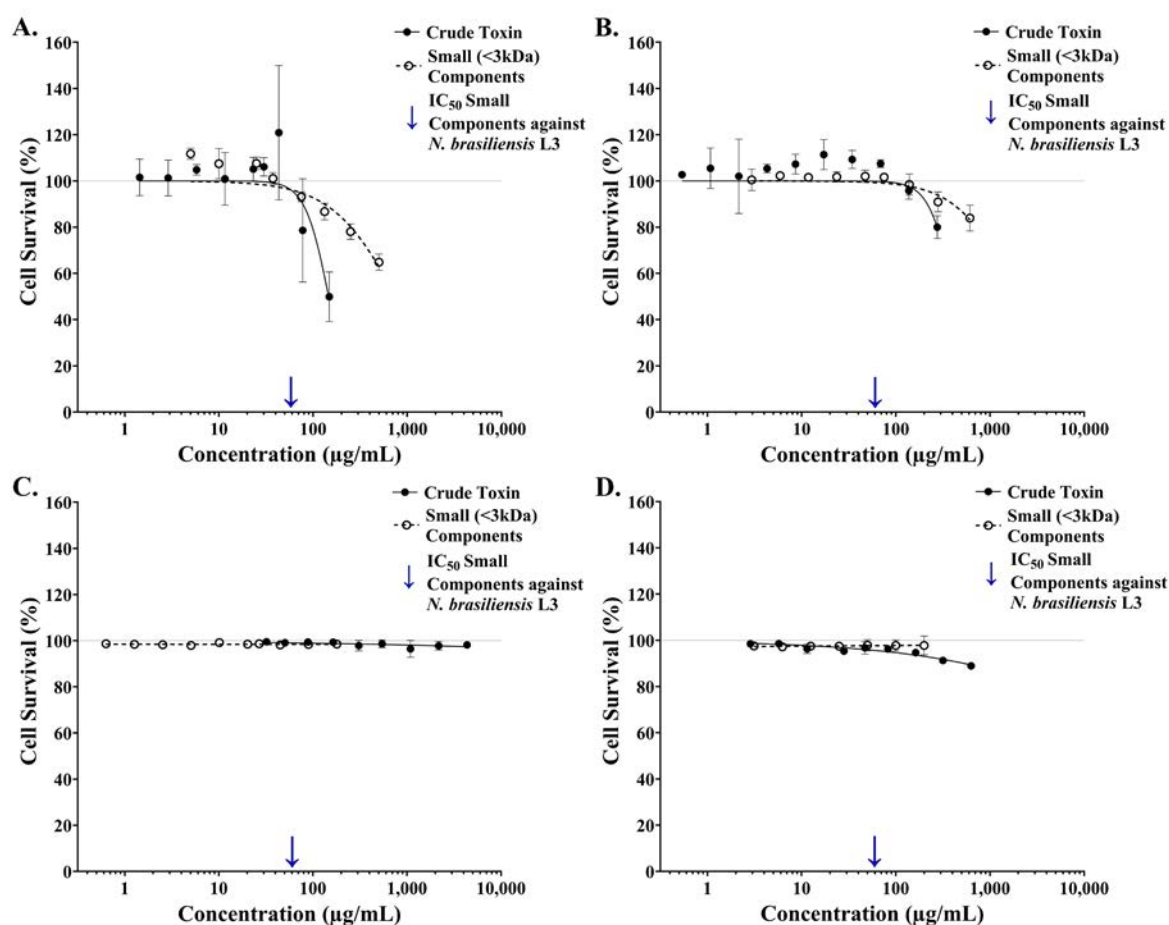


Figure 9.4 The Toxicity of Isolated Small Components from Estuarine Stonefish (*Synanceia horrida*) Ichthyocrinotoxin to Mammalian Cell Lines *In Vitro*. Cell adhesion relative to the negative (i.e., no toxin) control group was used as an indicator for the percentage of Human Normal Dermal Fibroblast Cells (**Panel A**), Human Bile Duct Epithelial Cells (**Panel B**), Human Red Blood Cell Erythrocytes (**Panel C**) and Rodent Red Blood Cell Erythrocytes (**Panel D**) that survived following one hour of exposure to varying concentrations of crude toxin (—●—) and isolated small (< 3kDa) components (- -○- -) from *S. horrida* ichthyocrinotoxin. Arrows (↓) mark the IC₅₀ value of the small components against *N. brasiliensis* L3. Error bars represent 95% confidence intervals.

9.4.5 Comparing the Toxicity of Crude and Isolated Small Component Samples from Estuarine Stonefish Ichthyocrinotoxin between Parasite and Cellular Targets

The toxicity of crude and isolated small components from *S. horrida* ichthyocrinotoxin were compared between parasite (*N. brasiliensis* L3) and various cellular (skin, bile duct, and red blood cells) targets using a two-way ANVOA (**Figure 9.5**). There was a significant interaction between toxin type (isolated small components vs. crude) and target (*N. brasiliensis* L3, human skin cells, etc.) on survival ($F_{4,23} = 10.89$, $p < 0.001$). There were also significant effects of target ($F_{4,23} = 75.78$, $p < 0.001$), and sample type ($F_{1,23} = 26.76$, $p < 0.001$) on survival.

Post-hoc LSD revealed that the small components isolated from *S. horrida* ichthyocrinotoxin had little effect on the survival of mammalian cells, with all cell lines displaying statistically similar survival rates exceeding 95% ($\pm 2\%$). In contrast, the small components exhibited a significantly greater toxicity against *N. brasiliensis* L3 ($\sim 50\% \pm 10\%$ decrease in survival) compared to mammalian cells. Furthermore, crude toxin also exerted minimal effects on the survival of human and rat red blood cells ($\sim 99\%$ and $95\% \pm 2\%$ survival, respectively) and had only a mild effect ($\sim 20\% \pm 5\%$ decrease in survival) on human bile duct cells. In contrast, the effect of crude toxin on the survival of human skin cells was comparable to that observed for *N. brasiliensis* L3, with a $\sim 50\% \pm 10\%$ decrease in survival observed across both targets.

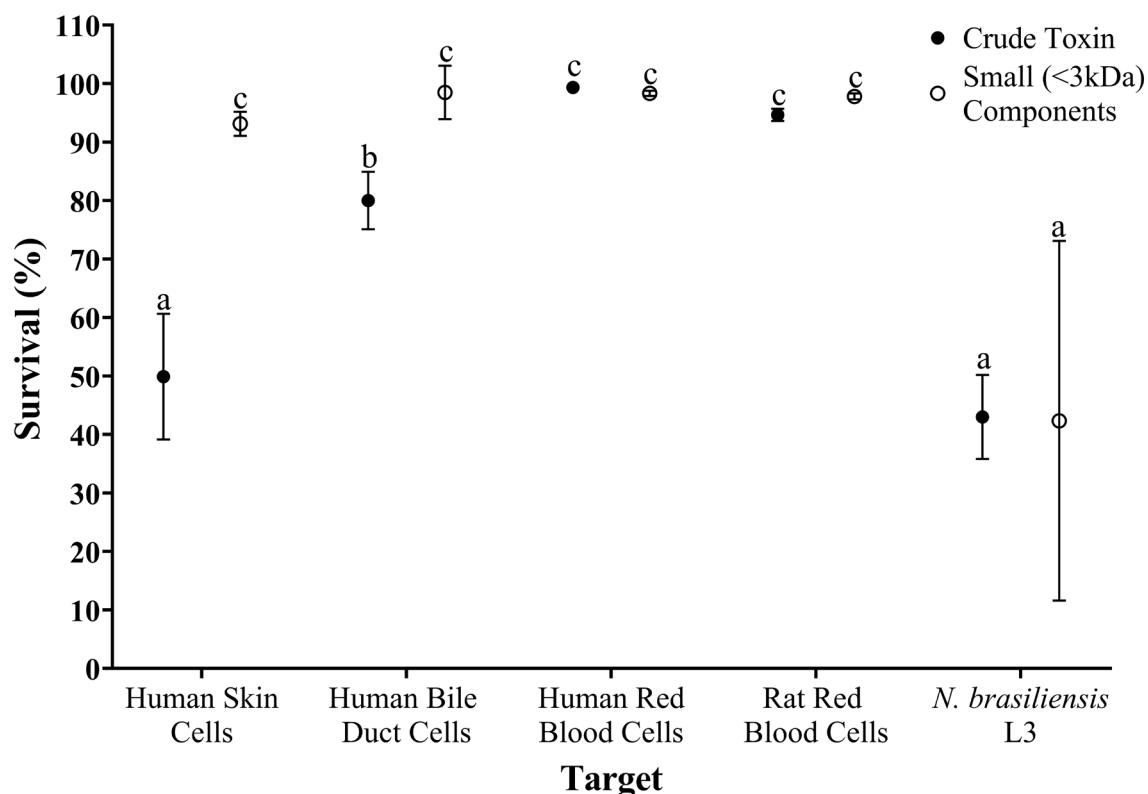


Figure 9.5 Comparative Toxicity Assessment of Crude and Isolated Small Components Samples from Estuarine Stonefish (*Synanceia horrida*) Ichthyocriotoxin between Parasite and Cellular Targets. Parasite motility (*N. brasiliensis* L3) and the adhesion of various mammalian cell lines (human skin and bile duct cells, human and rat red blood cells) relative to a negative (i.e., no toxin) control group have been used as an indicator for the survival of parasites/cells following one hour of exposure to crude toxin (●) and isolated small (<3 kDa) components (○) from *S. horrida* ichthyocriotoxin. For isolated small components, the highest achievable treatment concentration shared by all targets (100 µg/mL) was selected for comparison. For crude toxins, a treatment concentration of 200 µg/mL was chosen for comparison to account for the estimated 50% composition of small components. Target survival is shown relative to the negative control group (no toxin exposure). Letters (a – c) indicate statistically significant post-hoc LSD groupings. Error bars represent 95% confidence intervals.

9.5 Discussion

The present study investigated the anthelmintic activity of stonefish ichthyocrinotoxins and their potential applications as novel therapeutics. Stonefish ichthyocrinotoxins were found to elicit a dose-dependent toxic effect on *N. brasiliensis* L3. These findings further support the hypothesis that stonefish ichthyocrinotoxins may function, at least in part, in parasite defence. Additionally, previous research has shown that stonefish ichthyocrinotoxins also exhibited toxicity towards ciliated protozoans (Cameron *et al.*, 1981). Together, these findings suggest that stonefish ichthyocrinotoxins may provide protection against a broad spectrum of parasite taxa, ranging from single-celled protozoa to more complex multicellular organisms like nematodes. However, further investigation into the natural parasites of stonefish is required to be able to fully elucidate the ecological role of these toxins in parasite defence.

Interestingly, the potency of stonefish ichthyocrinotoxins to *N. brasiliensis* L3 differed between the two species. Specifically, crude ichthyocrinotoxin from *S. horrida* was found to be significantly more toxic to the parasite ($IC_{50} = 196.0 \mu\text{g/mL}$), than ichthyocrinotoxin from *S. verrucosa* ($IC_{50} = 329.0 \mu\text{g/mL}$). The components responsible for the anthelmintic activity were also found to differ between the two species. The anthelmintic properties of *S. verrucosa* ichthyocrinotoxin were lost upon separation of its larger and smaller components. This suggests that the anthelmintic activity of *S. verrucosa* ichthyocrinotoxin either arises from synergistic interactions (i.e., the combined action of multiple components of different sizes), or that the activity was lost during sample processing. In contrast, most of the anthelmintic activity of *S. horrida* ichthyocrinotoxin was associated with its smaller components. However, it remains unclear whether this activity is due to a single active compound or synergistic interactions between compounds.

Previous research has shown that *S. horrida* ichthyocrinotoxin has negligible protease, haemolytic, and haemagglutinating activities (Cameron *et al.*, 1981). Conversely, *S. horrida* ichthyocrinotoxin has also been shown to inhibit contractions in guinea pig ileum and vas deferens (Hoy & Cameron, 1986; Cameron *et al.*, 1981). Our study provides further support for the varied cytotoxic properties of stonefish ichthyocrinotoxins. Crude ichthyocrinotoxins from both *S. horrida* and *S. verrucosa* had little to no effect on human and rat red blood cells, even at high concentrations ($>250 \mu\text{g/mL}$). Additionally, ichthyocrinotoxin from *S. verrucosa* appeared to promote the proliferation of human skin and bile duct cells. In contrast, crude

ichthyocrinotoxin from *S. horrida* was highly toxic to human skin cells ($IC_{50} = 146.3 \mu\text{g/mL}$) and exhibited mild toxicity toward human bile duct cells ($IC_{20} = 274.6 \mu\text{g/mL}$).

