



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). 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, 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 ichthyocrotinotoxins that stonefish secrete onto their epidermis is limited. Therefore, this review aims to consolidate literature on ichthyocrotinotoxins 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 ichthyocrotinotoxins and their potential practical applications as therapeutics and/or industrial products.

2. The role of ichthyocrotinotoxins 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). Ichthyocrotinotoxins 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 ichthyocrotinotoxins 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 ichthyocrotinotoxins (Randall, 1967). This hypothesis was later supported by a study on the feeding responses of both wild and captive elasmobranchs to ichthyocrotinotoxins secreted by *Paradachirus 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, ichthyocrotinotoxic *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 ichthyocrotinotoxin, were observed to repel wild grey reef sharks (*Charcharhinus amblyhinchus*) 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* ichthyocrotinotoxins 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 ichthyocrotinotoxins 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) ichthyocrotinotoxic *Gobiiodons* (coral-dwelling gobies) outside of their coral host. Instead, one study has shown that *Gobiiodon* ichthyocrotinotoxins, 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 ichthyocrotinotoxic *Gobiiodons* compared to those that were not (Dirnwoeber and Herler, 2013). This gave rise to the hypothesis that *Gobiiodon* ichthyocrotinotoxins 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* ichthyocrotinotoxins 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 ichthyocrotinotoxins 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 ichthyocrotinotoxins 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 and Zlotkin, 2009; Marezki 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., ichthyocrotinotoxin) 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 ichthyocrotinotoxins could be noxious to other fish (Brock, 1956).

Studies conducted in a controlled aquarium environment demonstrated that when exposed to ichthyocrotinotoxins 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; Marezki and Del Castillo, 1967; Thomson, 1969). Recovery from these symptoms depended on the species of ichthyocrotinotoxin to which the fish were exposed (Marezki and Del Castillo, 1967; Thomson, 1969). For instance, exposure to *O. meleagris* ichthyocrotinotoxins led to irreparable illness and death within an hour, while exposure to *Rypticus saponaceus* (greater soapfish) ichthyocrotinotoxins could be reversed within one to two hours of transfer to clean, toxin-free water (Marezki and Del Castillo, 1967; Thomson, 1969).

2.3. Defensive toxins - mammals

Moreover, ichthyocrotinotoxins previously identified to be ichthyotoxic were also found to exhibit hemotoxic properties. Studies reported that the forced consumption or injection of ichthyocrotinotoxins 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) ichthyocrotinotoxin (Hashimoto and Kamiya, 1969). Likewise, mice injected with ichthyocrotinotoxins 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; Marezki 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' ichthyocrotinotoxins are primarily perpetrated by components with similar chemical properties.

For instance, pardaxin, pahutoxin, and grammistin, were isolated from the ichthyocrotinotoxins 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 (Kalmanzon 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; Kalmanzon and Zlotkin, 2009). The surfactant components within some ichthyocrotinotoxins 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 ichthyocrotinotoxins 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 ichthyocrotinotoxin 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 and 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 boxfishes own cell membranes (Kalmanzon et al., 1999, 2003). It is essential to note that although extensive compositional and structural data exist for the ichthyocrotinotoxins of some species, others, such as those belonging to members of *Synanceia*, remain relatively unexplored.

2.5. Harnessing both venom and ichthyocrotinotoxin

Contrary to common perception, not all ichthyocrotinotoxins 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 ichthyocrotinotoxins 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 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 in the mucosal secretions of all fish and those specific to ichthyocriotoxins hinges on the source of the secretion – i.e., general mucosal cells and specialised ichthyocriotoxin-producing glandular structures, respectively – it is important to consider the potential coexistence of both types within the epidermal environment of ichthyocriotoxin 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 ichthyocriotoxins 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 ichthyocriotoxins in mitigating integumentary interactions

In addition to exhibiting reduced or absent scales, many ichthyocriotoxin organisms also demonstrate a sedentary and/or site-attachment lifestyle (Cameron and Endean, 1973; Endean, 1961). This behaviour increases the vulnerability of ichthyocriotoxin 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 ichthyocriotoxins 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 ichthyocriotoxin 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, ichthyocriotoxin 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 ichthyocriotoxin 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* ichthyocriotoxin. 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 ichthyocriotoxin species, only one study by Munday et al. (2003) has directly investigated the possible anti-parasitic properties of ichthyocriotoxins.

Munday et al. (2003) examined the interactions between ichthyocriotoxin *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 ichthyocriotoxins 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 ichthyocrotinotoxin, to expedite the reattachment of these organisms. Whether stonefish ichthyocrotinotoxins 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 ichthyocrotinotoxin, 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* ichthyocrotinotoxins. The researchers discovered that a component within *S. horrida* ichthyocrotinotoxin, 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 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 scutum rostralis muscles (i.e., the muscle that controls the scuta lips) for up to 1 hour after immersion in *S. horrida* ichthyocrotinotoxin, 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* ichthyocrotinotoxins 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 ichthyocrotinotoxins

Ichthyocrotinotoxins 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 ichthyocrotinotoxins. Therefore, further research is required to determine the natural function of stonefish ichthyocrotinotoxins, as well as their potential therapeutic or industrial applications.

One area of research that warrants further exploration is the potential role of stonefish ichthyocrotinotoxins in predator defence. Although stonefish ichthyocrotinotoxins, 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 ichthyocrotinotoxins 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 ichthyocrotinotoxins 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 ichthyocrotinotoxins 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 ichthyocrotinotoxins 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 Serranidae 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 scutum 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 ichthyocrotinotoxins 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-Pradrón, 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 ichthyocrotinotoxins 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 ichthyocrotinotoxins 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 ichthyocrotinotoxins. 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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