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The toxins that time forgot:

Characterisation of marine toxins with emphasis on the neglected status of marine toxinology.

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Abstract

Toxins, both venom and poison, have great potential for pharmacological, ecological and evolutionary research. Yet despite this, many toxins, especially in marine organisms, have been relatively unstudied. This negligence within marine toxinology is surprising given the vast rise in proteomic and transcriptomic methods available to analyse toxins. This study attempts to characterise toxins of marine taxa of which little is currently known. In doing so, it provides a template for future studies to explore marine toxinology. The species chosen were lesser weever fish (*Echiichthys vipera*), greater weever fish (*Trachinus draco*) and a nemertean (*Lineus longissimus*). A proteomic and transcriptomic approach was taken, utilising methods as SDS-PAGE, HPLC, MS-MS and total RNA analysis to understand the composition of the toxins. Further, the morphological and ecological aspects of the toxin delivery systems were also investigated. Proteomic data obtained for both weever fish highlight key aspects of their venom composition which corroborate previous research, confirming the presence of both Trachinine and Dracotoxin along with their molecular masses. Here I have described novel venom delivery systems with unique structural and morphological features in weever fish, and have characterized three novel *L. longissimus* toxins, testing their effects on a natural predator. Transcriptomic analysis of *E. vipera* and *L. longissimus* toxins reveal a suite of matches to closely related toxic species indicating that gene duplication may play an important role in the evolution of marine toxins. This research has provided new insights into the evolution of marine toxins through family lineages and can potentially aid in further understanding the evolution of toxins throughout Animalia. The discoveries made in this study represent an ideal start to push marine toxinology from a neglected status and into the forefront of toxinological research.

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Figures & Tables

1. Introduction

Toxins, utilised as venom or poison, are complex mixtures of chemical compounds that have convergently evolved throughout the animal kingdom for defense, predation and competitive purposes (Casewell *et al*., 2013; Nelsen *et al*., 2013). Toxins were thought to have evolved first within the marine environment, with specialised algal species producing poisonous toxins (Rantala *et al*., 2004; Dittman *et al*., 2013) followed by early species of invertebrates as cnidarians, molluscs and echinoderms evolving venoms, with a later evolution in vertebrates as fish (Casewell *et al*., 2013). Such a large evolutionary time scale should allow for a greater refinement and specificity of marine toxins than that of their terrestrial counterparts, who evolved the use of toxins much later in evolutionary terms (Sunagar & Moran, 2015). Yet it appears that toxins within the marine environment are somewhat a neglected topic, in comparison with the countless studies on terrestrial organisms as reptiles, spiders, scorpions and amphibians. It is certainly unclear as to the exact reason why many toxic marine organisms are relatively understudied, especially given how many authors have brought this negligence to light, pinpointing specific marine groups as echinoderms, molluscs, crustaceans, nemerteans, polychetes (von Reumont *et al*., 2014a, b & c) and fish (Ziegman & Alewood, 2015; Campos *et al*., 2016). There are very few marine species that have had extensive research conducted on their toxins to date, e.g. cone snails (Olivera *et al*., 1985; Olivera *et al*., 1990; Olivera, 2002; Dutertre *et al*., 2014) and stone fish (Kreger, 1991; Garnier *et al*., 1997; Khoo, 2002). The studies on these two taxa have provided breakthrough research outputs within pharmacology (McIntosh *et al*., 1982; Clark, 1996), as well as evolutionary and toxinological fields (Ellisdon *et al*., 2015; Duterrtre *et al*., 2014). Yet despite these major breakthroughs studies on marine toxins still remain elusive within toxinology.

Toxinology is the study of naturally occurring biological toxins from animals, plants and microbes. The development of '-omic' techniques (transcriptomics and proteomics) has permitted this field to recently be at the forefront of cutting edge science. Thus, allowing for new areas such as venomics to be born, as well as developing interdisciplinary studies within pharmacology (Escoubas *et al*., 2008; Calvete *et al*., 2009; Vetter *et a*l., 2011). With these techniques, new tools have been developed to better identify and characterise toxins as well as explore the evolution of these toxins through multiple lineages. This has led to some ground-breaking evolutionary theories and debates on toxin evolution (Fry *et al*., 2006; Hargreaves *et al*., 2014) and to the development of new pharmaceuticals, e.g. Prialt, Exenatide and Captopril (Clark, 1996; Fernandez *et al*., 2004; Triplitt & Chiquette, 2006), that are widely used today and are currently the most effective drugs to treat their target diseases. New protocols for use with bioanalyzers have also been developed regarding venom research. This allows for the fast and effective

composition analysis of crude animal venoms (Zancolli *et al*., 2017). These methods are ideal for determining protein constituents of venoms without the laborious procedures of gel electrophoretic techniques, and can be used to further answer questions regarding the ecological and evolutionary facets of venom. This protocol however was designed using snake venom, and is yet to be tested with other species, particularly within the marine environment. Consequently, based on the vast development and utilisation of such techniques within toxinology, it seems odd still, that marine toxins are not being subject to such scrutinising research.

Thus, the question must be asked, why are toxin producing marine taxa neglected within toxinology? Most problems seem to arise in the difficulty in obtaining good samples of venom/ poison which subsequently cause issues with methodology and experimental designs. The extraction method of toxins can be difficult due to many marine species utilising unique venom/ poison apparatus, not found in terrestrial species. For example; nematocysts, beaks, harpoons, spines (Ziegman & Alewood, 2015; Fry *et al*., 2009; Smith & Wheeler, 2006; Smith *et al*., 2016) and toxin mucus secretions (Kem, 1985; Bakus *et al*., 1986; Mebs, 2000). Some of these structures are very delicate, which can be damaged by the process of milking, severely harming or reducing the fitness of the species involved. These structures are not as simplified or robust as fangs, claws or stingers found in many terrestrial taxa. Thus, terrestrial species can easily be milked multiple times with no detrimental effects. Another problem with sample extraction is the contamination of samples with water impurities and/ or mucus associated with toxin distribution and protection (Baumann *et al*., 2014). Many marine toxins, particularly fish toxins, are very delicate labile molecules, which are sensitive to pH, heat, lyophilisation and freeze-thaw processes (Baumann *et al*., 2014), making some protein analyses and assays difficult to conduct. Another barrier within the research is the inaccessibility of some marine species (Church and Hodgson, 2002; Jha and Zi-Rong, 2004; Smith and Wheeler, 2006). The marine environment is a specialised habitat in which some species may be difficult to locate or may reside at remote depths. Further relating to this is the difficulty in maintaining healthy organisms in an aquarium environment, with a limited time frame to conduct research before organisms become stressed and/or die (Barton & Iwama, 1991; Iwama, 1998).

Although the aforementioned issues seem to pose some drawbacks, advancing scientific methods and techniques can now be used to eliminate or reduce the problems in which some of these issues raise. There have been efforts to provide better purification methods of toxin samples in fish venoms for analysis using various proteomic methods (Baumann *et al*., 2014; Casewell, *et al*., 2017; Han *et al*., 2017). As well as methods of venom extraction in fish (Mohamadi, *et al*.,

2015; Almada, *et al*., 2016); which can potentially be applied to a range of marine taxa whilst avoiding ethical and animal welfare issues. Advancing technologies have allowed for the identity of protein sizes and the exact protein structures using cutting-edge procedures as liquid chromatography–tandem mass spectrometry (LC-MS/MS), Protein-NMR, HPLC and electrophoretic procedures. In combination with next-generation sequencing (NGS), these techniques vastly are becoming much more efficient at identifying toxins and being able to predict their functions, which ultimately can allow for novel drug developments. These modern venomic approaches that utilise high-throughput proteomics and transcriptomic methods to identify the function of toxin proteins and the genes that code for the toxins themselves. With this, not only can the identity of toxins to be investigated, but the evolution of these toxins through multiple lineages, bringing about comparable studies across multiple research fields. These advancing techniques are also perfect to reevaluate previous toxin research that was conducted in an era that had limited and now outdated biochemical and proteomic methods.

Therefore, given the importance of studying toxins using modern biochemical and -omic technologies, combined with the fact that toxic marine organisms have been neglected within current literature, we should attempt to target prime candidates by which the characterisation and analysis of their toxins could provide a basis for pushing forward research on marine toxins. We have chosen three marine species (two weever fish and one nemertean) as primary candidates to investigate their toxins. These species were chosen based on their ease of accessibility within UK waters and their overall lack of, or out dated research relating to their toxic arsenal.

1.1 Clearing things up: The difference between venom, poison and toxungens

Before the relevant species are introduced, we must ensure that the terminology is clear, and the distinct differences between venom, poison and toxungens are highlighted. Venoms are toxins that are purposefully injected into an organism by means of a specialised apparatus such as fangs, spines, claws, nematocysts etc., which provide a benefit to itself such as predation, defence, competition or communication (Nelsen *et al*., 2013; Fry *et al*., 2009; Arbuckle, 2015). Conversely, poisons are encountered passively by ingestion, inhalation or absorption across the body surface, via a bioaccumulation of external toxic compounds and without the need of a specialised delivery system (Nelsen *et al*., 2013). Toxungens slightly differ from that of a poison in the sense that are sprayed, spitted or smeared onto a predator (Nelsen *et al.,* 2013). In some instance toxungens can be classified as a poison, but due to some distinct delivery and mechanical differences they can be also classed as a different toxic weaponry. Poisons and

toxungens are used strictly for defensive purposes, unlike the multi-functionality of venoms (Nelsen *et al.,* 2013).