The observed interspecific differences in the anthelmintic activity and cytotoxicity of stonefish ichthyocrinotoxins are consistent with previously reported compositional variations (Lennox-Bulow *et al.*, 2023c). It was suggested that interspecific compositional variation in stonefish ichthyocrinotoxins may be driven by differences in the ecology of these species (Lennox-Bulow *et al.*, 2023c). While the biological significance of the observed cytotoxicity to some mammalian cell lines remains unclear, interspecific variation in the toxins anthelmintic activities may be driven by differing parasite dynamics in the respective habitats of these species. For instance, the greater anthelmintic potency of *S. horrida* ichthyocrinotoxin may reflect a higher abundance and/or diversity of parasites in estuarine habitats compared to coral reefs. Alternatively, the parasite taxa that these species encounter may be different between reef and estuarine environments, necessitating different compounds for an effective parasite defence. It is also plausible that the increased connectivity between terrestrial and estuarine environments could enhance the exposure of primarily estuary-dwelling stonefish species, such as *S. horrida*, to mammalian-hosted parasites. This potential exposure may also contribute to the observed interspecific differences in the anthelmintic activity of stonefish ichthyocrinotoxins to *N. brasiliensis* L3. However, it is important to note that *N. brasiliensis* is not a natural parasite of stonefish. Therefore, the observed differences in the anthelmintic properties between stonefish species might be an artefact rather than a reflection of true ecological scenarios.

Nevertheless, the observed anthelmintic activity of stonefish ichthyocrinotoxins towards a mammalian-hosted nematode suggests potential applications beyond their immediate ecological context, such as a novel anthelmintic therapeutic. From this perspective, ichthyocrinotoxin from *S. horrida* emerges as a more attractive candidate for therapeutic development due to the small size of the active component(s). Small molecules are often preferred for drug discovery due to their ease of characterisation, lower manufacturing costs, enhanced stability, and more predictable pharmacokinetic and pharmacodynamic properties (Gu *et al.*, 2023; Ngo & Garneau-Tsodikova, 2018). In clinical settings, this may translate to greater affordability for patients and healthcare systems, oral treatment options, and simpler dosing regimens (Gu *et al.*, 2023; Ngo & Garneau-Tsodikova, 2018). In contrast, due to the

potential synergistic nature of its anthelmintic activity, *S. verrucosa* ichthyocrinotoxin may pose greater challenges for downstream development and clinical implementation.

The therapeutic potential of the isolated small components from *S. horrida* ichthyocrinotoxin is further strengthened by their high potency against *N. brasiliensis* L3 and reduced toxicity to mammalian cells. At a concentration of 100 µg/mL, these components caused less than 10% cell death in all tested mammalian cell lines but inhibited *N. brasiliensis* L3 survival by over 50%. Additionally, while the small components did exhibit some dose-dependent toxicity to human skin and bile duct cells, they were significantly less cytotoxic than the crude toxin. Nearly twice the concentration of the small component sample was required to reduce the survival of human skin cells by only 20% ($IC_{20} = 250.4 \mu\text{g/mL}$) compared to the concentration of crude toxin that halved cell survival ($IC_{50} = 146.3 \mu\text{g/mL}$). Similar results were observed for bile duct cells, where more than twice the concentration of small components ($IC_{20} = 706.9 \mu\text{g/mL}$) was needed to achieve the same effect as the crude toxin ($IC_{20} = 274.6 \mu\text{g/mL}$). Furthermore, at biologically comparable concentrations, the small components demonstrated a weaker dose-dependent effect on human skin and bile duct cell survival compared to the crude toxin. This suggests that the cytotoxicity of *S. horrida* ichthyocrinotoxin is either driven by synergistic interactions between its small and large components, or that the toxins activity was lost through fractionation. Furthermore, the components responsible for the observed cytotoxicity may differ from those active against *N. brasiliensis* L3, potentially making them ideal candidates for further *in vivo* evaluation.

Despite showing promise for therapeutic development, the isolated small components from *S. horrida* ichthyocrinotoxin exhibit considerably lower *in vitro* potency to parasites ($IC_{50} = 65.80 \mu\text{g/mL}$) compared to established anthelmintics like ivermectin (Mahjoub *et al.*, 2024; Smout *et al.*, 2010). However, the isolated small components from *S. horrida* ichthyocrinotoxin are likely a multifarious mixture; thus, further isolation, as well as the structural and functional characterisation of the active component(s) is necessary to determine their true potency and clinical relevance. Additionally, future studies should determine the efficacy of these toxins against a wider range of parasite species, particularly medically significant hookworm species such as *Necator americanus* (Human Hookworm) and *Ancylostoma ceylanicum* (Canine and Human Hookworm), to evaluate their broad-spectrum potential.

In conclusion, this study demonstrates the anthelmintic properties of stonefish ichthyocrinotoxins and highlights their potential as novel therapeutics. While both *S. horrida*

and *S. verrucosa* ichthyocrinotoxins showed activity against skin-burrowing hookworm larvae, the isolated small component mixture from *S. horrida* ichthyocrinotoxin emerged as the most promising drug lead due to its increased potency to the parasite, favourable size for drug development, and reduced cytotoxicity compared to the crude extract. However, further isolation and the identification of the active component(s) within *S. horrida* ichthyocrinotoxin is needed to fully determine its clinical relevance.

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9.7 APPENDIX H

The Anthelmintic Activity of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins and their Potential as Novel Therapeutics

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Article

The Anthelmintic Activity of Stonefish (*Synanceia* spp.) Ichthyocrinotoxins and Their Potential as Novel Therapeutics

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Abstract: Parasitic gastrointestinal worms (i.e., helminths) remain a significant global health and economic burden. The increasing inefficacy of current anthelmintic drugs against parasitic diseases necessitates the discovery of novel therapeutic options. This study investigated the anthelmintic properties and therapeutic potential of stonefish ichthyocrinotoxins (i.e., secreted skin toxins). xWORM (xCELLigence Worm Real-Time Motility Assay) was used to evaluate the anthelmintic activity of ichthyocrinotoxins from two stonefish species, *Synanceia horrida* (Estuarine Stonefish) and *Synanceia verrucosa* (Reef Stonefish), against the infective third-stage larvae of *Nippostrongylus brasiliensis* (Rodent Hookworm). Both toxins demonstrated potent anthelmintic effects, with *S. horrida* ichthyocrinotoxin exhibiting greater potency ($IC_{50} = 196.0 \mu\text{g/mL}$) compared to ichthyocrinotoxin from *S. verrucosa* ($IC_{50} = 329.7 \mu\text{g/mL}$). Fractionation revealed that the anthelmintic activity of *S. verrucosa* is likely driven by synergistic interactions between the large (>3 kDa) and small (<3 kDa) components. In contrast, the small components isolated from *S. horrida* ichthyocrinotoxin were responsible for the majority of the observed activity, making them a more attractive therapeutic candidate. Furthermore, despite the cytotoxicity of crude *S. horrida* ichthyocrinotoxin against human skin and bile duct cell lines, the isolated small components exhibited potent anthelmintic effects ($IC_{50} = 70.5 \mu\text{g/mL}$) with negligible cytotoxicity (<10% decrease in survival at 100 $\mu\text{g/mL}$). While further research is necessary to fully characterise these compounds and assess their clinical suitability, this study highlights the potential of stonefish ichthyocrinotoxins as a novel source of anthelmintic therapeutics.

Keywords: novel compounds; stonefish; toxins; anthelmintic; therapeutics; ichthyocrinotoxin

Key Contribution: Stonefish skin toxins (ichthyocrinotoxins) may have practical applications as novel anthelmintic therapeutics.

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1. Introduction

Parasitic worms (or helminths) infect almost all taxa across the animal kingdom [1]. It is estimated that over two billion people, or a little over 24% of the global population, are currently infected with helminths [2,3]. The highest prevalence of helminth infections in humans is found in developing countries, particularly in places with poor sanitation and limited access to healthcare [2,3]. Different helminths cause distinct pathophysiological conditions, depending on the niches they occupy, including intestinal issues (i.e.,

diarrhoea, abdominal pain), organ damage (i.e., blindness, kidney disease), dermatological conditions (i.e., skin penetration, skin rashes), malnutrition, and impaired growth and physical development [4,5]. Beyond their toll on health and wellbeing, helminths also impose substantial economic losses, particularly in agricultural and aquacultural sectors, by compromising the productivity of livestock and the market value of animal products [6–8]. Such production losses can also affect consumers, as reduced supply can strain the market, which in turn, drives up retail prices [8,9].

The devastating impact of helminths on health and the economy is further compounded by the emergence of singular or multi-drug resistance across numerous parasite species. It is now well-established that resistance has developed against all classes of anthelmintic drugs used against helminth species that infect livestock [10]. Moreover, within the last decade, cases of drug resistance have also emerged for helminth species that infect companion animals [11,12], and concerning, humans [13,14]. Novel anthelmintic treatment options with distinct mechanisms of action are thus urgently needed to address this issue [15–17]. Nature has already provided a profusion of modern therapeutics, including analgesics (i.e., penicillin, opium, and aspirin), decongestants (i.e., pseudoephedrine/Sudafed), cholesterol-lowering medications (i.e., statins), anthelmintics (i.e., avermectin), and much more [15,18]. Yet, a vast reservoir of unexplored compounds remains, some of which are likely to have therapeutic potential. Animal toxins, which include venoms and poisons, are particularly promising for biodiscovery due to their highly diverse, dynamic, and often complex composition [19].

Stonefish (*Synanceia* spp.) stand out as being one of few organisms known to possess two distinct types of toxins: a potent defensive venom associated with each of their dorsal spines, and a secreted skin toxin called ichthyocrinotoxin [20,21]. While stonefish are widely distributed throughout the shallow, coastal waters of the Indo-Pacific, two species inhabit Queensland waters: *Synanceia horrida* (Estuarine Stonefish), which are primarily found inshore amongst rocky intertidal areas and estuaries, and *Synanceia verrucosa* (Reef Stonefish), which are commonly associated with coral reefs [22]. Stonefish generally adopt a cryptic, benthic, and predominantly sedentary lifestyle, a strategy that likely assists with prey capture and predator avoidance [22,23]. However, the intimate association that stonefish have with the benthos, combined with their lack of squamation (i.e., scales) [20,22], should render these animals highly susceptible to parasites, particularly those, such as helminths, that burrow through the skin.

Helminths are ubiquitous in the aquatic realm, and as such, their occurrence in fish is common and widely reported, particularly for species targeted or reared for consumption [24,25]. Yet, members of the genus *Synanceia* (including stonefish) appear to harbour a surprisingly low diversity of internal parasite taxa [26]. One hypothesis for the paucity of internal parasites in stonefish may be that their ichthyocrinotoxins afford the skin some protection [27]. Although the role of stonefish ichthyocrinotoxins in parasite defence is currently unknown [28], a previous study on ichthyocrinotoxic *Gobiodon* species supports the hypothesis that some ichthyocrinotoxins may aid in preventing parasitism [29]. This study observed that *Gobiodon* ichthyocrinotoxins influenced the attachment sites of external parasites (gnathiid isopods), with parasites demonstrating a preference for areas, such as the fins, where the toxin glands were less abundant [29].

Furthermore, a previous study demonstrated the broad-spectrum toxicity of stonefish ichthyocrinotoxins against invertebrates. Specifically, *S. horrida* ichthyocrinotoxin was found to inhibit the cilia of invertebrates, including those associated with mussel gills, and protozoan locomotory appendages [20]. The toxin was also found to increase the tone of barnacle depressor scutorum rostralis muscles (i.e., the muscle that controls the scuta lips) for up to one hour after immersion [20]. Additionally, while ichthyocrinotoxins secreted by other fish commonly exhibit both ichthyotoxic and hemotoxic properties [28], stonefish

ichthyocrinotoxins do not appear to follow suit. Ichthyocrinotoxins from *S. horrida* were found to demonstrate negligible protease, haemolytic and haemagglutinating activity, and were only mildly toxic to mosquito fishes and blennies [20]. However, *S. horrida* ichthyocrinotoxin was found to inhibit contractions in guinea pig ileum and vas deferens [20,30]. This suggests that while the toxin may have some effect on vertebrates, it appears to be more potent against invertebrates, which potentially include helminths. Moreover, the apparent limited toxicity of stonefish ichthyocrinotoxins to vertebrates warrants further investigation into its therapeutic potential.

Therefore, the aim of this study was to investigate the anthelmintic properties of stonefish (*Synanceia* spp.) ichthyocrinotoxins as a possible explanation for the paucity of endoparasites observed in these animals. Furthermore, this study evaluated the therapeutic potential of stonefish ichthyocrinotoxins by (1) identifying the components responsible for the toxin's anthelmintic activity against *Nippostrongylus brasiliensis* L3 (infective Rodent Hookworm larvae), and (2) determining the cytotoxicity of these components against various mammalian cell lines (human skin cells, human bile duct cells, as well as human and rat red blood cells) in vitro.

2. Results

2.1. Assessing the Toxicity of Crude Stonefish Ichthyocrinotoxins to Infective Rodent Hookworm Larvae

A four-parameter logistic regression model (log inhibitor vs. normalised response, variable slope) was used to investigate the dose-dependent effect of crude stonefish ichthyocrinotoxins on the survival of *N. brasiliensis* L3 after one hour of exposure to treatment (Figure 1). A strong inhibitory dose–response relationship was observed for both *S. horrida* (Figure 1 $R^2 = 0.952$, $AIC_c = 199.5$, $HillSlope = -2.60$) and *S. verrucosa* (Figure 1, $R^2 = 0.893$, $AIC_c = 214.1$, $HillSlope = -3.11$) ichthyocrinotoxin treatments. A two-tailed *t*-test revealed a significant difference in the IC_{50} values between *S. horrida* and *S. verrucosa* ichthyocrinotoxins ($t_{0.05(2),36} = 4.32$, $p = 0.0001$), indicating that the anthelmintic activity of these toxins varies between the species. Specifically, ichthyocrinotoxin from *S. horrida* was significantly more toxic to *N. brasiliensis* L3 ($IC_{50} = 196.0 \mu\text{g/mL}$, 95% CI: 175.1–221.4 $\mu\text{g/mL}$) compared to ichthyocrinotoxin from *S. verrucosa* ($IC_{50} = 329.7 \mu\text{g/mL}$, 95% CI: 279.9–391.5 $\mu\text{g/mL}$).

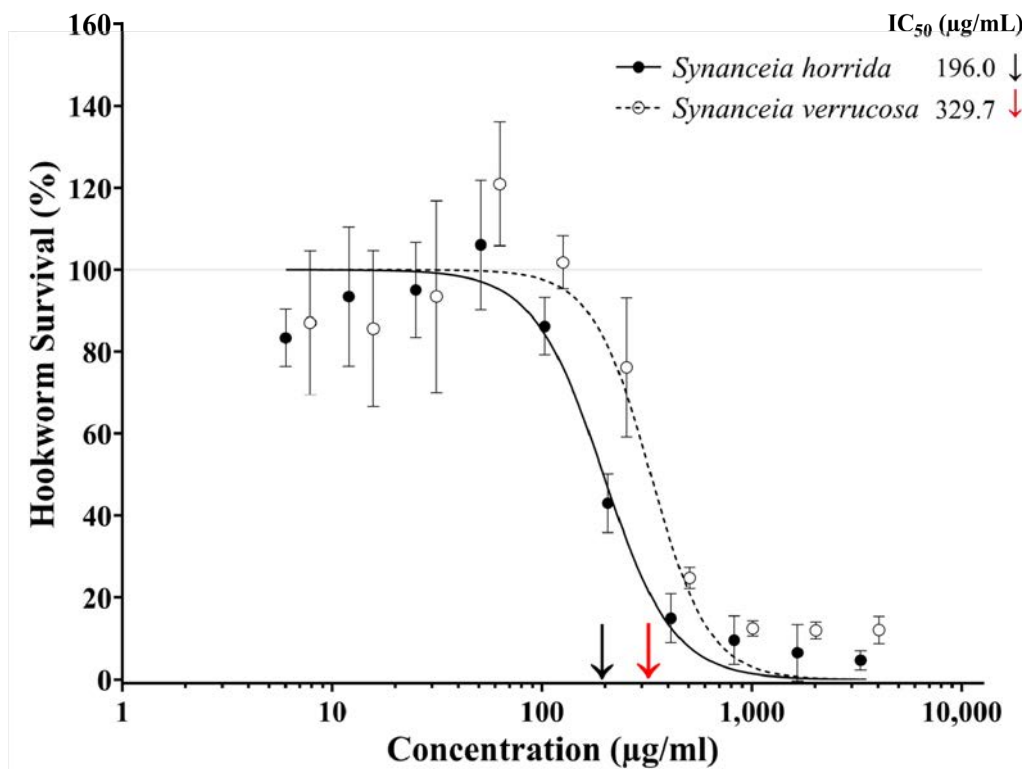


Figure 1. Dose-dependent effect of crude stonefish (*Synanceia* spp.) ichthyocrinotoxins on hookworm larvae survival. The motility of *N. brasiliensis* L3 (infective larval stage 3 Rodent Hookworm) relative to the negative (i.e., no toxin) control group has been used as an indicator for the percentage of hookworm larvae that survived following one hour of exposure to varying concentrations of *Synanceia horrida* (Estuarine Stonefish, —●—) or *Synanceia verrucosa* (Reef Stonefish, ---○---) ichthyocrinotoxins. The IC₅₀ values for each species toxin were calculated from the fitted curve and marked on the x-axis with ↓ for *S. horrida*, and ↓ for *S. verrucosa*. Error bars represent 95% confidence intervals.

2.2. Identifying Components in Stonefish Ichthyocrinotoxins with Anthelmintic Activity

A four-parameter logistic regression model (log inhibitor vs. normalised response, variable slope) was used to investigate the dose-dependent effect of the large (>3 kDa) and small (<3 kDa) components isolated from stonefish ichthyocrinotoxins on the survival of *N. brasiliensis* L3 after one hour of exposure to treatment (Figure 2). A strong inhibitory dose–response relationship was observed for the small (>3 kDa) components isolated from *S. horrida* ichthyocrinotoxin (Figure 2A, $R^2 = 0.78$, AICc = 238.6, HillSlope = −3.83). Given that these small components constitute approximately 50% of the crude *S. horrida* ichthyocrinotoxin mixture, their potency to *N. brasiliensis* L3 (IC₅₀ = 70.5 µg/mL, 95% CI: 55.9–92.6 µg/mL) was comparable to that of the crude toxin (IC₅₀ = 196.0 µg/mL, 95% CI: 173.9–222.1 µg/mL). In contrast, much higher concentrations (ten times greater) of the large components isolated from *S. horrida* ichthyocrinotoxin were required to induce a modest 20% inhibitory effect on *N. brasiliensis* L3 survival (IC₂₀ = 990.0 µg/mL, 95% CI: 806.2–1270.0 µg/mL, $R^2 = 0.74$, AICc = 45.7, HillSlope = −1.85) compared to the crude toxin. While crude *S. verrucosa* ichthyocrinotoxin elicited a strong dose-dependent inhibitory effect on hookworm survival (IC₅₀ = 329.7 µg/mL, 95% CI: 279.9–391.5 µg/mL), both the isolated large and small components had minimal effect across all tested treatment dosages (Figure 2B). Only high concentrations of the large components (>500 µg/mL) resulted in a slight decrease (~10%) in hookworm survival.

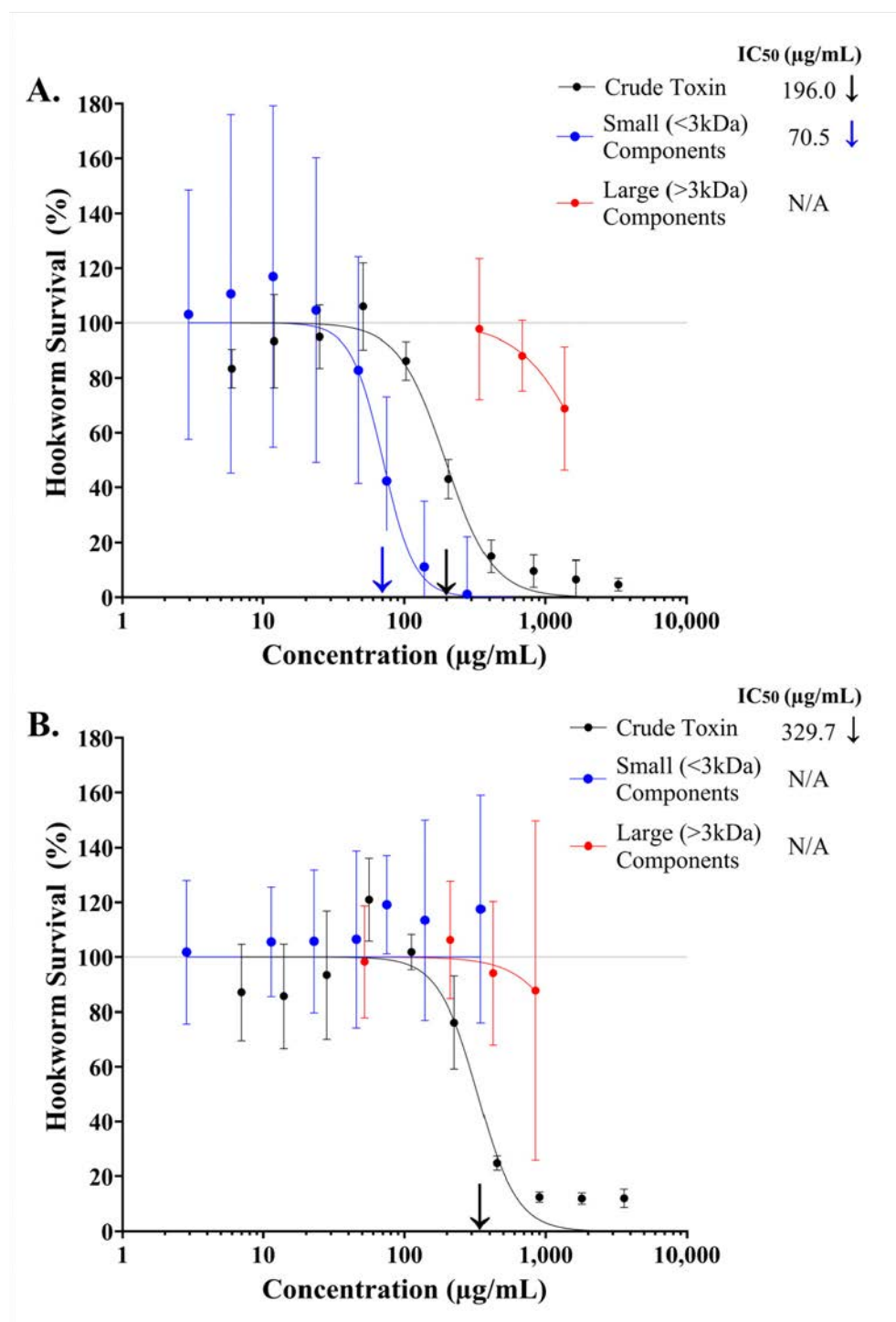


Figure 2. The anthelmintic activity of the isolated components from stonefish (*Synanceia* spp.) ichthyocrotoxins. Dose–response analysis was used to determine the anthelmintic activity of the small (<3 kDa, —●—) and large (>3 kDa, —●—) components isolated from *Synanceia horrida* (Estuarine Stonefish, Panel (A)) and *S. verrucosa* (Reef Stonefish, Panel (B)) ichthyocrotoxins against crude toxin (i.e., unprocessed, —●—). Hookworm motility relative to the negative (i.e., no toxin) control group has been used as an indicator for the percentage of *N. brasiliensis* L3 that survived following one hour of exposure to each treatment. Error bars represent 95% confidence intervals. Arrows mark the IC₅₀ values for crude (↓) and isolated small components (↓) from stonefish ichthyocrotoxins on *N. brasiliensis* L3.

2.3. Assessing the Cytotoxicity of Crude Stonefish Ichthyocrinotoxins to Mammalian Cell Lines In Vitro

A four-parameter logistic regression model (log inhibitor vs. response, variable slope) was used to investigate the dose-dependent effect of crude stonefish ichthyocrinotoxins on the survival of several mammalian cell lines in vitro, following one hour of exposure to treatment (Figure 3). Both *S. horrida* (Figure 3A) and *S. verrucosa* (Figure 3B) ichthyocrinotoxins had minimal effect on the survival of human red blood cells across all tested concentrations (*S. horrida*: $R^2 = 0.32$, AICc = 2.35, HillSlope = -0.23 , and *S. verrucosa*: $R^2 = 0.88$, AICc = -42.25 , HillSlope = -1.570). Similarly, both species toxins also had minimal effect on rodent red blood cells (*S. horrida*: $R^2 = 0.90$, AICc = 6.12, HillSlope = -0.41 , and *S. verrucosa*: $R^2 = 0.80$, AICc = 29.37, HillSlope = -0.399); however, high treatment concentrations ($>250 \mu\text{g/mL}$) elicited a non-significant decrease in survival (up to 10%). Additionally, crude *S. verrucosa* ichthyocrinotoxin exhibited no inhibitory effect on the survival of human skin cells ($R^2 = 0.66$, AICc = 141.6, HillSlope = 0.068) or human bile duct cells ($R^2 = 0.65$, AICc = 94.03, HillSlope = 0.22) across all tested concentrations. Instead, the toxin appeared to stimulate the proliferation of both cell types, with a maximum effect of approximately 40% and 20% for skin and bile duct cells, respectively. In contrast, crude *S. horrida* ichthyocrinotoxin exhibited a strong dose-dependent inhibitory effect on the survival of human skin cells ($R^2 = 0.75$, AICc = 135.9, HillSlope = -3.479), with an IC_{50} of $146.3 \mu\text{g/mL}$ (95% CI: $128.7\text{--}170.7 \mu\text{g/mL}$). While crude *S. horrida* ichthyocrinotoxin also exerted an inhibitory effect on human bile duct cell survival ($R^2 = 0.43$, AICc = 129.5, HillSlope = -3.361), its potency against these cells was notably lower, requiring a concentration of $274.6 \mu\text{g/mL}$ (95% CI: $242.5\text{--}324.9 \mu\text{g/mL}$) to achieve only a 20% reduction in survival. Furthermore, at a concentration that resulted in 50% parasite mortality (IC_{50}), crude *S. verrucosa* ichthyocrinotoxin had no negative effect on the survival of human bile duct and skin cells and had minimal effect on human and rat red blood cells. Similarly, crude *S. horrida* ichthyocrinotoxin exerted a minimal effect ($>10\%$ reduction in survival) on the survival of human or rat red blood cells, or human bile duct cells at the concentration that resulted in 50% hookworm death. In contrast, *S. horrida* ichthyocrinotoxin exerted a similar toxic effect on human skin cells, as it did on *N. brasiliensis* L3.