1.2 Relevant species

1.2.1 Weever fish: *Trachinus draco & Echiichthys vipera*

Weever fish are members of the family Trachinidae (Scorpaeniformes) and their distribution range covers from the eastern Atlantic shores of Europe, the Mediterranean and North Africa (Russell & Emery, 1960; Cain, 1983; Tortonese, 1986). The term 'weever' is thought to come from the Anglo-Saxon word 'Wivre' which translates to 'viper', a seemingly apt description of these fish (Russel & Emery, 1960). The lesser weever (*Echiichthys vipera,* cited in previous literature as *Trachinus vipera*) ranges between 6-12cm in body length. Whilst the greater weever (*Trancinus draco*) is much larger, between 28-50cm as adults (Russel & Emery, 1960). The weevers usually have a yellow-grey dorsal colouration, with a white underside, and sometimes have an iridescent sheen to their mottled pattern. *E. vipera* has a very characteristically unique yellow and black caudal fin, which is distinctly different from other species of weever fish. Both species are benthic burrowers, burying their bodies in the sediment, with just their dorsally positioned eyes protruding. *E. vipera* inhabits shallower sandy coastal waters and can pose a problem to beach tourists, whilst *T. draco* inhabits deeper waters and is usually only in contact with fishermen and deep-sea divers (Evans, 1907; Russel & Emery, 1960; Briars & Gordon, 1992). Both species use similar venomous spines as a defensive mechanism against predators (in depth detail in section 1.3.1), and it is these spines that can cause problems for both fishermen and beach tourists.

1.2.2 Nemertean*: Lineus longissimus*

Lineus longissimus is a ribbon worm of the phylum Nemertea (Anopla, Heteronemertea). *L. longissimus* is considered the world's longest animal with a recorded length of up to 60m (Gittenberger & Schipper, 1992), and therefore by default it would also be the world's longest toxic organism. However, there is no museum or voucher specimen of this size, and this evidence comes from a written occurrence and the true accuracy is somewhat uncertain. Most specimens do not reach such great lengths, as the average length is 5-10m and their width is 5-10mm (Gittenberger & Schipper, 1992). Such a long, thin shape gives them their resemblance to and common name of 'Bootlace worm'. They inhabit coastal waters, hiding in rocks and crevices of tide pools and sandy sublittoral zones. They range from Northern and Western European-Atlantic shores to the Baltic sea regions, with some reports of specimen found around the Mediterranean

(Gibson, 1995). They are predominantly black/ dark-brown in colouration, sometimes with lighter striations along the body. When they are disturbed they produce a copious mucus which contains the toxins (in depth detail in sections 1.3.2 & 1.4.2).

Fig. 1. *A) Lesser weever fish (Echiichthys vipera) ©Robert Pillion – B) Greater weever fish (Trachinus draco) ©Hernández-González, C.L. – C) Bootlace worm (Lineus longissimus) ©Gwyln.*

1.3 Apparatus and mechanism of toxin delivery

1.3.1 *Trachinus draco & Echiichthys vipera*

Both species of weever fish, *T. draco* & *E. vipera,* have very similar toxin delivery systems, utilising an array of venomous spines located on their dorsal fin. The use of venomous fin spines as a method of venom delivery is almost synonymous throughout the majority venomous fish clades, with few rare exceptions as fang blennies (*Meiacanthus spp*.), utilising venomous teeth (Casewell *et al*., 2017) and rays containing a venomous tail barb (Smith & Wheeler, 2006; Smith *et al*., 2016). Both weever fish species have approximately four to eight dorsal spines and two opercular spines - one on either side of the body (Russel & Emery, 1960) (*Fig. 2*.). The dorsal spines of weevers are motile, thus when the fish detects the presence of a predator the dorsal fin is simply erected, awaiting the inevitable attack (Russel & Emery, 1960). The spines are covered by an integumentary sheath consisting of holocrine cells, in which contains the venom (Davies & Evans, 1996). When a predator attacks or the fish is stepped on, the sheath is ruptured, as the spines penetrate the sheath and venom discharges into the spines puncture wound (Russel & Emery, 1960; Briars & Gordon, 1992). The spine structures themselves are thought to contain anterolateral grooves located on the external surface (Smith & Wheeler, 2006; Smith *et al*., 2016). These grooves allow venom to move to the tip of the spine in a capillary-like mechanism as the integumentary sheath ruptures.

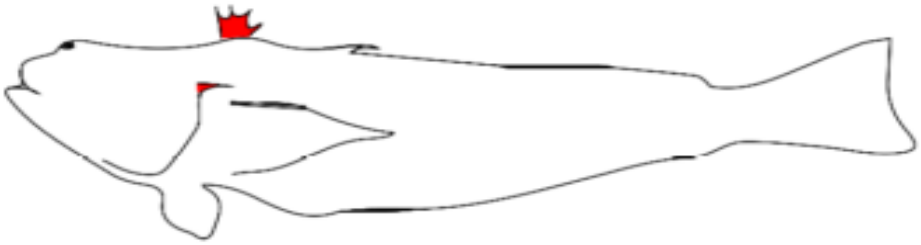

Fig. 2. *Diagram of 'weever fish' spines highlighted in red, indicating the dorsal fin spines and opercular spine(s). It is unknown which species of weever fish this diagram represents. Adapted from Ziegman & Alewood, 2015.*

It is still unclear whether the opercular spines on weever fish are venomous or are simply there as a non-venomous defence. Some literature suggests that all weever opercular spines are venomous (Russel & Emery, 1960; Smith & Wheeler, 2006), yet only *T. araneus* spines have been depicted in these studies. No isolation of toxic components has been attempted. Opercular spines are relatively common in fish, and are used in defence by 'flaring' the operculum open to prevent being eaten by predators. Despite this, it is unknown as to whether opercular spines of most fish are venomous or not. Given the energetic demands of venom maintenance, it would be easy to assume most opercular spines are non-venomous, but with a lack of observed data for this kind of defensive trait, it remains unanswered. Some species of Toadfish (Batrachoididae), which are known to have venomous opercular spines, have been recently shown to have a loss/ reduction of their opercular venom gland (Smith & Wheeler, 2006). This may suggest that the use of venom in opercular spines is no longer needed as non-venomous spines still provide an adequate defense. This is also certainly possible with weever fish, as there is no direct evidence of venom within their opercular spines. Only 1% of venomous fish seem to utilise venomous opercular defences (Smith *et al*., 2016), which suggests that venomous opercular spines have no greater effect on defense than non-venomous spines. However, these suggestions are based purely on first principles, and further study is needed for verification.

Despite there being some literature on the structural features of weever fish spines, there is no actual macroscopic data within the literature that adequately illustrates the structure of both dorsal and opercular venomous spines of *E. vipera* and *T. draco*. Even some diagrams within the literature seem to be inaccurate (Fig.2.), it is uncertain which species of weever fish this diagram

represents. Thus, a need to provide reliable evidence illustrating their venom apparatus is needed to form a consistent agreement on the structural features.

1.3.2 *Lineus longissimus*

Lineus longissimus, uses its toxins as both a poison and toxungen (Nelsen *et al*., 2013). They are secreted within a thick copious mucus from the integumentary tissues of the body. The toxins are accumulated and secreted via many different types of body cells (Bacq, 1936 & 1937; Kem, 1985), and the exact purpose and mechanism of secretion is still not fully understood (Kem, 1985). It is unclear if *L. longissimus* biosynthesises the toxins itself, or whether it sequesters the toxins from another organism. Some evidence suggests a possible symbiotic partnership with toxin-secreting bacterium species (McEvoy *et al.,* 1998; Carroll *et al*., 2003). However, recent reports indicate this may not in fact hold true as previous literature had produced a false positive for testing this symbiotic relationship, thus it is still highly debated if the toxins are produced symbiotically by a bacterial symbiont of *L. longissimus* (Strand *et al*., 2016).

The mucus seems to act as a double anti-predatory defence mechanism. Not only are the toxins concealed in the mucus when the nemerteans are provoked (Kem, 1985; McDermott & Roe, 1985), but the mucus appears to act like hagfish mucus, allowing an easy escape from predators getting a tight grip on their body (Zintzen *et al*., 2011). Yet, although the toxic mucus is more likely to play a defensive role than an offensive, nemerteans are still preyed upon by crustacean predators, which appears contradictory as their toxins were shown to have a high target specificity only for crustacean neuron receptors (Kem, 1985). Consequently, the exact ecological function of *L. longissimus* toxins remains elusive and a need to provide this data is vital in better understanding their toxin uses.

1.4 Biochemistry of toxins

1.4.1 *Trachinus draco*, *Echiichthys vipera* and the Scorpaeniformes family

The venom toxins of both weever fish seem to act in very similar ways causing severe pain at the site of envenomation as well as neuromuscular and cardiotoxic affects (Evans, 1907; Russel & Emery, 1960; Skeie, 1962; Chhatwal & Dreyer, 1992a & b). However, the basic biochemistry of their crude venom extracts seems to contain different toxic compounds (Russel & Emery, 1960; Carlisle, 1962; Chhatwal & Dreyer, 1992a & b).

Some elementary analyses conducted by Carlisle (1962), on the dorsal venom of *E. vipera* found compounds as 5-hydroxytryptamine (Serotonin), histamine releasers, kinin-like molecules

and muccopolysaccharides. Serotonin is a nocioreceptive compound known for its pain producing effects and its ability to facilitate the absorption of other toxic components of venom (Carlisle, 1962). It is also found in other venoms across the animal kingdom such as snakes, scorpions, bees and even some plants. Serotonin accounts for 0.1-2% of the dry weight of *E. vipera* venom, which is in excess of 0.2-0.4% in scorpion venoms (Adam & Weiss, 1956, 1958 & 1959; Carlisle, 1962). Serotonin causes paralysis in crustaceans when used by cephalopods (Erspamer, 1954). Other molecules found in venom, histamines and kinin-like compounds, simply work to facilitate the movement of serotonin and other toxins, by causing anti-inflammatory responses and vasodilation (Greisbacher *et al*., 1998; Bonnet, 2000). A later study discovered two lethal protein fractions (Perriere *et al*., 1988); the first was a large protein structure, aptly named 'Trachinine' that seemed to cause the cardiotoxic affects noted in previous literature (Russel & Emery, 1960; Perriere *et al*., 1988). Trachinine has a molecular weight of 324kDa and is estimated to consist of four identical subunits each of 81kDa. However, this protein tends to lose its toxicity as the molecular structure degrades over the 24 hours following extraction (Perriere *et al*., 1988), making some analyses difficult to conduct. The second was identified simply as 'non-migrating' fraction, as it did not migrate during electrophoresis. This non-migrating fraction might not be a single protein, and is suggested to be an aggregation of lethal proteins that act synergistically. These proteins have a range of molecular weights from 40-92kDa (Perriere *et al*., 1988). It seems the role of Serotonin is to disrupt neuronal input and cause server pain, whilst Tranchinine attacks the pulmonary and cardiovascular system. The 'non-migratory' fraction effects remain unknown. The combination of these components all appear to work in unison to create the lethal venom effects. The studies of *E. vipera* did not attempt to isolate any toxins from the opercular spines (Russel & Emery, 1960; Carlisle, 1962), which potentially could contain differing toxic compounds to the dorsal spines, if found to distribute venom at all.

The crude venom of *T. draco* was also analysed using basic biochemical techniques (Skeie, 1962; Chhatwal & Dreyer, 1992a & b). It was found to contain a Hyaluronidase type enzyme (Skeie, 1962) which is also found in many other fish venoms (Ziegman & Alewood, 2015). Hyaluronidase allows for the facilitation and distribution of other toxic compounds into the system by breaking down hyaluronan around the envenomation site (Ziegman & Alewood, 2015). A toxic protein of a molecular weight of 105kDa was isolated from a crude venom extract in another study, named 'Dracotoxin' (Chhatwal & Dreyer, 1992a & b). Dracotoxin was found to cause haemolysis via membrane depolarisation and has a high target specificity for rabbit erythrocytes (Chhatwal & Dreyer, 1992a). Unlike *E. vipera*, the previous literature has not attempted to test if Dracotoxin consists of sub-units, or whether it is related to the Trachinine toxin. Other than these known

components, nothing much else has been identified in relation to the biochemistry of *T. draco* venom, with very few published studies conducted.

From what little research has been produced on the characterisation of Scorpaeniformes toxins, the venomous species within the family all share similar characteristics between their venom composition. Based on the diversity of venomous fish species within Scorpaeniformes (Smith & Wheeler, 2006; Smith *et al*., 2016) only a small minority of species have had their toxin bioactivities identified. They are as follows; *Synanceia verrucosa, S. horrida, S. trachynis* (Kreger, 1991; Garnier *et al*., 1997; Khoo, 2002), *Scorpaena guttata, S. plumieri, Notesthes robusta, Hypodytes rubripinnis* (Schaeffer *et al*., 1971; Hahn & O'connor, 2000; Nagasaka *et al*., 2009; Andrich *et al*., 2010), *Pterois antennata, P. volitans, P. lunulata* (Shiomi *et al*., 1989; Kiriake & Shiomi, 2011), *Inimicus japonicas and Dendrochirus zebra* (Shiomi *et al*., 1989). All these toxins share similarities in haemolytic and cytolytic activities e.g pore-formation, as well as structural and functional parallels between toxin molecules. The toxins belong to a novel protein family that are composed of 2 subunits (Ghadessy *et al*., 1996; Garnier *et al*., 1997; Ueda *et al*., 2006; Kiriake & Shiomi, 2011; Kiriake *et al*., 2013), which based on protein sequencing, show similarities between all characterised toxins from Scorpaeniformes (Chuang & Shiao, 2014). It is further thought that these toxins all evolved from a single common ancestor toxin (Ghadessy *et al*., 1996; Ueda *et al*., 2006; Kiriake & Shiomi, 2011). Recent research indicates that the toxin genes present within Scorpaeniformes are under negative selection pressures and that gene duplication may have played a key role in the diversification and evolution of some of these toxins (Chaung & Shiao, 2014). This negative selection is also consistent with research conducted on ancient venomous lineages (Sunagar & Moran, 2015), suggesting that Scorpaeniform venoms have remained within the family for a large evolutionary timescale.

The elucidation of the toxic compounds from *E. vipera* and *T. draco* were conducted using basic and what now would be deemed as ‘outdated’ biochemical techniques. The attempts to isolate the venom toxins from *E. vipera* were conducted over 30 years ago (Russel & Emery, 1960; Carlisle, 1962; Perriere et al., 1988), whilst studies conducted on *T. draco*, were as recent as 24 years (Chhatwal & Dreyer, 1992a & b). Because of the lack of data on both species, it is difficult to determine whether they conform to the toxin similarities of Scorpaeniformes, and hence why recent studies like Chaung & Shiao (2014) have not included them within the family analysis. From what we already know of *E. vipera* and *T. draco* venom, it seems that there are some fundamental similarities as haemolytic and cytolytic activities, along with *E. vipera* producing a toxic molecule which is composed of subunits. However, without transcriptomic analysis of toxin

sequences it is difficult to conclude on such similarities by structural and functional comparisons alone.

1.4.2 *Lineus longissimus* and other nemertean toxins

Unlike both species of weever fish, there has been no attempt to elucidate the toxins of *L. longissimus.* This is surprising since *L. lonissimus* is known for its toxic mucus and as it is questionably considered the longest toxic organism recorded to date (Gittenberger & Schipper, 2008). The best attempt at understanding the toxic arsenal of *L. longissimus* was a study investigating the transcriptomic sequencing of multiple nemertean species (Whelan *et al*., 2014). Although, this study attempted to identify putative toxin genes of nemerteans and compare these to other known toxin genes, it does not highlight the overall percent of gene matches. Therefore, we remain uncertain how closely related these genes are. Still, one thing to note from this study is the diversity of toxin gene matches between multiple marine and terrestrial species, which suggests that toxins in nemerteans may have also convergently evolved from non-toxic proteins found throughout Animalia (Fry *et al*., 2009; Whelan *et al*., 2014). Despite this study, no further research has investigated the biochemical make-up of *L. longissimus* to understand the bioactivities or structural aspects of the toxins. There have been some in-depth studies conducted on other nemertean species from the class Enopla (Bacq, 1936 & 1937) and even more closely related species in the class Anopla (Kem, 1976, 1978 & 1985), however the research is still lacking on nemerteans. Therefore, we can only currently analyse the toxins from other closely related nemerteans and attempt to hypothesise where *L. longissimus* fits in with the other species.

Enoplans are known as ‘armed’ nemerteans as they use both toxic mucus and a venomous proboscis for defensive and predatory functions. The toxins of Enoplans have been shown to cause paralysis and neuromuscular nerve blocking (Roark, 1947; Kem, 1971 & 1988; Lee *et al*., 2006). Members of the order hoplonemertea, seem to possess active toxins primarily composed of pyridine-based alkaloid compounds, most notably anabasine (3-(2-pyridyl)-3,4,5,6-tetra-hydropyridine) and nemertelline (2-(Pyridin-3-yl)-4-[2-(pyridin-3-yl)-pyridin-3-yl]-pyridine) both with similar effects (Kem, 1971; Lee *et al*., 2006). Anabasine, is a nicotinic alkaloid that has a high potency for activating cholinergic nicotinic receptors, eliciting nerve action potentials (Kem, 1985; Lee *et al*., 2006). Anabasine is commonly found in various species of wild tobacco as *Nicotiana glauca*, and is used as an insect deterrent (Roark, 1947). This suggests that it is utilised by the Enoplans in a similar way to the insecticidal properties of anabasine in wild tobacco, and has evolved as a similar defensive deterrent via convergent evolution (Daly, 2004).

In contrast with Enoplans, members of the class Anopla (the class *L. longissimus* belongs), known as 'unarmed' nemerteans, only seem to use mucus as a form of defence, they lack a venomous proboscis. They have also been shown to produce polypeptide based toxins such as neuro- and cytotoxins within their mucus (Kem, 1971, 1976 & 1985). These neurotoxins, similarly to anabasine, affect the neuromuscular system causing paralysis, whilst cytotoxins cause localised tissue damage. These types of peptide compounds are seen in most venoms found throughout the animal kingdom (Casewell *et al.,* 2013). Heteronemerteans of the class Anopla, produce peptide based neurotoxins, and are assumed to be four times more potent than the pyridine alkaloid toxins of the hoplonemerteans (Enopla) (Kem, 1971 & 1973). Certain species of the genera *Lineus* and *Cerebratulus* have toxins which evoke repetitive action potentials and cause prolonged repolarisation of crustacean neurons. The effects seem to consist of convulsions, stiffening, loss of limb control and eventually death (Kem, 1985). The characterised *Cerebratulus* toxin, B-IV, was shown to specifically bind to unique receptor sites in crustacean nerves (Kem, 1976). This suggests that the toxins have evolved with a high specificity to target crustacean neuron receptors.

The best attempt to understand the toxins of *L. longissimus* have been studies seeking the presence of tetrodotoxin (TTX) and the symbiotic species of bacteria that produce it (McEvoy *et al*., 1998; Carroll *et al*., 2003; Strand *et al*., 2016). TTX is very potent pyridine-based alkaloid neurotoxin with no known receptor antagonist to diminish its neuronal disrupting properties. TTX binds to voltage-gated Na^+ channels, degrading all action potentials across nerve cell membranes (Hwang & Noguchi, 2007). TTX is produced by a marine bacterium, of the genera *Pseudomonas* and *Vibrio* (Simidu *et al*., 1990; Yu *et al*., 2006). The bacterium utilise a symbiotic relationship with its selected host species, most notable examples are that of the Puffer fish (Tetraodontidae) (Noguchi *et al*., 1987) and Blue-ringed octopus (*Hapalochlaena spp*.) (Sheurnack *et al*., 1978). Current research has effectively identified some species of nemerteans e.g. *Cephalothrix spp.* that utilise this symbiotic relationship with TTX producing bacteria, *Vibrio alginoliticus* (Askawa *et al*., 2003; Askawa & Kajihara, 2013). Many other nemertean species, including such genus as *Lineus, Amphiporus* and *Cerebratulus*, are suggested to utilise TTX producing *Vibrio spp*. (McEvoy *et al*., 1998; Carroll *et al*., 2003). However, it is not clear if the bacterium is the producer of the toxic compounds or if the nemerteans produce their own toxins. These studies did not attempt to isolate and biochemically identify specific TTX-like compounds from individual species, in particular, *L. longissimus.* This evidence of TTX toxins also contradicts previous suggestions that heteronemertean toxins are peptide based compounds (Kem, 1971, 1976 & 1985). Recent evidence proposes that the studies conducted on *L. longissimus* utilising TTX may have shown

false-positive results, therefore there is still a lot of uncertainty surround the toxins in which *L. longissimus* use (Strand *et al*., 2016).