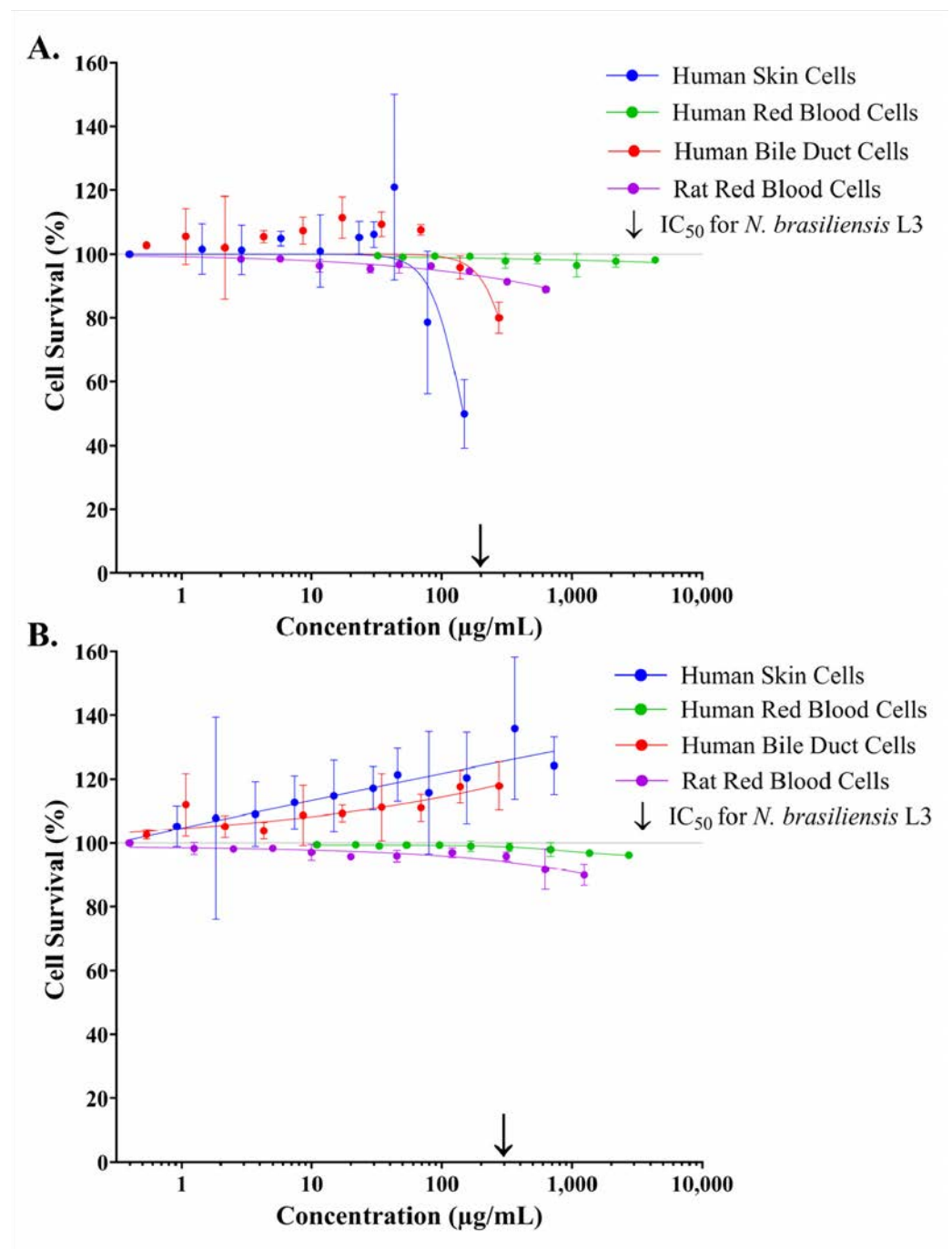


Figure 3. The effect of crude stonefish (*Synanceia* spp.) ichthyocrinotoxins on the survival of several mammalian cell lines maintained *in vitro*. Percent survival (%) of Human Normal Dermal Fibroblast Cells (—●—), Human Bile Duct Epithelial Cells (—●—), Human Red Blood Cell Erythrocytes (—●—), and Rodent Red Blood Cell Erythrocytes (—●—) was assessed one hour after the administration of crude *Synanceia horrida* (Estuarine Stonefish, Panel (A)) or *Synanceia verrucosa* (Reef Stonefish, Panel (B)) ichthyocrinotoxin at varying concentrations. Cell adhesion relative to the negative (i.e., no toxin) control group has been used as an indicator for the percentage of cells that survived following one hour of exposure to treatment. Arrows (↓) mark the IC_{50} values for crude stonefish ichthyocrinotoxins on *N. brasiliensis* L3. Error bars represent 95% confidence intervals.

2.4. Assessing the Cytotoxicity of Isolated Small Components from Estuarine Stonefish Ichthyocrinotoxins to Mammalian Cell Lines *In Vitro*

A four-parameter logistic regression model (log inhibitor vs. normalised response, variable slope) was used to determine the dose-dependent effect of the small components

isolated from *S. horrida* ichthyocrinotoxin on the survival of several mammalian cell lines *in vitro* following one hour of exposure to treatment (Figure 4). Like the crude toxin, the isolated small components (<3 kDa) from *S. horrida* ichthyocrinotoxin had minimal effect on the survival of human or rat red blood cell erythrocytes, with ~99% cell survival observed across all tested concentrations (Figure 4C,D). Conversely, a strong inhibitory dose–response relationship was observed for the survival of human skin cells (Figure 4A, $R_2 = 0.82$, $AICc = 96.4$, $HillSlope = -1.25$) and human bile duct cells (Figure 4B, $R_2 = 0.86$, $AICc = 51.25$, $HillSlope = -1.44$). However, the small components demonstrated a weaker inhibitory effect on the survival of both cell lines compared to the crude toxin. While accurate IC_{50} values for the effect of the small components on mammalian cell lines could not be calculated from the available data, the IC_{20} was determined to be 250.4 $\mu\text{g/mL}$ (95% CI: 204.6–314.0 $\mu\text{g/mL}$) for human skin cells and 706.9 $\mu\text{g/mL}$ (95% CI: 628.9–807.3 $\mu\text{g/mL}$) for bile duct cells. Furthermore, at a concentration that resulted in 50% mortality of *N. brasiliensis* L3, the isolated small components from *S. horrida* ichthyocrinotoxin had minimal effect on the survival (>10% reduction in survival) of all tested cell lines.

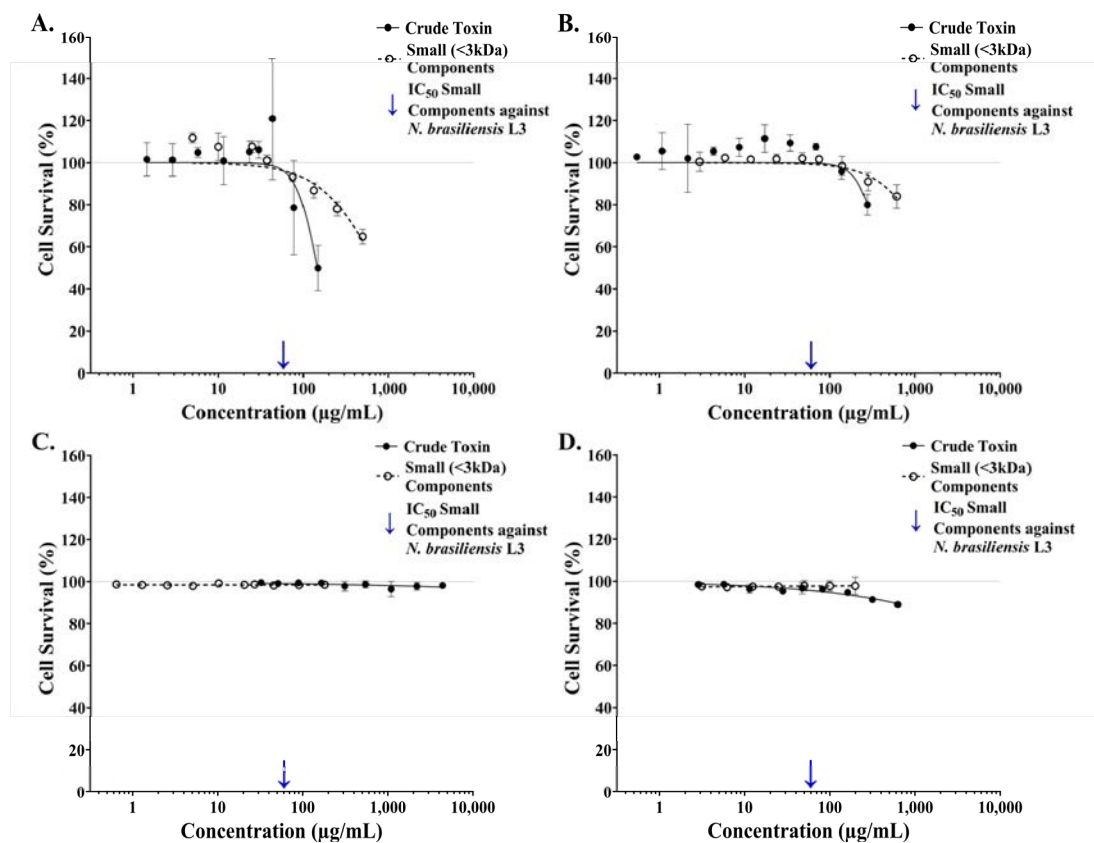


Figure 4. The toxicity of isolated small components from Estuarine Stonefish (*Synanceia horrida*) ichthyocrinotoxin to mammalian cell lines *in vitro*. Cell adhesion relative to the negative (i.e., no toxin) control group as an indicator of the percentage of Human Normal Dermal Fibroblast Cells (Panel (A)), Human Bile Duct Epithelial Cells (Panel (B)), Human Red Blood Cell Erythrocytes (Panel (C)), and Rodent Red Blood Cell Erythrocytes (Panel (D)) that survived following one hour of exposure to varying concentrations of crude toxin (—●—) and isolated small (<3 kDa) components (---○---) from *Synanceia horrida* (Estuarine Stonefish) ichthyocrinotoxin. Arrows (↓) mark the IC_{50} value of the small components isolated from *S. horrida* ichthyocrinotoxin against *N. brasiliensis* L3. Error bars represent 95% confidence intervals.

2.5. Comparing the Toxicity of Crude and Isolated Small-Component Samples from Estuarine Stonefish Ichthyocrinotoxins Between Parasite and Cellular Targets

The toxicity of crude and isolated small components from *S. horrida* ichthyocrinotoxin were compared between parasite (*N. brasiliensis* L3) and various cellular (skin, bile duct, and red blood cell) targets using two-way ANOVA (Figure 5). There was a significant interaction between toxin type (isolated small components vs. crude) and target (*N. brasiliensis* L3, human skin cells, etc.), on survival ($F_{4,23} = 10.89$, $p < 0.001$). There were also significant effects of target ($F_{4,23} = 75.78$, $p < 0.001$) and sample type ($F_{1,23} = 26.76$, $p < 0.001$) on survival.

Post hoc LSD revealed that the small components isolated from *S. horrida* ichthyocrinotoxin had little effect on the survival of mammalian cells, with all cell lines displaying statistically similar survival rates exceeding 95% ($\pm 2\%$). In contrast, the small components exhibited a significantly greater toxicity against *N. brasiliensis* L3 (~50% $\pm 10\%$ decrease in survival) compared to mammalian cells. Furthermore, crude toxin also exerted minimal effects on the survival of human and rat red blood cells (~99% and 95% $\pm 2\%$ survival, respectively) and had only a mild effect (~20% $\pm 5\%$ decrease in survival) on human bile duct cells. In contrast, the effect of crude toxin on the survival of human skin cells was comparable to that observed for *N. brasiliensis* L3, with a ~50% $\pm 10\%$ decrease in survival observed across both targets.