Thus, it seems that an understanding of *L. longissimus* toxin biochemistry is severely neglected in comparison to closely related species. Until further analyses are conducted, it is uncertain as to whether *L. longissimus* toxins adhere to the suggestion that the class of Heteronemertea (Anopla) produce only peptide based toxins, or if the toxins resemble more distant species of the class Hoplonemertea (Enopla), that produce pyridine-based alkaloids. We are still uncertain as to the reliability of research that has attempted to identify *L. longissimus* toxins as TTX-like compounds, produced by *Vibrio spp.* of bacteria. Therefore, a thorough biochemical analysis of *L. longissimus* toxins is needed to support or reject such conflicting suggestions within current literature (McEvoy *et al*., 1998; Carroll *et al*., 2003; Kem, 1971, 1976 & 1985).

1.5 Symptoms and first aid of envenomation/ poisoning in humans

1.5.1 *Trachinus draco & Echiichthys vipera*

Stings from weever fish mostly occur when fishermen pick them out of their nets as bycatch and beach tourists attempt to handle or unknowingly step on them (Dehaan *et al*., 1991; Davies & Evans, 1996; Bonnet, 2000). Descriptions of envenomations describe a sharp and immediate painful stab, along with excruciating pain which can last for around 20-50 minutes before it begins to decrease in intensity, whilst it may be up to 24 hours before the pain fully subsides (Evans, 1907; Davies & Evans, 1996). Localised necrosis and swelling of the envenomation site have also been observed in some cases (Russell & Emery, 1960; Dehaan *et al*., 1991). However, the necrotic nature of the wound does not seem to be severe, healing up with minimal scarring. Other symptoms may occur, such as agitation, drop of blood pressure, headaches/ nausea, vomiting, sweating, brachycardia and fainting (Davies & Evans, 1996; Bonnet, 2000). There is an extreme case report of a six-week pregnant woman developing vaginal bleeding after a weever sting, whilst after three weeks spontaneously aborting the fetus (Gonzalo, 1985; Dehaan *et al*., 1991). However, it is unclear as to whether the effects of the venom directly caused this much damage to her and the fetus.

The most effective first aid response to weever fish envenomations is hot water immersion (HWI) of the affected area, with the water at a temperature of around 40°C (Russell, 1965; Dehaan *et al*., 1991; Briars & Gordon, 1992; Davies & Evans, 1996). This treatment has been cited countlessly throughout the literature and is even a first aid recommendation by organisations such

as International Life Saving Federation, British Marine Life Study Society and Toxicology databases as Toxbase (Atkinson *et al*., 2006). Although this method seems to be widely accepted, there is little evidence to suggest that this method does in fact work. Most data published has been based on case reports and standard follow up patient questionnaires. There seems to be a lack of methodological detail, small test groups, survey bias in some case reports and no attempt to produce randomised controlled trials to test the hypothesis of HWI (Atkinson *et al.,* 2006). There has also been no experimental data on testing the thermostability of weever toxins, to identify if they are denatured within a heat range tolerable to human skin. Some authors question the efficacy of HWI, as such quick denaturation of proteins requires temperatures that would result in burns and damage to the skin (Muirhead, 2002). It seems HWI does not relieve pain by denaturing proteins, but rather by phenomenon such as Gate Control theory and Diffuse Noxious Inhibitory Control theory. Simply, these theories suggest that a response to painful stimuli can be inhibited by other stimulus, for example the sensation of applied heat would override the sensation of pain (Kakigi & Watanabe, 1996; Muirhead, 2002), thus the patients pain perception is altered.

1.5.2 *Lineus longissimus*

There is no direct evidence of a human ingesting the poisonous mucus of any nemertean, thus the toxic effects on humans are unknown. There is one documented case of tasting the mucus, It was described as leaving an “acrid taste in the whole mouth, remaining for a long period of time” (Wilson, 1900). Therefore, it seems the mucus is designed to taste very bad to any potential predator, acting as a defensive deterrent, but the effects of the toxins once ingested are unknown and need further investigating.

1.6 Aims and justification of analyses

The aims are to utilise a suite of proteomic and transcriptomic methods, to characterise the toxins from *E. vipera*, *T. draco* and *L. longissimus*.

The aims are as follows:

1) The venom from both weever fish will be subject to SDS-PAGE to attempt to identify the molecular weight of toxins in concurrence with previous research.

2) High Performance Liquid Chromatography (HPLC) will be used to isolate and identify the toxins within *L. longissimus* mucus.

3) We will attempt to understand the ecological functions and mechanical aspects of their toxin delivery systems, by conducting a bioassay of *L. longissimus* toxins on a common nemertean predator (*Carcinus maenas*) and investigating spine morphology in weever fish using macroscopy techniques.

4) Total RNA analysis will be investigated, evaluating DNA and protein sequence alignments for *E. vipera* and *L. longissimus*, comparing to known toxin sequences.

Once the toxins have been characterised, re-evaluated and a greater understanding of their biochemistry and functional aspects analysed, we can hope to provide reasons for why and how this field of research can be pushed forward to reduce the 'neglected' status in which it has been labelled with.

2. Methods

2.1 Specimen collections

Both *E. vipera* and *L. longissimus* were collected from the shores of the Menai Strait, Beaumaris, N. Wales (53˚16'43.3"N 4˚04'43.1"W). *L. longissimus* were caught during a spring tide from rock pools using a standard hand net. *E. vipera* were collected from sand banks using seine nets. *T. draco* were caught from Cardigan bay, Aberdovey (52°31'57.1"N 4°04'57.1"W). Only dead specimen, (kept on ice) were available from local fishermen, which were thawed for further analysis. All live individuals were maintained under aquarium conditions for approx. a ten-month period at Bangor University marine aquarium facilities. Aquarium conditions were maintained with filtered seawater at a temperature that matched the local sea surface temperatures over the 10-month period (13-15˚C).

2.2 *Trachinus draco & Echiichthys vipera*

2.2.1 Venomous spines macroscopy

Spines of both *E. vipera* and *T. draco* were examined and images were taken using a Canon EOS 1200D camera set up to a Wild Heerbrugg M400 photo-macroscope. Camera settings were as follows; ISO 3200 at a focal length of 50mm.

2.2.2 Venom extraction

Initial venom extraction attempts euthanised the fish using Tricaine methanesulfonate (MS-222), and the spines cut away from the body. The spines were then placed into distilled H_2O

and the venom sac was then ruptured to release the venom. The venom content was dried and resuspended into 1.5ml Eppendorf tubes. This method was used for both *E. vipera* and *T. draco*.

By refinement, a second method of extraction, using a synthetic sponge, worked much better, adapted from Almada *et al*., (2016). A small (1cm^3) non-detergent synthetic sponge was placed onto the spines, allowing them to penetrate deep into the sponge membrane. The sponge was carefully massaged around the spines, allowing for adequate absorption of any venom. The sponge was then dripped with small quantities of distilled H_2O, until fully soaked, without any excess run off. Then placed into a 1.5ml Eppendorf and centrifuged at 3000RPM, allowing the solution to separate from the sponge. The remaining sponge was then removed and the solution was used for proteomic analysis. This method allows for the fish to remain alive for venom extraction, adhering to ethical and animal welfare protocols. This method was only tested on *E. vipera*, as live *T. draco* specimen were not available.

2.2.3 SDS-PAGE

The dried toxin samples were added to 15µl of PBS (Phosphate-Buffered Saline). A further 15µl of 2x Laemmli Sample Buffer was added to the solution. Samples were heated at 95°C for 5 minutes. The samples were then loaded onto a Bio-Rad 7% mini-PROTEAN TGX™ Precast Protein Gel with a molecular weight marker (Precision Plus Protein® Standards, BioRad, USA) loaded for direct protein weight comparison. SDS buffer was prepared using a TGS Bio-rad buffer (25mM Tris, 192mM glycine, 0.14 (w/v) SDS, pH 8.3) at a 1:9 ratio with distilled H_2O. The gels were run at 120V for 3 hours. The separated gel proteins were stained with InstaBlue protein stain™ (Sigma-Aldridge) for a 30-minute period.

2.2.4 Phylogenetic analysis of Scorpaeniform toxins

The evolutionary history of the toxin sequences was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). The bootstrap consensus tree inferred from 500 replicates. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The analysis involved 28 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 217 positions in the final dataset. All sequence alignments, albeit the Trinity sequences from our data, was obtained via the NCBI GenBank database. The outgroup selection and toxin sequence alignments were based on the sequences used by Chuang & Shiao (2014) phylogenetic analysis of scorpaeniform toxins.

The phylogenetic analyses and tree was produced using MEGA7 (Kumar, *et al*., 2016). Only dorsal toxin sequences were used, as there is no sequence alignments for toxins from opercular spines in scorpaeniform fish. All GenBank accession identifiers for scorpaeniform toxin sequences are listed in Table 2 & 3.

2.3 Lineus longissimus

2.3.1 Mucus extraction

Individuals were placed into a beaker with 5ml of sea water. They were then gently shaken and agitated until they released copious amount of mucus. Once enough mucus was secreted, *L. longissimus* was removed and the remaining mucus solution was collected and frozen.

2.3.2 Toxin purification

Raw mucus samples were centrifuged at 20000G for 5 minutes. The toxins were separated using a Phenomenex® Strata-X® SPE cartridges along with a Phenomenex® vacuum manifold system. 6ml Acetonitrile 100% (ACN) initially passed through the SPE cartridge. Followed by a further 6ml of distilled H_2O to allow for re-calibration. 8ml of the raw mucus extract was then slowly dripped through the SPE cartridge. A further 8ml of distilled H_2O was passed through. The final solution containing the toxin molecules was then collected by passing though 3ml ACN 25%. The final solutions were heat centrifuged, until and the crystalline toxin was left.

2.3.3 High Performance Liquid Chromatography (HPLC)

The toxins were diluted in 200µl of distilled water containing 0.1% trifluoroacetic acid (TFA), fractionated by HPLC on a Jupiter wide pore C18 column (Phenomenex; 300 x 4.6mm). Elution was performed at 1 ml/min by applying a gradient towards solution B (acetonitrile, containing 0.1% TFA). The sample was run over 10-80% B gradient for 42 min at 210nm. The 1ml fractions were collected with the first 2-minute interval being discarded as it contained the running buffer elution. All fractions were heat centrifuged for 3hrs.