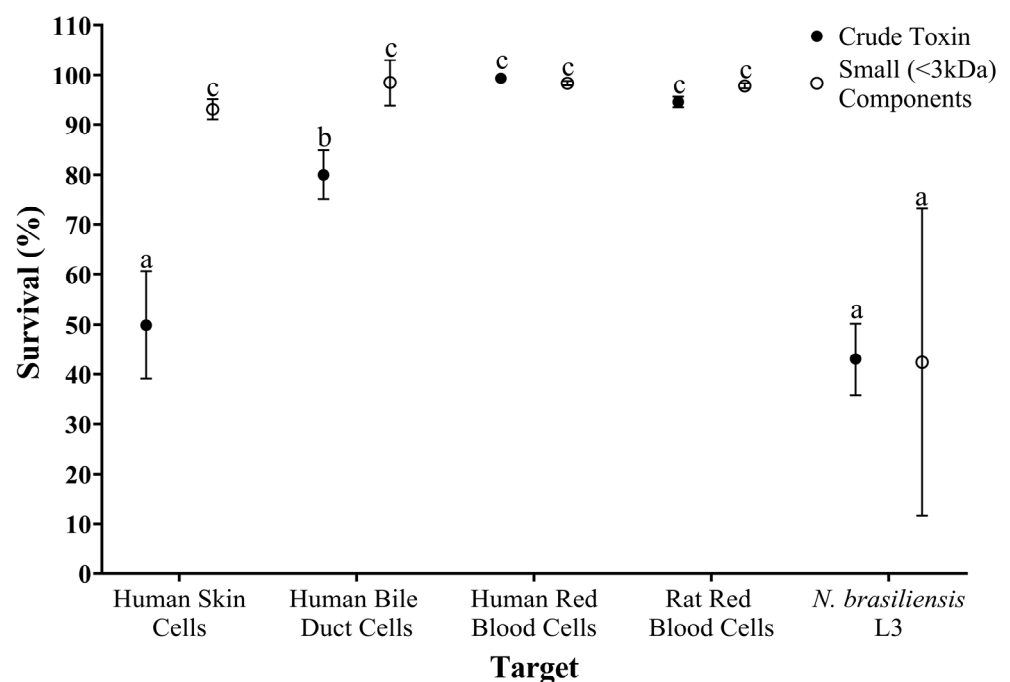


Figure 5. Comparative toxicity assessment of crude and isolated small components samples from Estuarine Stonefish ichthyocrinotoxin between parasite and cellular targets. Parasite motility (*N. brasiliensis* L3) and the adhesion of various mammalian cell lines (human skin and bile duct cells, human and rat red blood cells) relative to a negative (i.e., no toxin) control group as an indicator for the survival of parasites/cells following one hour of exposure to crude toxin (●) and isolated small (<3 kDa) components (○) from *Synanceia horrida* (Estuarine Stonefish) ichthyocrinotoxin. For isolated small components, the highest achievable treatment concentration shared by all targets (100 $\mu\text{g/mL}$) was selected for comparison. For crude toxins, a treatment concentration of 200 $\mu\text{g/mL}$ was chosen for comparison to account for the estimated 50% composition of small components. Target survival is shown relative to the negative control group (no toxin exposure). Letters (a–c) indicate statistically significant post hoc LSD groupings. Error bars represent 95% confidence intervals.

3. Discussion

The present study investigated the anthelmintic activity of stonefish ichthyocrinotoxins and their potential applications as novel therapeutics. Stonefish ichthyocrinotoxins were found to elicit a dose-dependent toxic effect on *N. brasiliensis* L3. These findings further support the hypothesis that stonefish ichthyocrinotoxins may function, at least in part, in parasite defence. Additionally, previous research has shown that stonefish ichthyocrinotoxins also exhibited toxicity towards ciliated protozoans [20]. Together, these findings suggest that stonefish ichthyocrinotoxins may provide protection against a broad spectrum of parasite taxa, ranging from single-celled protozoa to more complex multicellular organisms like nematodes.

Interestingly, the potency of stonefish ichthyocrinotoxins to *N. brasiliensis* L3 differed between the two species. Specifically, crude ichthyocrinotoxin from *S. horrida* was found to be significantly more toxic to the parasite ($IC_{50} = 196.0 \mu\text{g/mL}$) than ichthyocrinotoxin from *S. verrucosa* ($IC_{50} = 329.0 \mu\text{g/mL}$). The components responsible for the anthelmintic activity were also found to differ between the two species. The anthelmintic properties of *S. verrucosa* ichthyocrinotoxin were lost upon separation of its larger and smaller components. This suggests that the anthelmintic activity of *S. verrucosa* ichthyocrinotoxin either arises from synergistic interactions (i.e., the combined action of multiple components of different sizes), or that the activity was lost during sample processing. In contrast, most of the anthelmintic activity of *S. horrida* ichthyocrinotoxin was associated with its smaller components. However, it remains unclear whether this activity is due to a single active compound or synergistic interactions between compounds.

Previous research has shown that *S. horrida* ichthyocrinotoxin has negligible protease, haemolytic, and haemagglutinating activities [20]. Conversely, *S. horrida* ichthyocrinotoxin has also been shown to inhibit contractions in guinea pig ileum and vas deferens [20,30]. Our study provides further support for the varied cytotoxic properties of stonefish ichthyocrinotoxins. Crude ichthyocrinotoxins from both *S. horrida* and *S. verrucosa* had little to no effect on human and rat red blood cells, even at high concentrations ($>250 \mu\text{g/mL}$). Additionally, ichthyocrinotoxin from *S. verrucosa* appeared to promote the proliferation of human skin and bile duct cells. In contrast, crude ichthyocrinotoxin from *S. horrida* was highly toxic to human skin cells ($IC_{50} = 146.3 \mu\text{g/mL}$) and exhibited mild toxicity toward human bile duct cells ($IC_{20} = 274.6 \mu\text{g/mL}$).

The observed interspecific differences in the anthelmintic activity and cytotoxicity of stonefish ichthyocrinotoxins are consistent with previously reported compositional variations [31]. It was suggested that interspecific compositional variation in stonefish ichthyocrinotoxins may be driven by differences in the ecology of these species [31]. While the biological significance of the observed cytotoxicity to some mammalian cell lines remains unclear, interspecific variation in the toxins' anthelmintic activities may be driven by differing parasite dynamics in the respective habitats of these species. For instance, the greater anthelmintic potency of *S. horrida* ichthyocrinotoxin may reflect a higher abundance and/or diversity of parasites in estuarine habitats compared to coral reefs. Alternatively, the parasite taxa that these species encounter may be different between reef and estuarine environments, necessitating different compounds for an effective parasite defence. It is also plausible that the increased connectivity between terrestrial and estuarine environments could enhance the exposure of primarily estuary-dwelling stonefish species, such as *S. horrida*, to mammalian-hosted parasites. This potential exposure may also contribute to the observed interspecific differences in the anthelmintic activity of stonefish ichthyocrinotoxins to *N. brasiliensis* L3. However, it is important to note that *N. brasiliensis* is not a natural parasite of stonefish. Therefore, the observed differences in the anthelmintic properties between stonefish species might be an artefact rather than a reflection of true ecological scenarios.

Nevertheless, the observed anthelmintic activity of stonefish ichthyocrinotoxins towards a mammalian-hosted nematode suggests potential applications beyond their immediate ecological context, such as a novel anthelmintic therapeutic. From this perspective, ichthyocrinotoxin from *S. horrida* emerges as a more attractive candidate for therapeutic development due to the small size of the active component(s). Small molecules are often preferred for drug discovery due to their ease of characterisation, lower manufacturing costs, enhanced stability, and more predictable pharmacokinetic and pharmacodynamic properties [32,33]. In clinical settings, this may translate to greater affordability for patients and healthcare systems, oral treatment options, and simpler dosing regimens [32,33]. In contrast, due to the potential synergistic nature of its anthelmintic activity, *S. verrucosa* ichthyocrinotoxin may pose greater challenges for downstream development and clinical implementation.

The therapeutic potential of the isolated small components from *S. horrida* ichthyocrinotoxin is further strengthened by their high potency against *N. brasiliensis* L3 and reduced toxicity to mammalian cells. At a concentration of 100 µg/mL, these components caused less than 10% cell death in all tested mammalian cell lines but inhibited *N. brasiliensis* L3 survival by over 50%. Additionally, while the small components did exhibit some dose-dependent toxicity to human skin and bile duct cells, they were significantly less cytotoxic than the crude toxin. Nearly twice the concentration of the small component sample was required to reduce the survival of human skin cells by only 20% ($IC_{20} = 250.4$ µg/mL) compared to the concentration of crude toxin that halved cell survival ($IC_{50} = 146.3$ µg/mL). Similar results were observed for bile duct cells, where more than twice the concentration of small components ($IC_{20} = 706.9$ µg/mL) was needed to achieve the same effect as the crude toxin ($IC_{20} = 274.6$ µg/mL). Furthermore, at biologically comparable concentrations, the small components demonstrated a weaker dose-dependent effect on human skin and bile duct cell survival compared to the crude toxin. This suggests that the cytotoxicity of *S. horrida* ichthyocrinotoxin is either driven by synergistic interactions between its small and large components, or that the toxins activity was lost through fractionation. Furthermore, the components responsible for the observed cytotoxicity may differ from those active against *N. brasiliensis* L3, potentially making them ideal candidates for further in vivo evaluation.

Despite showing promise for therapeutic development, the isolated small components from *S. horrida* ichthyocrinotoxin exhibit considerably lower in vitro potency to parasites ($IC_{50} = 65.80$ µg/mL) compared to established anthelmintics like ivermectin [34,35]. However, the isolated small components from *S. horrida* ichthyocrinotoxin are likely a multifarious mixture; thus, further isolation, as well as the structural and functional characterisation of the active component(s), is necessary to determine their true potency and clinical relevance. Additionally, future studies should determine the efficacy of these toxins against a wider range of parasite species, particularly medically significant hookworm species such as *Necator americanus* (human hookworm) and *Ancylostoma ceylanicum* (canine and human hookworm), to evaluate their broad-spectrum potential.

4. Conclusions

In conclusion, this study demonstrates the anthelmintic properties of stonefish ichthyocrinotoxins and highlights their potential as novel therapeutics. While both *S. horrida* and *S. verrucosa* ichthyocrinotoxins showed activity against skin-burrowing hookworm larvae, the isolated small component mixture from *S. horrida* ichthyocrinotoxin emerged as the most promising drug lead due to its increased potency to the parasite, favourable size for drug development, and reduced cytotoxicity compared to the crude extract. However, further isolation and identification of the active component(s) within *S. horrida* ichthyocrinotoxin are needed to fully determine its clinical relevance.

5. Materials and Methods

5.1. Ichthyocrinotoxin Collection and Parasite Cultivation

5.1.1. Ichthyocrinotoxin Collection

Ichthyocrinotoxins from *S. verrucosa* and *S. horrida* were collected following the protocol outlined by [31]. A single specimen (*S. horrida* or *S. verrucosa*, 100 to 400 mm body length) was transferred to a damp surface and secured by gently holding either side of the pectoral fins. A saltwater-dampened cloth was placed over the animal's eyes to minimise stress throughout the process. Ichthyocrinotoxin was then extracted from the skin tubercles using targeted mechanical massage under a controlled vacuum. This vacuum was generated by a purpose-built extraction apparatus consisting of a 100 mm segment of clear vinyl tubing affixed to a 50 mL syringe. To account for potential individual variation in toxin composition, ichthyocrinotoxin was extracted and pooled from three to five individuals of each species. Pooled ichthyocrinotoxin samples were stored in 1.5 mL Eppendorf tubes at -80°C until required. The concentration of crude ichthyocrinotoxin was determined for both species using a standard Pierce™ Bicinchoninic Acid (BCA) Protein Assay kit, following the manufacturer's instructions.

5.1.2. Parasite Cultivation

Infective stage larval instar three (L3) *Nippostrongylus brasiliensis* (Rodent Hookworm) were cultivated following a protocol adapted from Camberis, et al. [36]. Briefly, *N. brasiliensis* L3 were suspended in 1× Phosphate-Buffered Saline (PBS) at a density of 2500 to 3000 L3/250 μL . Two female Sprague–Dawley rats were then subcutaneously injected between the shoulder blades with 250 μL of the larval suspension in accordance with James Cook University Animal Ethics approval (A2626). The rats were then monitored daily for any physical or behavioural abnormalities, and on the fifth day post-infection, they were transferred to new cages with clean bedding. Infected faecal pellets were collected on the sixth and seventh days post-infection. The faecal pellets were saturated in reverse-osmosis (R/O) water and blended into a slurry. The slurry was then combined with granulated and fine particulate activated charcoal at a ratio of roughly 1 part faecal slurry: 2 parts granulated charcoal: 1 part fine particulate charcoal, or until the mixture resembled natural soil. This mixture was evenly spread across sterile 90 mm plastic petri dishes lined with R/O water-dampened filter paper (Whatman® Buckinghamshire, United Kingdom, 80 mm rounded, Grade 4, 20–25 μm pore size). The Petri dishes containing the faecal culture were then set to incubate undisturbed in the dark at 26°C for five days to allow the larvae to hatch and migrate to the periphery of the plates. *Nippostrongylus brasiliensis* L3 were gently washed from the plate sides and lid with a Pasteur pipette and R/O water. The suspension was transferred to a 50 mL Falcon tube and brought to the nearest 10 mL gradation with R/O water. To quantify the number of *N. brasiliensis* L3 harvested, 100 μL of larval suspension was diluted 1:10 with R/O water. Then, 100 μL of diluted larval suspension was aliquoted on to a clean 90 mm plastic Petri dish, and larvae were counted under a top light microscope. The total number of larvae harvested was extrapolated by multiplying counts by the dilution factor (DF = 100).