2.3.4 Mass spectrometry

Isolated toxins from HPLC peaks were sent to Aberystwyth University for high resolution Mass Spectrometry of the compounds.

2.3.5 *Carcinus maenas* bioassay

The effects of the toxins were tested on *Carcinus maenas*, the green shore crab. This was based on observations that nemertean toxins are crustacean specific neurotoxins (Kem, 1976). A preliminary test was set up to determine if the raw mucus extract affected *C. maenas*. This involved injecting three individuals with 0.1ml of raw mucus into the soft tissue of the basis/ coxa area where the pereopods join to the carapace. From this series of injections, a simple ethogram was designed to characterise the behaviour of *C. maenas* in response to the effects of the toxic mucus.

A *Carcinus Maenas* saline stock solution was created as a control. This consisted of 55.3M distilled H_2O, 433mM NaCl, 12mM KCl, 12mM $CaCl_2.H_2O$, 12mM $MgCl_2.6H_2O$, 10mM HEPES. Further NaOH was added to adjust to pH 7.6. Solution was then autoclaved.

For the bioassay series of injection, test groups were designed as follows; a control group injected with *C. maenas* saline solution, a raw mucus extract group and a HPLC pooled fractions group (in minutes) of 2-22, 23-26, 27-29, 30-33, 34-42. The pooled sample time intervals were designed to allow for any major peaks identified in the HPLC chromatogram to be isolated. The HPLC pooled fractions were mixed with *C. maenas* saline. All individuals were injected with 0.1ml solutions of each group. Test groups were split into male and female (n=12, 6 individuals of each sex) for each category and their carapace width measured to ensure all specimens were of a similar size range of 5-6cm. *C. maenas* were not anesthetized but carefully handled and restrained for bioassay injections.

All test groups behaviour was observed and noted, based on the results of the preliminary raw mucus injections, and the timing of each occurrence of observed behaviour was recorded. The data was statistically analysed using a one-way ANOVA and post-hoc LSD tests.

2.4 Transcriptomics

Animals were killed using a Schedule 1 method, and tissue samples collected directly into RNAlater (Ambion). Total RNA was extracted using the Qiagen RNeasy kit with on-column DNase treatment. RNAseq libraries were made with the TruSeq Stranded mRNA Library Prep Kit (Cat. No. RS-122-2101). Libraries were pooled and sequenced on one lane of the Illumina HiSeq platform, using v4 chemistry. Raw reads were quality trimmed with trimmomatic (Bolger *et al*., 2014) to assure high quality (i.e. average PHRED>30). Tissue-specific and pooled transcriptomes were assembled with Trinity (Grabherr *et al*., 2011) using default parameters. I identified

candidate venom-encoding contigs from these transcriptomes with local BLAST (BLAST+ (Camacho *et al*., 2009)), using known venom sequences from 14 species (accessions; KF156777.1, KJ689807.1, KJ689805.1, KJ689803.1, AB262392.1, AB775457.1, AB775455.1, U36237.1, KJ689800.1, AB623222.1, AB623223.1, AB775453.1, AB775454.1, AB623220.1) as queries**.** Reads were then assembled using Trinity Trinity assemblies were then assessed using and matched known toxins were identified and compared.

3. Results

3.1 ***Trachinus draco* & *Echiichthys vipera* spine morphology**

The morphological examination of both *E. vipera* and *T. draco* spines (opercular and dorsal), shows key features that have not been described before in these species. Figure 3 highlights the full body images of both *E. vipera* and *T. draco*, with the dorsal spines erect. These images clearly highlight the positions of the dorsal and opercular spines, and give an adequate size comparison of full body and spines. The images allow a direct comparison of the closely related species in terms of their camouflage and/or aposematic colouration. Opercular spines have been highlighted in red as they are difficult to observe on the images. Spines are numbered and consistent in later images. All size measurements were conducted on the individuals imaged, thus the measurements will differ between all specimen.

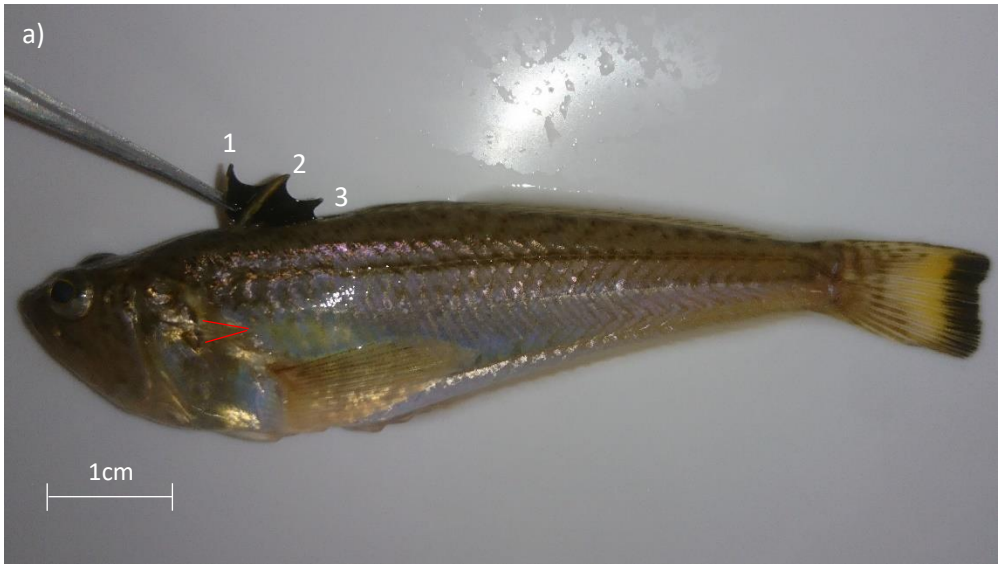

Fig.3. *Image comparisons of E. vipera (a) and T. draco (b). Numbers indicate the spine positioning from head to caudal fin direction. Total length of E. vipera and T. draco specimen imaged were 8.3cm & 28.7cm respectively.*

The macroscopic examination of both *E. vipera* and *T. draco* dorsal spines (Fig. 4.) indicate that there is very little morphological difference between their apparatus and method of venom deposition. There are many morphological features associated with each apparatus and this is consistent with previous literature (albeit regarding the venom 'gland') and across both species. However, observing the opercular spines (Fig.5.) there is a distinct difference between species, with *T. draco* (Fig.5c & d) having a venom sac (VS) surrounding the spine, as well as anterolateral grooves on the spine surface, whereas *E. vipera* spines (Fig. 5a & b) do not contain these features.

Cross sections of spines with AG (Fig. 6.) seemed to all be distinctly different, in the sense that the AG had no obvious standard number from one individual to the next. The AG were randomly distributed on each spine and the number of AG differed, some individuals having as few as one with others having up to three. However, the base of each spine was a consistent solid circular foundation, most likely for structural stability when injected into an organism. All AG tapered towards the tip of each spine, and terminate there. All cross sections were observed from approx. the middle of the spine length, with the exception of the base cross section.

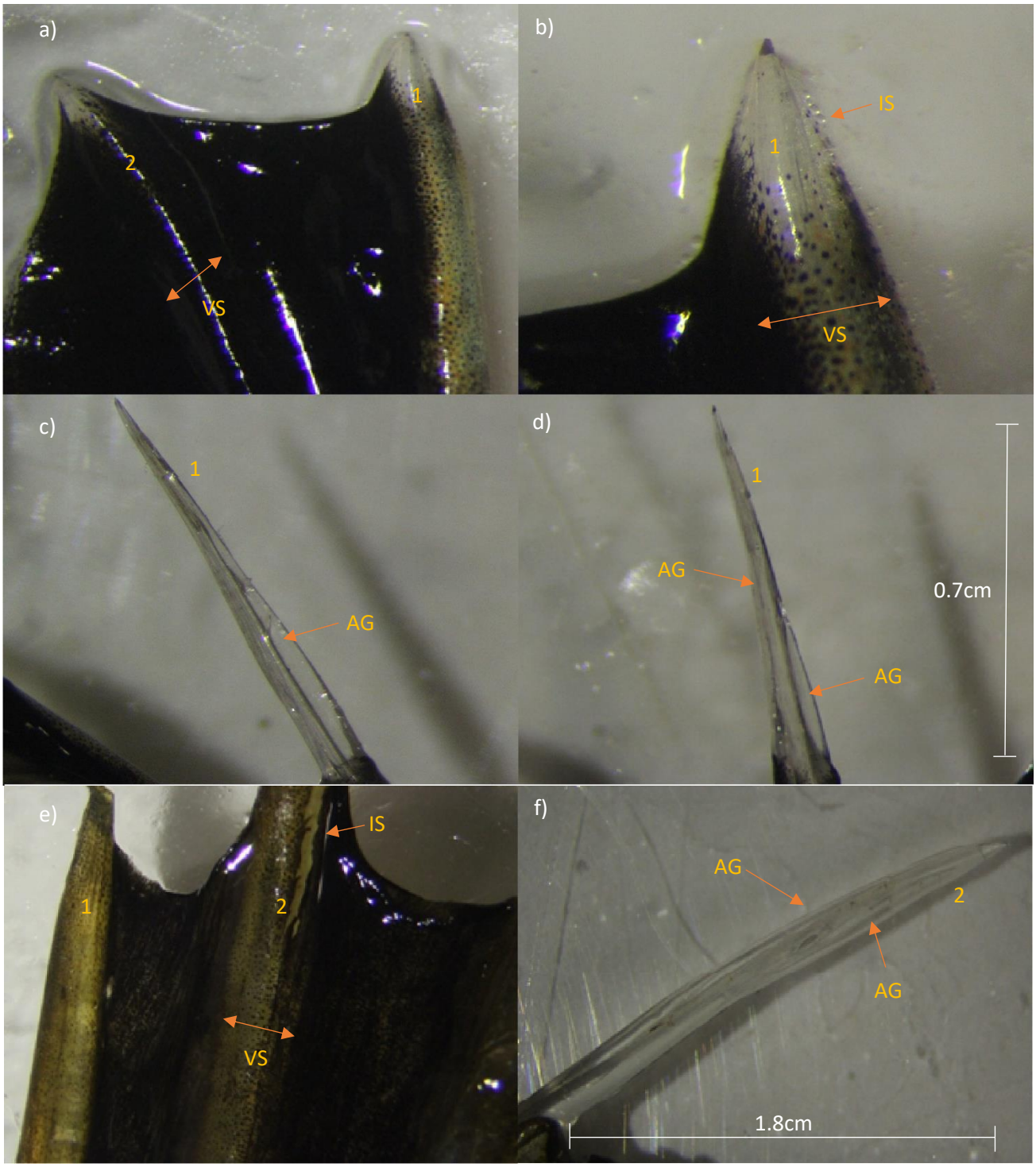

Fig. 4. *Macroscopy images of the dorsal spine of E. vipera (a - d) and T. draco (e & f). Abbreviations highlight the venom sac (VS), integumentary sheath (IS) and anterolateral grooves (AG). Numbers indicate spine positioning (c.f. Fig.3.).*

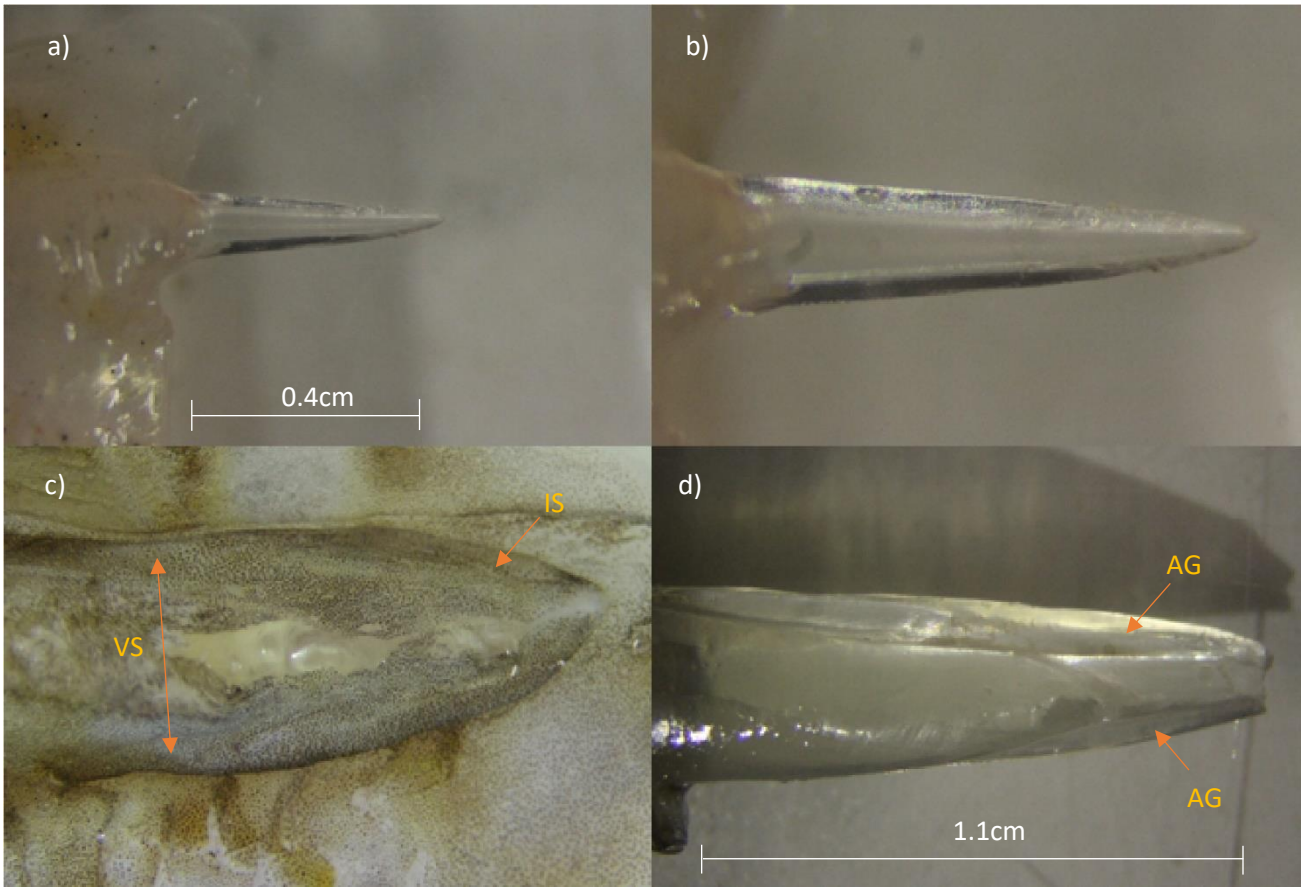

Fig. 5. *Macroscopy images of the opercular spines of E. vipera (A & B) and T. draco (C & D). Abbreviations are consistent with previous figures.*

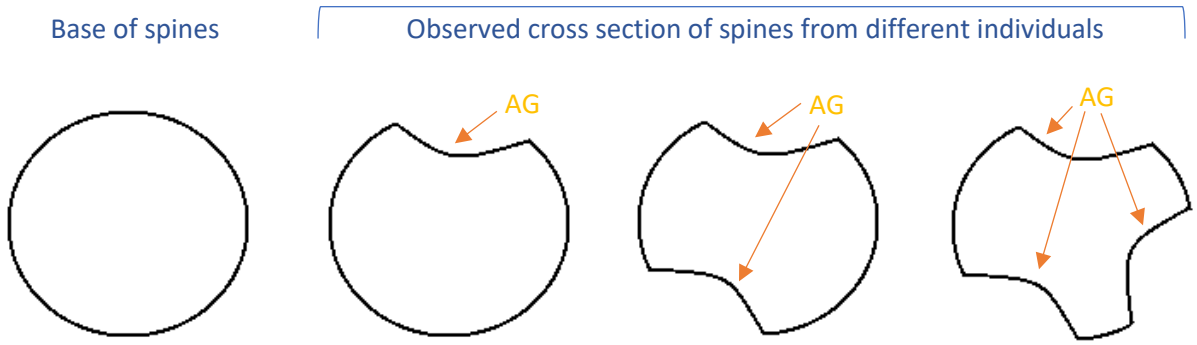

Fig. 6*. A standard cross section diagram of differently observed spines (both dorsal and opercular) with Anterolateral groove (AG). This represents any spine from both species (E. vipera & T. draco) that have AG. All cross sections were observed from approx. the middle of the spine length.*

3.2 SDS-PAGE

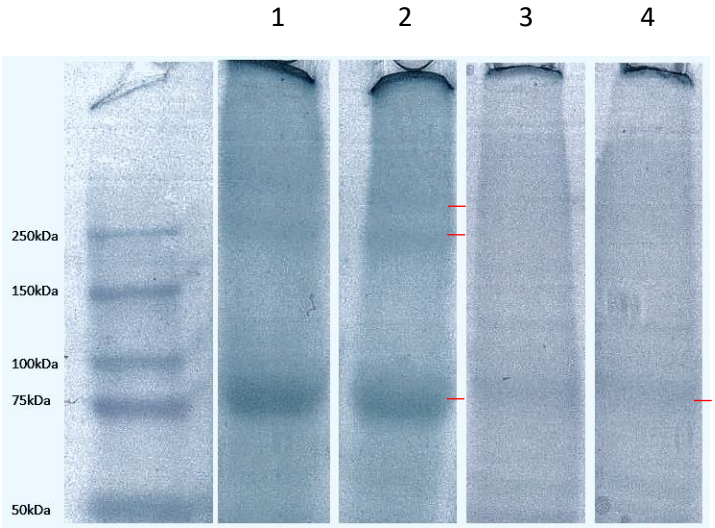

Fig. 7. *Venom profile of E. vipera fractioned using SDS-PAGE, venom was obtained by the removal of dorsal spines. Lanes 1 & 2 were venom extracted from dorsal spines of two different individual specimen of E. vipera using the euthanised extraction method. Lanes 3 & 4 were venom extracted from dorsal spines using the sponge method. Three bands can be seen in lanes 1 & 2, one just above 75kDa and two above 250kDa. Only one band is present in lanes 3 & 4 located just above 75kDa.*

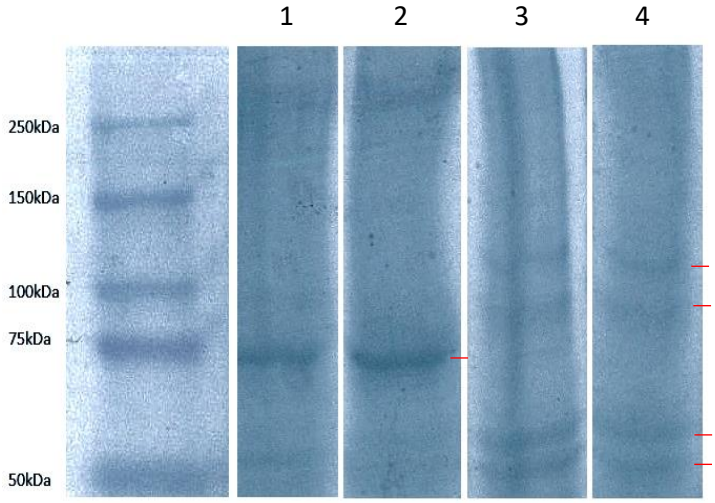

Fig.8. *Venom profile of T. draco fractioned using SDS-PAGE, venom was obtained by the removal of spines of freeze-thawed specimen. Lanes 1 & 2 were venom extracted from the opercular spines of two different individuals, whilst lanes 3 & 4 were venom extracted from the dorsal spines. There is a single band present just below 75kDa in lanes 1 & 2. Four bands can be seen in lanes 3 & 4, two above 50kDa, one below 100kDa and one above 100kDa respectively.*

The venom samples of *E. vipera* that were subject to SDS-PAGE (Fig.7.) show a clear distinct band marginally above 75kDa on all 4 lanes. Lanes 3 and 4, although matched the same

75kDa, the banding was not as clearly stained compared to lanes 1 and 2. There are also two weak bands to be seen, one located at around the maximum 250kDa and the other is above this in lanes 1 and 2. However these bands cannot be seen in lanes 3 and 4. It is difficult to determine the molecular weight as it is above the maximum marker. There is a very strongly stained band of high molecular weight (HMW) that remained within the loading well on all 4 gel lanes and has not been able to pass through the gel matrix.