5.2. Measuring Hookworm Larvae Motility with xWORM

5.2.1. Measuring Hookworm Larvae Motility

The effect of stonefish ichthyocrocinotoxins on the motility of *N. brasiliensis* L3 was assessed using a modified version of xWORM (xCELLigence Worm Real-Time Motility Assay) adapted from [37]. Assay parameters including media type, media concentration, and in-well parasite density were selected for *N. brasiliensis* L3 based on the findings from Lennox-Bulow, et al. [38]. In brief, two 96-well E-plates were pre-loaded with 100 µL of 0.25× PBS and 2% Antibiotic–Antimycotic (AA) solution in each well. The E-plates were then placed on an xCELLigence Real-Time-Cell-Analyser (RTCA) single-plate (SP) instrument to undergo an initial background measurement. The xCELLigence SP instrument was incubated in a temperature-controlled cabinet maintained at 37 °C and 5% CO₂ for the duration of the experiment to simulate the internal conditions of a mammalian host, and thus stimulate L3 motility. For each E-plate, a total of 48 wells were loaded with 50 µL of live *N. brasiliensis* L3 suspension (300 L3/50 µL of 0.25× PBS larval suspension), and a further 24 wells received 50 µL of heat-killed (95 °C for 5 min) L3 suspension at the same density. To mitigate the effect of media evaporation during incubation, parasites were only added to the inner wells of the E-plates. Wells along the outer perimeter of the plates received 50 µL 0.25× PBS with 2% AA and were excluded from subsequent analysis. The motility of *N. brasiliensis* L3, which was used as a proxy for hookworm survival, was continuously monitored at 25 kHz with regular user-defined readings taken every 15 s over a 72 h period. The larvae were acclimated to the experimental conditions for 20 h prior to the administration of treatment.

5.2.2. Data Transformation to Motility Index/Percent Parasite Survival

Raw Cell Index readings from RTCA Software Pro version 2.0 were exported for processing into a motility index, a measure of the standard deviation of the difference from the rolling Cell Index mean over time, as described by [35]. To account for background noise, motility index data were blank-adjusted by subtracting each data point of each treatment group from the mean of its corresponding positive control group (i.e., heat-killed L3 that had received the same dosage of *S. horrida* or *S. verrucosa* ichthyocrocinotoxin). Variations in baseline motility between live *N. brasiliensis* L3 wells were compensated for by identifying the highest motility value across all wells at a single pre-treatment time point (19 h post assay commencement) and dividing this by the motility index recorded for each individual well at that time point. This yielded an adjustment factor for each well, which was then applied by multiplying this factor by all proceeding motility index values (post-treatment). Adjusted motility index values were then converted to percent motility, herein referred to as hookworm survival, by calculating the relative motility index of each treatment group compared to the negative control (wells containing live L3 that received 0.25× PBS only) and multiplying by 100.

5.3. Assessing the Anthelmintic Activity of Stonefish Ichthyocrocinotoxins and Identifying Hookworm-Toxic Components

xWORM and data transformation procedures detailed in Section 5.2 were employed for all experiments outlined in this section. To evaluate the effect of ichthyocrocinotoxin samples on the survival of *N. brasiliensis* L3, we exposed the parasites to toxins for one hour prior to all subsequent analyses. This exposure duration was chosen to mimic a plausible infection scenario, as the parasite is expected to penetrate a host's skin within this timeframe.

5.3.1. Determining the Effect of Crude Stonefish Ichthyocrocinotoxins on Hookworm Larvae Survival

To determine the effect of stonefish ichthyocrinotoxins on the survival of *N. brasiliensis* L3, crude samples of ichthyocrinotoxin from *S. horrida* and *S. verrucosa* were serially diluted (1 part toxin: 2 parts 0.25× PBS) to eleven concentrations that were four times more concentrated than the final in-well treatment concentration. Treatment involved the addition of 50 µL of crude 4× concentrated *S. horrida* or *S. verrucosa* ichthyocrinotoxin samples to 150 µL of parasite/0.25× PBS mixture (total well volume 200 µL). The final in-well ichthyocrinotoxin treatment concentrations applied to *N. brasiliensis* L3 ranged from 6 to 7200 µg/mL for both species' toxins. Each condition (i.e., toxin concentration and stonefish species combination) was replicated four times on live parasite wells, which were assigned as the treatment group, and two times on heat-killed parasite wells, which served as a positive control group (i.e., 0% survival). An additional four wells containing live parasites served as the negative control group (i.e., 100% survival) and received 50 µL of 0.25× PBS with 2% AA in lieu of ichthyocrinotoxin. A four-parameter logistic regression model (log-inhibitor vs. normalised response, variable slope) was used to analyse the relationship between ichthyocrinotoxin concentration and *N. brasiliensis* L3 survival. Hookworm survival (%) one hour after receiving treatment was used as the dependent variable, log₁₀-transformed ichthyocrinotoxin concentration was assigned as the independent variable, and stonefish species (*S. horrida* and *S. verrucosa*) that the ichthyocrinotoxin was sourced from was the covariate. The concentration of ichthyocrinotoxin required to inhibit parasite survival by 50% (IC₅₀) was calculated for each stonefish species using the fitted relationship. A two-tailed *t*-test was then used to compare IC₅₀ values between *S. horrida* and *S. verrucosa* ichthyocrinotoxin treatments following a method outlined by Motulsky [39].

5.3.2. Isolating the Large (>3 kDa) and Small (<3 kDa) Components from Crude Toxin

To begin to identify the components responsible for the anthelmintic activity, crude ichthyocrinotoxin samples from *S. horrida* and *S. verrucosa* were each fractionated into two component groups: large (>3 kDa) components and small (<3 kDa) components. In brief, crude ichthyocrinotoxin (400 µL) was diluted in 4 mL of Milli-Q water and placed in the filter compartment of a centrifugal filter device (Amicon® Millipore Ultra 15, Merck, Darmstadt Germany) with a 3 kDa molecular weight cutoff. The device was then centrifuged at 4000× *g* at 4 °C for 10 min intervals until the sample volume in the filter compartment was reduced to 100 µL. Both the retained sample (large components) and the filtrate (small components) were collected. The filtrate was subsequently lyophilised to remove excess water. Both large and small component samples were then diluted/rehydrated to a final volume of 500 µL each. Protein concentrations were quantified using a standard Pierce™ BCA Protein Assay kit, following the manufacturer's instructions. The protein concentrations of the isolated small and large component groups were used to estimate their relative abundance within the original crude toxin mixture. The abundance of each component group within the crude toxin was determined to be approximately equal.

5.3.3. Measuring the Dose-Dependent Effect of Isolated Toxin Components and Comparing Their Activity Against Crude Toxin

xWORM was used to determine whether the large and small components isolated from stonefish ichthyocrinotoxins exhibited a dose-dependent effect on the survival of *N. brasiliensis* L3. The small (<3 kDa) components isolated from stonefish ichthyocrinotoxins were serially diluted (1 part toxin: 2 parts 0.25× PBS) to yield nine treatment concentrations ranging from 3 to 600 µg/mL for *S. horrida*, and eight treatment concentrations ranging from 3 to 345 µg/mL for *S. verrucosa*. The large (>3 kDa) components isolated from stonefish ichthyocrinotoxins were also serially diluted (1 part toxin: 2 parts 0.25× PBS) to three treatment concentrations ranging from 341 to 1368 µg/mL for *S. horrida*, and four

treatment concentrations ranging from 51 to 843 µg/mL for *S. verrucosa*. Treatment was administered to *N. brasiliensis* L3 as described in Section 5.3.1.

Separate four-parameter logistic regression models (log-inhibitor vs. normalised response, variable slope) for each species toxin (*S. horrida* and *S. verrucosa*) were performed to analyse the relationship between ichthyocrinotoxin concentration and *N. brasiliensis* L3 survival. Parasite survival (%) one hour after receiving treatment was used as the dependent variable, log₁₀-transformed ichthyocrinotoxin concentration was assigned as the independent variable, and toxin type (isolated small components, isolated large components, crude toxin) was the covariate. The concentration of ichthyocrinotoxin required to inhibit parasite survival by 50% (IC₅₀) was calculated for each toxin type and stonefish species using the fitted relationship.

5.4. Determining the Cytotoxicity of Stonefish Ichthyocrinotoxins to Mammalian Cell Lines In Vitro

5.4.1. Culturing Mammalian Cell Lines In Vitro

Human normal dermal fibroblast (1BR.3.GN) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC, product code 90020509). The cholangiocyte cell line (H69), an SV40-transformed bile duct epithelial cell line derived from a non-cancerous human liver, was obtained in 2010 from Dr. Gregory J. Gores, Mayo Clinic, Rochester, Minnesota [40]. These cell lines will herein be referred to as human skin cells for 1BR.3.GN, and human bile duct cells for H69. Both cell lines were cultured according to manufacturer protocols. In brief, cells were seeded in 75 cm² filter top flasks with 15 mL of an appropriate complete growth medium and incubated at 37 °C with 5% CO₂. The complete growth medium for human skin cells consisted of DMEM/Nutrient Mixture F12 with 10% Foetal Bovine Serum (FBS) and 1% Antibiotic–Antimycotic (AA). The complete growth medium for human bile duct cells comprised high-glucose DMEM/Nutrient Mixture F12 with 10% FBS, 1% AA, Adenine (25 µg/mL), Insulin (5 µg/mL), Epinephrine (1 µg/mL), Holo-transferrin (8.3 µg/mL), Hydrocortisone (0.62 µg/mL), T3 enzyme mix (13.6 ng/mL), and Epidermal Growth Factor (10 ng/mL).

Cell lines were left to form a monolayer until reaching approximately 80% confluence before being passaged. Cells were then gently washed in 10 mL 1× PBS to remove debris, followed by detachment using 3 mL of 1× Tryple reagent (Gibco). Harvested cells were combined with 12 mL of their respective complete growth medium and centrifuged at 150× g for 5 min (Hettich ROTINA 420R Centrifuge). The resulting supernatant was discarded, and the remaining cell pellet was resuspended in fresh complete growth medium. The concentration of all cell lines was determined using a haemocytometer.

5.4.2. xCELLigence Cell Viability Assay

The cytotoxicity of *S. horrida* and *S. verrucosa* ichthyocrinotoxin samples (crude and isolated small components) to human skin cells and human bile duct cells in vitro was assessed using a cell adhesion assay. Cell adhesion, which was used as a proxy for cell survival, was monitored in real-time using an xCELLigence RTCA (Agilent) system. E-Plates were loaded with 50 µL of the appropriate complete growth medium and then placed on an xCELLigence RTCA single-plate (SP) instrument to undergo an initial background measurement. Then, either 100 µL of human skin cell suspension or 100 µL of human bile duct cell suspension was aliquoted in each well of a 96-well E-plate at a density of 5000 cells/100 µL. Eight E-plates were set up with one cell type (human skin cells or human bile duct cells), stonefish species ichthyocrinotoxin (*S. horrida* or *S. verrucosa* ichthyocrinotoxin), and sample type (crude sample or isolated small components) combination allocated per plate. All E-plates were then incubated at 37 °C and 5% CO₂ throughout the duration of the experiment to mimic the internal thermal conditions of a mammal.

Cells were left to form a monolayer on the bottom of the wells for 19 h prior to the addition of treatment. Samples of crude *S. horrida* and *S. verrucosa* ichthyocrinotoxin and small (<3 kDa) components isolated from *S. horrida* ichthyocrinotoxin were prepared by serial dilution (1 part toxin: 2 parts complete growth medium). Treatment involved the addition of 50 µL of 4× concentrated samples of crude or isolated small components from *S. horrida* or *S. verrucosa* ichthyocrinotoxin to wells containing 150 µL of human skin or bile duct cells in their relevant complete growth medium. The final in-well treatment conditions were as follows: eleven concentrations of crude samples from *S. horrida* or *S. verrucosa* ichthyocrinotoxin ranging from 0.9 to 725 µg/mL were applied to human skin cells; ten concentrations of crude samples of *S. horrida* or *S. verrucosa* ichthyocrinotoxin ranging from 0.5 to 275 µg/mL were applied to human bile duct cells; eight concentrations of isolated small components samples from *S. horrida* ichthyocrinotoxin ranging from 5 to 600 µg/mL were applied to human skin cells; and nine concentrations of isolated small components samples from *S. horrida* ichthyocrinotoxin ranging from 3 to 700 µg/mL were applied to human bile duct cells. Each treatment condition was conducted in triplicate. To establish a reference for 100% cell survival, three wells on each E-plate were designated as a negative control group and received 50 µL of the relevant complete growth medium in lieu of ichthyocrinotoxin. Following treatment, cell progression was continuously monitored over 72 hours using xCELLigence RTCA (Agilent). The system performed 50 sweeps of the E-plate at intervals of 15 s for the first hour, 2 min for the second hour, 15 min for six hours, and 60 min thereafter, generating a continuous Cell Index trace (RTCA Software Pro version 2). Pre-treatment (19 h post assay commencement) Normalised Cell Index values were used to quantify the percent change in cell adhesion in response to treatment. Percent cell survival was calculated by dividing the Normalised Cell Index values of each treatment condition by the average normalised Cell Index value of the negative control group and multiplying by 100. Percent survival data were then exported to GraphPad Prism version 10 for dose–response analysis.