Venom from both dorsal and opercular spines were observed using SDS-PAGE from *T. draco* (Fig. 8.). Opercular venom (Fig.8. - lanes 1 & 2), show one distinct banding just below the 75kDa marker, which is consistent from both lanes of different individuals. Dorsal venom (Fig. 8. - lanes 3 & 4) highlight four separate bands. The highest banding is slightly above 100kDa and another is just below the 100kDa marker. Two other bands appear above 50kDa. Also note that the lanes of *T. draco* (Fig.8.) also contained a HMW banding, similarly to *E. vipera* lanes (Fig.7.) that did not pass out of the loading well, however, due to the re-sizing of images the loading well banding had to be edited out.

3.3 ***Echiichthys vipera*** RNA Sequencing

Table 1*. Trinity Transcriptome assembly statistics for E. vipera based on all reads (Global), only dorsal spine reads and only operculum spine reads.*

Transcriptome	Total number of 150bp reads	Total assembled bases	N50	GC%	Mean contig length	Median contig length	Number of contigs
Global	102406566	185830496	1408	46.22	769.36	381	188062
Dorsal	50445444	124401609	1501	46.54	818.44	408	120232
Operculum	51961122	139046193	1398	46.53	770.40	386	145303

Table 2. *Toxin genes identified from E. vipera dorsal spine RNA Trinity sequence alignments when searched in NCBI GenBank database.*

Trinity sequence alignment	GenBank identified toxins	Species	% matched identities	% Query cover	E value	GenBank Accession identifier
DN30045	Echiitoxin a-subunit	*Echiichthys vipera*	97	100	0.0	KF156777.1
	Tx alpha-subunit (Tx A)	*Sebastiscus marmoratus*	78	100	1e-67	KJ689807.1
	Tx alpha-subunit (Tx A)	*Sebastiscus oxycephala*	78	94	2e-58	KJ689805.1
	Tx alpha-subunit (Tx A)	*Sebastapistes strongia*	77	94	1e-56	KJ689803.1
	neoVTX-a subunit	*Synanceia verrucosa*	77	94	1e-56	AB262392.1
	HrTx-a subunit	*Hypodytes rubripinnis*	82	65	3e-54	AB775457.1

	IjTx-a subunit	*Inimicus japonicus*	77	93	8e-51	AB775455.1
	Stonustoxin alpha-subunit	*Synanceia horrida*	77	90	8e-51	U36237.1
	Tx alpha-subunit (Tx A)	*Dendrochirus zebra*	74	94	7e-33	KJ689800.1
DN30331	Tx beta-subunit (Tx B)	*Sebastiscus marmoratus*	92	98	2e-75	KJ689808.1
	Tx beta-subunit (Tx B)	*Sebastapistes strongia*	91	98	1e-73	KJ689804.1
	neoVTX-b neoverruscotoxin b-subunit	*Synanceia verrucosa*	91	100	1e-73	AB262393.1
	Stonustoxin beta-subunit	*Synanceia horrida*	91	100	1e-73	U32516.1
	Tx beta-subunit (Tx B)	*Scorpaenopsis oxycephala*	91	98	6e-72	KJ689806.1
	HrTx-b subunit	*Hypodytes rubripinnis*	90	100	6e-72	AB775458.1
	Tx beta-subunit (Tx B)	*Dendrochirus zebra*	90	98	3e-70	KJ689801.1
	Tx gamma-subunit (Tx C)	*Dendrochirus zebra*	90	98	2e-68	KJ689802.1
	IjTx-b subunit	*Inimicus japonicus*	89	100	2e-68	AB775456.1
	PvTx-a subunit	*Pterois volitans*	90	98	2e-68	AB623222.1
	PvTX-b subunit	*Pterois volitans*	90	98	2e-68	AB623223.1
	PlTx-a subunit	*Pterois lunulata*	89	98	5e-65	AB775453.1
	PlTx-b subunit	*Pterois lunulata*	89	98	9e-67	AB775454.1
	PaTx-a subunit	*Pterois antennata*	89	98	9e-67	AB623220.1
	PaTx-b subunit	*Pterois antennata*	89	98	9e-67	AB623221.1
DN29664	Hyaluronidase	*Pterois volitans*	83	59	0.0	AB759698.1
	Hyaluronidase	*Pterois antennata*	84	57	0.0	AB759697.1
	Hyaluronidase	*Synanceia verrucosa*	82	63	0.0	AB607856.1

Trinity sequence alignment	GenBank identified toxins	Species	% matched identities	% Query cover	E value	GenBank Accession identifier
DN34197	Echiitoxin a-subunit	*Echiichthys vipera*	95	81	0.0	KF156777.1
	Tx alpha-subunit (Tx A)	*Sebastiscus marmoratus*	76	43	2e-75	KJ689807.1
	Tx alpha-subunit (Tx A)	*Sebastiscus oxycephala*	74	37	2e-49	KJ689805.1
	Tx alpha-subunit (Tx A)	*Dendrochirus zebra*	73	28	1e-47	KJ689803.1
	neoVTX-a subunit	*Synanceia verrucosa*	73	30	1e-46	AB262392.1
	Tx alpha-subunit (Tx A)	*Sebastapistes strongia*	79	25	4e-36	KJ689803.1

	IjTx-a subunit	*Inimicus japonicus*	73	24	2e-34	AB775455.1
	Stonustoxin alpha-subunit	*Synanceia horrida*	72	27	1e-25	U36237.1
	HrTx-a subunit	*Hypodytes rubripinnis*	76	15	3e-15	AB775457.1
DN32404	Tx beta-subunit (Tx B)	Sebastiscus marmoratus	83	24	2e-75	KJ689799.1
	Tx beta-subunit (Tx B)	Sebastiscus oxycephala	83	30	2e-48	KJ689797.1
	Tx beta-subunit (Tx B)	Sebastapistes strongia	81	32	4e-36	KJ689795.1

Table 3. *Toxin genes identified from E. vipera opercular spine RNA Trinity sequence alignments when searched on NCBI GenBank database.*

The assembled transcriptome has 241539 transcripts from 188062 genes, with an N50 of 1408 bp (Table 1). The transcriptome was assembled with data from both dorsal and opercular total RNA extractions, and include many matched toxin orthologues from NCBI GenBank database (Table 2 & 3). Percent query cover indicates the amount of sequence bases that are compared/ matched, whilst percent matched identities, indicates the amount of that sequence queried that matches the database alignments. Toxins from dorsal spines have a greater percent identity and query cover than that of opercular spines, though similar toxins were found overall.

3.4 Phylogenetic analysis of Scorpaeniform toxins

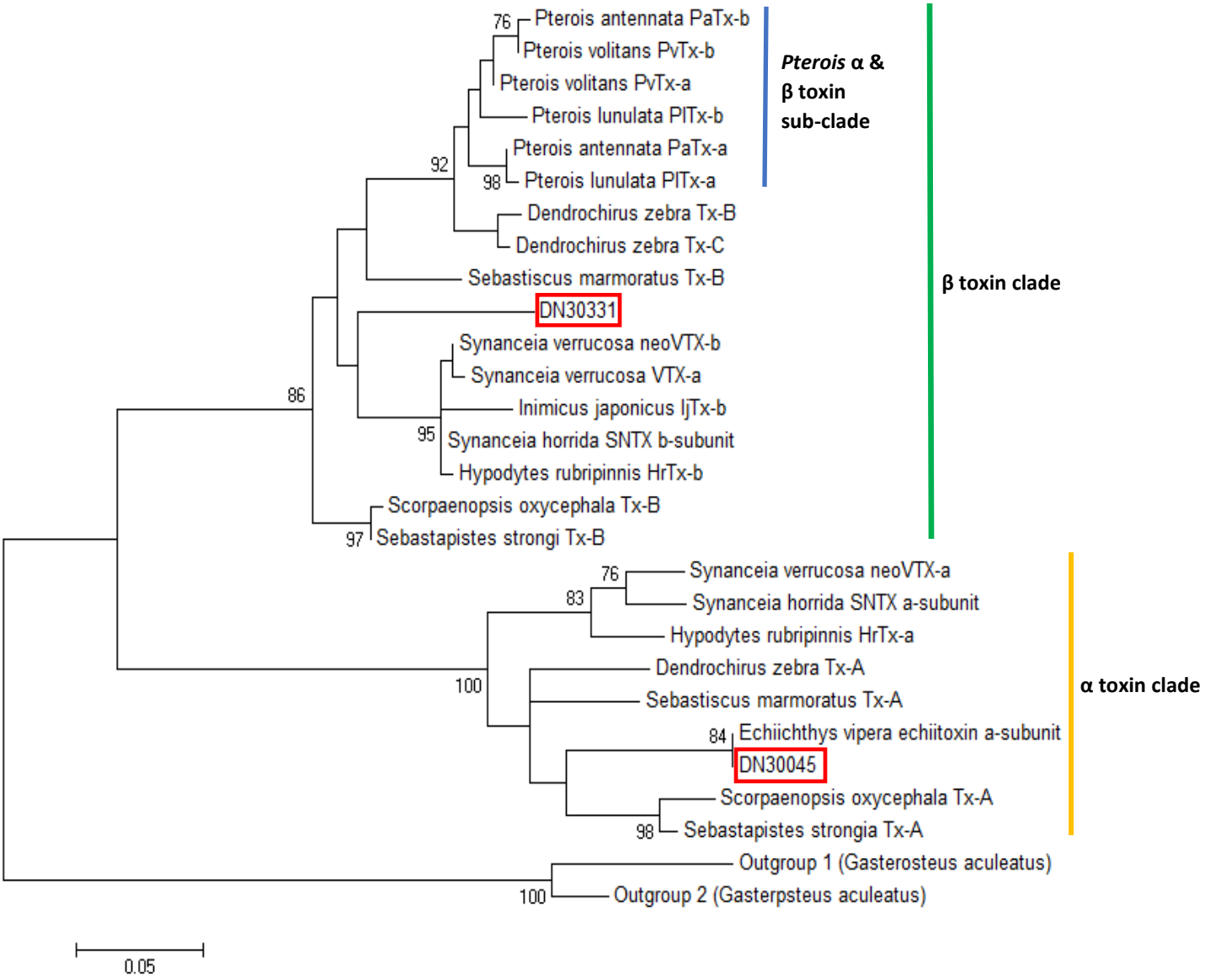

Fig.9. *Molecular phylogenetic analysis by Maximum Likelihood of Scorpaeniformes toxin gene sequences.E. vipera toxin sequences are highlighted in red. Only dorsal spine toxin sequences were analysed.*

Phylogenetic analysis of Scorpaeniformes toxins (Fig.9.), including Trinity sequence data from *E. vipera*, indicate multiple distinct clades that highlight different divergences of α and β toxin sub-units. A sub-clade is also derived, which consists of both α and β toxin sub-units of the *Pterois* genus. These seem to be separately evolved from the distinct α toxin clade. Bootstrapping of >70% was only highlighted.