5.4.3. Haemotoxicity Assay

The haemotoxicity of *S. horrida* and *S. verrucosa* ichthyocrinotoxin to human and rat red blood cell erythrocytes was assessed using an absorbance-based lysis assay outlined by Brinkman, et al. [41]. Rat blood was collected via cardiac puncture from two female Sprague–Dawley rats that had been recently euthanised (James Cook University Animal Ethics Approval A2626). Human blood was drawn from the vein (venipuncture) of a healthy donor with human research and ethics approvals provided by James Cook University (James Cook University Human Ethics Approval H8523). To isolate red blood cell erythrocytes, whole-blood samples were centrifuged at 3000× g for 10 min at 4 °C. Following centrifugation, the resulting supernatant was discarded, and the sedimented erythrocytes were resuspended in sterile 1× PBS. This process was repeated five times to thoroughly wash the erythrocytes and ensure the removal of any remanent plasma. A 1% erythrocyte suspension (approximately 42×10^6 cells/450 µL), one each for human and rat erythrocytes, was prepared in 1× PBS. Human and rat erythrocyte suspensions (450 µL) were each aliquoted into 1.5 mL Eppendorf tubes.

Treatment involved the addition of 150 µL of crude *S. horrida* or *S. verrucosa* ichthyocrinotoxin, or isolated small (<3 kDa) components from *S. horrida* ichthyocrinotoxin at various concentrations to individual 1.5 mL Eppendorf tubes containing 450 µL of human or rat red blood cell erythrocytes ($n = 51$ tubes). Samples of crude *S. horrida* and *S. verrucosa* ichthyocrinotoxin and isolated small components from *S. horrida* ichthyocrinotoxin were first prepared by serial dilution (1 part toxin: 2 parts 1× PBS) to a series of samples that were 4 times more concentrated than the final in-well treatment conditions. The following final in-well treatment concentrations were tested for haemolytic activity to human and

rat red blood cell erythrocytes: ten concentrations of crude *S. horrida* or *S. verrucosa* ichthyocrinotoxin ranging from 1 to 1243 µg/mL were administered to rat red blood cells; eight concentrations of crude *S. horrida* or *S. verrucosa* ichthyocrinotoxin ranging from 10 to 4400 µg/mL were administered to human red blood cells; ten concentrations of isolated small component samples from *S. horrida* ichthyocrinotoxin ranging from 0.6 to 180 µg/mL were administered to human blood cells; and seven concentrations of isolated small components samples from *S. horrida* ichthyocrinotoxin ranging from 7.5 to 5000 µg/mL were administered to rat red blood cells. Additionally, one tube per cell type (i.e., human/rat cells) and treatment group (i.e., crude *S. horrida* ichthyocrinotoxin, isolated small components from *S. horrida* ichthyocrinotoxin, etc.) served as a positive control group (i.e., 100% cell lysis), which received 150 µL of lysis solution (1% Triton X-100 in 1× PBS) in lieu of toxin ($n = 8$ tubes). Another tube per cell type and treatment group was designated as a negative control group (i.e., 100% cell survival), which received 150 µL of 1× PBS ($n = 8$ tubes). All samples were then incubated for one hour at 37 °C with gentle agitation.

After the incubation period, samples were chilled on ice for 5 min, then centrifuged at 3000× g for 10 min at room temperature. Then, 200 µL of supernatant from each tube was transferred in triplicate to flat-bottomed 96-well plates. To account for background interference in absorbance readings, 200 µL of 1× PBS (media only wells) was aliquoted into three empty wells. Liberated haemoglobin was quantified by measuring its absorbance at 540 nm using a POLARstar Omega microplate reader. Raw absorbance data were blank adjusted by subtracting the average absorbance of the media-only wells. Percent lysis was determined by dividing each blank-adjusted absorbance value of the treatment group by the average of the positive control group and multiplying by 100. Percent survival was calculated by subtracting percent lysis from 100.

5.4.4. Dose–Response Analysis—Mammalian Cell Lines

Separate four-parameter logistic regression models (log-inhibitor vs. response, variable slope) for each species toxin (*S. horrida* and *S. verrucosa*) were performed to analyse the relationship between crude ichthyocrinotoxin concentration and cell survival one hour after treatment administration. The same regression model was used to analyse the relationship between the concentration of the small (<3 kDa) components from *S. horrida* ichthyocrinotoxin administered and cell survival one hour post-treatment. Cell survival (%) one hour post-treatment was used as the dependent variable, log₁₀-transformed ichthyocrinotoxin concentration was used as the independent variable, and cell type (i.e., human skin cells, human bile duct cells, human red blood cells, and rat red blood cells) was assigned as the co-variate. Where possible, the half maximal inhibitory concentration (IC₅₀) of ichthyocrinotoxin for cell survival was calculated using the fitted relationship. In cases where only mild cytotoxicity was observed (i.e., where cell survival remained above 50%), the treatment concentration that resulted in a 20% inhibition (IC₂₀) in cell survival was determined using a nonlinear regression model (log agonist vs. response—Find ECan-thing) with the following variables as constants: HillSlope = −1.0, F = 80, Bottom = 0, Top = 100.

5.5. Comparing the Activity of Crude- and Small-Component Samples from *S. horrida* Ichthyocrinotoxin Across Targets

The effects of crude toxin and isolated small (<3 kDa) components from *S. horrida* ichthyocrinotoxin on survival rates were compared between parasite and cellular targets using a two-way ANOVA (IBM SPSS version 27). Percent target survival after one hour of exposure to treatment served as the dependent variable, while target (human skin cells, human bile duct cells, human red blood cells, rat red blood cells, and *N. brasiliensis* L3) and toxin type (crude toxin, isolated small components) were assigned as the independent

variables. For the isolated small components, the highest achievable treatment concentration shared by all targets (100 µg/mL) was selected for comparison. For the crude toxin, a treatment concentration of 200 µg/mL was chosen for comparison to account for the estimated 50% composition of small components. Prior to analysis, the assumptions of normality and homoscedasticity were confirmed using Kolmogorov–Smirnov and Levene’s Statistic, respectively. Following a significant ANOVA result, the post hoc least significant difference (LSD) was used to identify specific target (cells/parasites) and toxin type (crude/small component) groups that exhibited statistically significant differences in survival. Analyses were conducted in IBM SPSS version 10.

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Chapter 10

Discussion

Stonefish (*Synanceia* spp.) are one of few animals that harness both a defensive venom (associated with their dorsal spines), and a secreted skin toxin (ichthyocrinotoxin). Prior to the commencement of this project, most of the research surrounding stonefish focussed on the medical significance of their venom. Resultingly, there was a significant gap in our understanding of the ecology of these animals and their toxins, especially their ichthyocrinotoxins. Further to this, the biodiscovery potential of toxins produced by stonefish was completely unconsidered. Numerous innovations that exist in the modern world, from medicines to industrial compounds, have been derived from nature. Arguably, one of the fastest and most effective ways to support biodiscovery is to better understand the ecology of the organism being targeted. Yet, this is seldom the approach of modern research, and there appears to be a disconnect between disciplines, especially between ecology and medicine. As such, using stonefish as a model organism, the focus of this thesis was to demonstrate the advantages of cross-disciplinary research by improving the current understanding of the ecology of these animals and their toxins, and leveraging this knowledge to highlight and explore potential biodiscovery pathways.

The primary aims of this research were to:

- ix) Investigate the diel activity patterns and habitat preferences of stonefish to gain a better understanding of how the animal interacts with its environment and how its toxin ecology is integrated into this.
- x) Assess the effect of species and geographic location on the composition of venom from *Synanceia horrida* (estuarine stonefish) and *Synanceia verrucosa* (reef stonefish).
- xi) Determine whether the composition of stonefish ichthyocrinotoxins vary between typically reef-dwelling (*S. verrucosa*) and estuary-dwelling (*S. horrida*) species.
- xii) Determine whether the composition of *S. horrida* ichthyocrinotoxin varies between geographically isolated populations.
- xiii) Quantify the palatability of *S. horrida* and *S. verrucosa* ichthyocrinotoxins to potential predators (e.g., *Dendrochirus zebra*, *Gymnothorax pseudothyrsoides*) to evaluate their role in predator defence.
- xiv) Identify the optimum xWORM assay conditions (i.e., media type, media concentration, and parasite density) for measuring the motility of *Nippostrongylus brasiliensis* L3

(infective rodent hookworm larvae) and *Necator americanus* L3 (infective human hookworm larvae).

- xv) Investigate the potential role of *S. horrida* and *S. verrucosa* ichthyocrinotoxins in parasite defence by measuring their toxicity to *N. brasiliensis* L3.
- xvi) Isolate and identify the anthelmintic components within the ichthyocrinotoxins of *S. horrida* and *S. verrucosa*, and evaluate their cytotoxicity against human skin and bile duct cells, as well as human and rat red blood cells *in vitro*, to assess their therapeutic potential.

Stonefish are widely perceived as a cryptic benthic ambush predator (Endean, 1961). The findings of this research have provided additional evidence to support this. However, the way stonefish utilise/interact with their environment may be more complex than previously recognised, which may drive their toxin ecology. Although stonefish were found to spend majority of their time sedentary in complex (i.e., structured) habitat, they were observed to move into simple (i.e., sand-only) habitat and had a notable increase in activity within this habitat type, particularly in the early morning. It is possible that stonefish traverse simple habitats to move to locations that increase their chances of encountering prey or reduce the likelihood of prey detection. Alternatively, stonefish may employ a different hunting strategy in the morning, moving out in the open in a bid to be perceived as a resource and/or refuge to early morning foraging prey.

The possibility that stonefish traverse and/or occupy habitats that increase their visibility to predators, coupled with their weak swimming abilities (Endean, 1961), exemplifies their need for a defensive venom. While *S. verrucosa* and *S. horrida* typically occupy different habitats – those being coral reefs, and inshore intertidal/estuarine environments, respectively – the transitional environments where their defensive venom is required may harbour the same or similar predator dynamics. This might explain why the composition of stonefish venom is largely conserved between species, particularly in places like Kota Kinabalu, where there is a greater overlap between reef and estuarine environments. However, considering their wide distribution across the Indo-Pacific, and thus, the broad spectrum of environmental conditions that they likely encounter, it is unsurprising that stonefish venoms also exhibited some degree of compositional variation.

Variation in the composition of stonefish venom was evident both between species in a single location, as well as within species across geographically isolated populations.

Geographic variation in stonefish venom composition was also observed at all spatial scales ranging from hundreds to thousands of kilometres. However, this research did not explore the ecological factors that could be driving this variation. Additionally, it is unknown whether the observed variation represents plasticity (i.e., changes in toxin composition within a lifetime driven by a certain condition or set of conditions), or whether it arose from genetic drift (i.e., generational/evolutionary change). It would be beneficial to conduct a set of experiments under controlled conditions to elucidate the effect of various factors that have previously been identified as potential drivers of venom plasticity in other taxa, such as diet (Daltry *et al.*, 1996), predation pressure (Dutertre *et al.*, 2014), temperature (Smith *et al.*, 2023; O'Hara *et al.*, 2018), and salinity (O'Hara *et al.*, 2021; Sachkova *et al.*, 2020), on the composition of stonefish venom. Additionally, determining whether physiological factors, such as sex and ontogeny, influence stonefish venom composition may provide further insight into the ecology of the animal. Whole genome sequencing of each stonefish species is also necessary to determine the contribution of genetic drift on compositional variation in stonefish venom between geographically isolated populations. Understanding how variations in venom composition are produced may help researchers to manipulate the animal to target, and/or select specific populations that produce the desired variations of components for biodiscovery applications. It is also important to identify the factors that influence venom composition so that more informed research can target venom variation in other taxa.

Several compounds within stonefish venom, including proteins (Kato *et al.*, 2016; Garnier *et al.*, 1997; Abe *et al.*, 1996; Ghadessy *et al.*, 1996; Kreger, 1991), enzymes (Madokoro *et al.*, 2011; Hopkins & Hodgson, 1998; Poh *et al.*, 1992; Austin *et al.*, 1965), and small molecules (Saggiomo *et al.*, 2024), have been structurally and functionally characterised. However, this research highlights that these represent only a fraction of the total diversity of components that comprise the venom of stonefish. This agrees with recent transcriptomic and proteomic analysis of components found in *S. horrida* venom, which indicate the presence of numerous uncharacterised and unique compounds (Ziegman *et al.*, 2019). Compositional variation in stonefish venom between species and locations might explain the diversity of clinical symptoms associated with envenomation in people (Armstrong *et al.*, 2023; Hifumi *et al.*, 2020; S. Y. Ngo *et al.*, 2009). However, the multifarious and dynamic composition of stonefish venom also highlights that beyond its deleterious nature to people, it also constitutes a rich resource of novel components, boasting huge potential for biodiscovery.

Future research on stonefish venom should focus on isolating and characterising additional components to better understand their ecological roles and medical significance. The mass of some components could undergo preliminary investigation using liquid chromatography mass spectrometry (LCMS), or Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF). Building on this, nuclear magnetic resonance (NMR), proteomic techniques, and bioinformatics could then be applied to extract structural and functional information about the venom components. This knowledge would likely contribute to the development of more effective treatments for stonefish stings, as well as potentially reveal the practical applications of some venom compounds.

This work also provided the first compositional profiles of ichthyocrinotoxins secreted by *S. horrida* and *S. verrucosa*. Like the venom, the composition of stonefish ichthyocrinotoxins also exhibited similarities and differences both between species (*S. horrida* and *S. verrucosa*), as well as within species (*S. horrida*) across geographically isolated Australian populations. However, the ecological factors driving variation in stonefish ichthyocrinotoxin composition were also not explored by this research. Despite significant advancements in venom ecology across numerous taxa, the ecological factors influencing the composition of secreted toxins, including poisons and ichthyocrinotoxins, remain poorly understood. This is particularly true for secreted toxins that are produced *de novo*, which, given their glandular origin, likely includes stonefish ichthyocrinotoxins. The strongest force acting upon the composition of venoms often relates to its ecological function, specifically the organism/s that it acts upon. As such, it is plausible that this might also be true for stonefish ichthyocrinotoxins.

Prior to the commencement of this research, very little was known about the ecological function of stonefish ichthyocrinotoxins. Based on the existing evidence from other ichthyocrinotoxic taxa (Abdul-Haqq & Shier, 2008; Randall, 1967), ostensibly, one of the roles of stonefish ichthyocrinotoxins was thought to be predator defence. However, stonefish ichthyocrinotoxins were not found to be distasteful or repellent towards two potential predator species, which broadly suggests that the toxin likely does not function solely as an anti-feedant defence. This corroborates the findings of earlier studies evidencing that stonefish ichthyocrinotoxins lack ichthyotoxicity, and thus do not function as a defensive toxin (Cameron *et al.*, 1981). Given that stonefish possess a potent defensive venom, it is unsurprising that their ichthyocrinotoxins do not play a major role in predator defence.

Nevertheless, stonefish ichthyocrinotoxins still potentially serve, at least in part, a defensive role, particularly against organisms that may interact with their skin. Stonefish lack body scales and have a close association with the benthos, often burrowing into the substratum (Cameron & Endean, 1973; Endean, 1961). As such, their skin is presumably highly susceptible to a range of benthic flora and fauna, especially those that attach to the skin surface or burrow through it. Stonefish may benefit from some of these interactions, such as the recruitment of algae on the skin which would aid in crypsis, and potentially increase their success as an ambush predator by luring herbivorous prey into strike range. Alternatively, others could be detrimental, such as the attachment styles of certain epibionts (i.e., bivalves), which could cause wounds, or the invasion of parasites and certain protozoans that cause illness or morbidity. The wide range of organisms that likely interact with stonefish skin, which ichthyocrinotoxins may serve to regulate, could explain the multifarious and proteinaceous nature of these toxins.

This research investigated the effect of stonefish ichthyocrinotoxins against one group of organisms that are likely being regulated by this toxin, these being parasitic gastrointestinal worms, or helminths. A key step in this process was to optimise a method, the xWORM assay, to accurately measure the toxicity of stonefish ichthyocrinotoxins against the specific parasite species and life stage being targeted. While the xWORM assay had been previously established, the influence of assay conditions on parasite vitality had not been considered. This research quantified the effects of media type, media concentration, and in-well parasite density on the vitality of *Nippostrongylus brasiliensis* L3 (infective rodent hookworm larvae), and *Necator americanus* L3 (infective human hookworm larvae) to optimise the xWORM assay for measuring the anthelmintic activity of stonefish ichthyocrinotoxins against these species. The optimal conditions that supported parasite vitality while balancing the requirements of the measuring equipment were found to be unique for each parasite species tested. As such, it is reasonable to suspect that the optimal xWORM assay conditions also differ for other parasite species, and potentially, life stages. This highlights the importance of establishing a dedicated optimisation phase for each parasite species and life stage prior to the commencement of novel drug trials. These findings further emphasise the significance of not only considering the ecology of the organism that is being bioprospected, but also that of the living organism being targeted for disruption.

The xWORM assay could be further improved by exploring other potentially crucial assay parameters such as the rate of oxygen depletion over time (as an indication of metabolic rate),

and whether this changes in response to different temperatures. Additionally, both adult and larval hookworms are known to produce excretory and secretory products, however, it is unknown whether a build-up of these products could potentially have a negative effect on parasite vitality. As such, future studies could also investigate the dose-dependent effects of excretory/secretory products on parasite vitality. This will allow for a better understanding of the factors that may influence the vitality of target parasites under *in vitro* assay conditions which is essential in the pursuit of novel anthelmintic drug leads.

The optimised xWORM assay was then used to determine whether stonefish ichthyocrinotoxins influenced the vitality of skin-penetrating larval (L3) *N. brasiliensis*. Both *S. horrida* and *S. verrucosa* ichthyocrinotoxins were found to be toxic to *N. brasiliensis* L3. This supports the hypothesis that one of the functions of stonefish ichthyocrinotoxins may be to prevent parasites from penetrating their skin. This could explain previous reports of an unusually low diversity of parasite taxa observed within these animals (Miller *et al.*, 2018). However, as stonefish are currently under-sampled for parasites, future studies should consider conducting comprehensive internal examinations, encompassing various tissues and multiple stonefish. This may reveal that stonefish harbour a higher diversity of parasites than previously thought. This task might become less laborious through the application of eDNA technology, whereby whole tissue samples from expired stonefish could be extracted and analysed for parasite biota. Investigating the internal parasite biota of stonefish could provide some insight into the parasite taxa that stonefish ichthyocrinotoxins may be selective against, or alternatively, identify those that the toxin does not target.

Strikingly, stonefish ichthyocrinotoxins also exhibited intraspecific differences in anthelmintic activity, whereby ichthyocrinotoxin from *S. horrida* was more potent to *N. brasiliensis* L3 than that of *S. verrucosa*. Moreover, the components responsible for the anthelmintic activity also differed between the two species ichthyocrinotoxins. For *S. horrida* ichthyocrinotoxin, the majority of the anthelmintic activity was associated with the small (<3 kDa) components. In contrast, *S. verrucosa* ichthyocrinotoxin lost activity when the large (>3 kDa) and small components within the toxin were isolated. While the mechanism underlying the anthelmintic activity of *S. horrida* ichthyocrinotoxin remains unclear, it is possible that *S. verrucosa* ichthyocrinotoxins activity relies on synergistic interactions between its components. Alternatively, the purification process may have inactivated this toxin. To test for synergism in

S. verrucosa ichthyocrinotoxin, the small and large components would need to be recombined following separation and retested for anthelmintic activity.

Intraspecific differences in the potency and source of the anthelmintic activity of stonefish ichthyocrinotoxins further highlights the differences in the ecology of these species, which could also potentially extend to individual organisms or geographically isolated populations. For instance, estuarine environments may harbour a greater diversity and/or abundance of parasite taxa compared to coral reefs. This may necessitate *S. horrida* wielding a toxin with greater potency or versatility against parasites, which may explain why *S. horrida* ichthyocrinotoxins employ different components than *S. verrucosa* for parasite defence. It is important to note that *N. brasiliensis* are not likely to be a natural parasite of stonefish. As such, future research should aim to determine whether stonefish ichthyocrinotoxins exhibit anti-parasitic qualities against naturally fish-hosted parasites. However, it may also be worthwhile considering that the connectivity between terrestrial and estuarine environments could potentially enhance the exposure of predominantly estuary-dwelling stonefish species, like *S. horrida*, to mammalian-hosted parasites. This might explain the greater potency of *S. horrida* ichthyocrinotoxin to *N. brasiliensis* L3 compared to the predominantly reef-dwelling *S. verrucosa*. This, again, exemplifies the importance of developing a better understanding of the ecology of the organism to aid biodiversity research efforts.

Presumably, the species assemblages, diversity, and/or abundance of parasite taxa also varies between locations separated by larger distances. This may be another factor contributing to intraspecific variation in the composition of *S. horrida* ichthyocrinotoxins between geographically isolated populations, particularly between the tropical and subtropical regions of Australia. As such, future studies should consider employing eDNA technology to investigate the parasite biodiversity within different environments and/or locations. This could provide further insight into which parasite taxa may be naturally targeted by, and/or shaping the composition of, stonefish ichthyocrinotoxins. Understanding the parasite dynamics associated with stonefish and their environment would also help to guide biodiversity research efforts for novel antiparasitic agents.

From a therapeutic perspective, ichthyocrinotoxins from *S. horrida* emerged as a more attractive novel drug candidate due to the small size of the active components. This is because components of smaller size generally offer simpler characterisation, cheaper manufacturing, and are often highly stable with predictable pharmacological properties (Gu *et al.*, 2023; H. X.

Ngo & Garneau-Tsodikova, 2018). The therapeutic potential of the small components isolated from *S. horrida* ichthyocrinotoxin is further strengthened by their minimal toxicity to mammalian cells. While the small components exhibited some toxicity to human skin and bile duct cells, this was significantly lower than that of the whole (crude) toxin. This suggested that the components responsible for cell toxicity may be distinct from those driving the anthelmintic activity.

Importantly, at a treatment concentration of 100 µg/mL, the small components from *S. horrida* ichthyocrinotoxin were found to be at least five times more toxic to *N. brasiliensis* L3 than any mammalian cell line tested. Further purification and the identification of the component/s in *S. horrida* ichthyocrinotoxin that are responsible for its anthelmintic activity is now necessary to progress this research down the drug development pipeline. This could involve chromatography techniques that have a high separation resolution such as High-Performance Liquid Chromatography. Additionally, sequencing the stonefish genome, followed by RNA transcriptomics on the venom bulb and skin tubercles would enable both the structural and functional characterisation of identified component/s of interest, as well as the recombinant production or synthesis of the component/s in the laboratory.

To assess the broad-spectrum potential of the component/s, it is crucial to evaluate their efficacy against a wider range of parasite species, particularly medically significant hookworm species like *N. americanus* and *Ancylostoma ceylanicum*, and different life stages of these parasites. Utilising a combination of *in vitro* and *in vivo* studies would also provide insight as to whether these component/s could be applied as both a preventative and curative treatment. Progressing this research could potentially lead to the development of a novel anthelmintic drug, offering a new tool in the fight against parasitic infections in livestock, domestic pets, and humans, as well as addressing the growing challenge of anthelmintic drug resistance.

Continuing to build a better understanding of the ecology of stonefish and their toxins could also reveal additional opportunities for biodiscovery. Currently, the function of the larger components within *S. horrida* ichthyocrinotoxin remain undetermined. One obvious avenue for future research with this species may be to determine whether these other components play a role in regulating epibiosis, specifically by preventing the attachment of certain invertebrates, while facilitating the attachment of certain plants or algae. Furthermore, comparative studies between stonefish species, as well as between geographically isolated populations of a single species, could reveal how toxin-mediated epibiosis regulation may be tailored for different

ecological conditions or strategies. Such insights could lead to the development of a natural antifoulant for maritime structures or vessels, or a fertiliser for aquaculture to boost the growth of consumable or medicinal aquatic flora.

In conclusion, by studying stonefish, this thesis has demonstrated how an improved understanding of an animal's ecology can inspire novel applications and guide biodiscovery research efforts. Numerous aspects of the ecology of stonefish and their toxins were investigated, including: i) the habitat preferences and activity patterns of the animal; ii) how their ecological background (in this case species and geographic location) can influence the composition of their toxins; and iii) using deductive reasoning, explored how the animal might utilise its toxins. Finally, drawing upon the ecological insights that were established within this body of work, this thesis investigated the potential therapeutic applications of stonefish ichthyocrinotoxins, and in doing so, culminated in the discovery of a promising new anthelmintic drug lead. To pursue this prospective novel drug lead would require further purification, followed by the structural and functional elucidation of the active component/s, and finally, an assessment of the component/s broad-spectrum potential under both *in vitro* and *in vivo* models. Perhaps exploring other aspects of stonefish toxin ecology, such as the role of their ichthyocrinotoxins in regulating epibiont interactions with the skin, or the functions of other venom components, could uncover additional avenues and opportunities for biodiscovery. This research has generated baseline data and provided a foundation for future studies aimed at biodiscovery of stonefish toxins. The challenges now lie in designing and conducting statistically robust experiments that provide deeper insight into the ecological factors driving variations in the toxins of these species. Only then can we confidently stride forward in the search for novel compounds with practical applications derived from these toxins.

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