3.5 ***Lineus longissimus*** **HPLC**

HPLC (Fig.10.) indicates the presence of three major peaks at; 23 (peak 1), 28 (peak 2) and 30 (peak 3) minutes respectively. These major peaks suggest the presence of three peptide based components to the purified mucus. Peaks around the 5-minute elution time are caused by the fractionation of the running buffer solution and therefore are not correspondent to any component of the mucus sample.

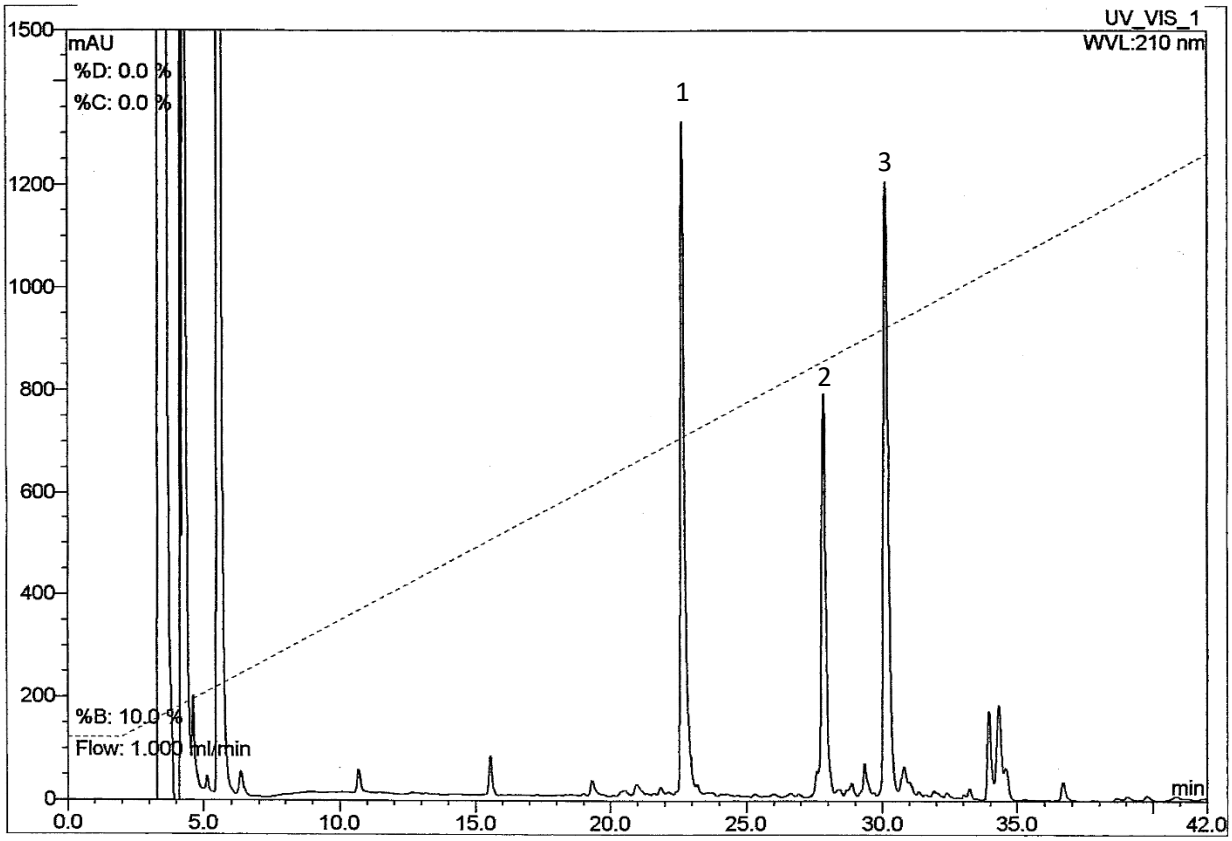

Fig.10*. HPLC chromatogram of L. longissimus purified mucus*. There are three main peaks at 23, 28 and 30 minutes.

3.6 ***Carcinus maenas*** **Bioassay behavioural ethogram**

Table 4*. C. maenas behavioural ethogram when injected with 0.1ml of L. longissimus raw mucus, saline and the isolated HPLC fractions (minutes). A ✓indicated that the described behaviour was observed, whilst a **X** indicates the lack of the described behaviour.*

	0.1ml injections						
Ethogram	Raw mucus	saline	2-22	23-26 **Peak 1**	27-29 **Peak 2**	30-33 **Peak 3**	34-42
Convulsions • Violent and rapid thrashing of limbs • Unable to proceed in normal functions as walking/ righting orientation	✓	X	X	✓	X	✓	X
Loss of limb control and neuronal twitching of dactyl (last segment of the pereopod/ leg furthest from the body) • Limbs stiffen and tend to fold inwards towards the abdominal region • Twitching occurs of the dactyl region of the pereopods/legs	✓	X	X	✓	✓	X	X
Death • Complete lack of movement of all body parts – including antennae and dactyl twitching • Abdominal flap usually open in both male and female individuals	✓	X	X	✓	X	✓	X
Other (only some individuals) • Ejection of brown liquid from mouth • Release of faeces	✓	X	X	✓	X	✓	X

Behavioral observations of *L. longissimus* injections (Table 4) indicates that the saline and fractions 2-22 and 34-42 had no effect on the behaviour of *C. maenas.* Whilst the raw mucus

along with fractions 23-26, 27-29 and 30-33 all affected the behaviour of *C. maenas*, albeit, with differing affects to each other in some instances. Peak 2 seems to only elicit neuronal activity, whilst peak 1 & 3 both cause death and convulsions to some degree.

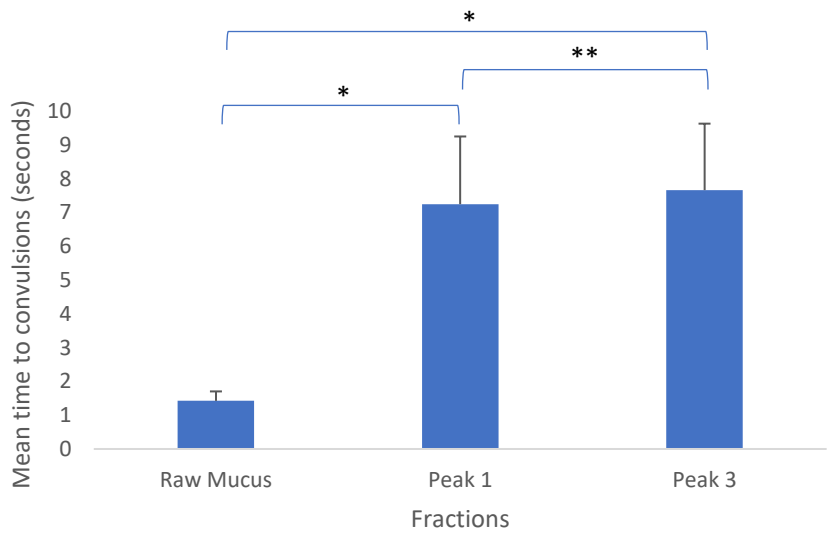

Fig.11. *The mean time (seconds) for convulsions to occur (+/- 95% CI) in C. maenas individuals (N=12) when injected with 0.1ml of different fractions. Only raw mucus and peak 1 & 3 produced any affects. Post-hoc LSD results are indicated by * (p<0.05) and ** (p>0.05).*

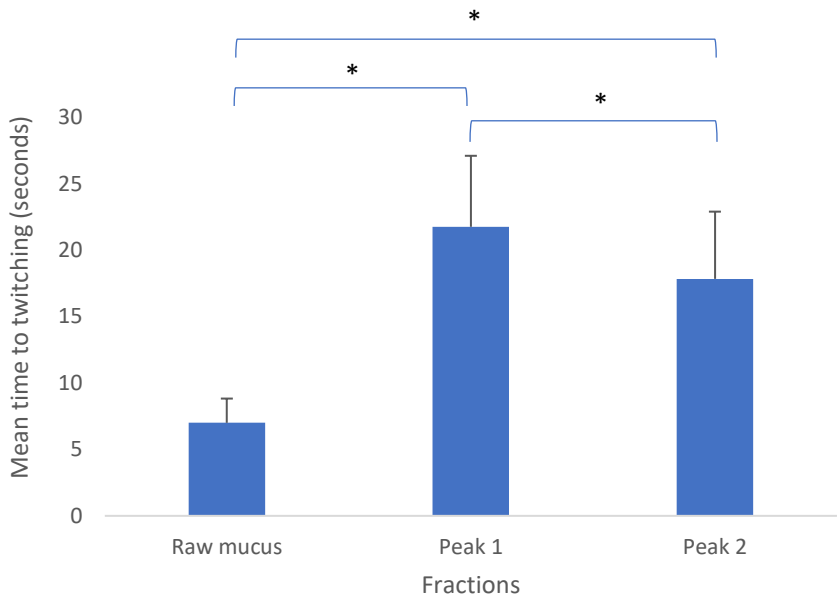

Fig.12*. The mean time (seconds) for twitching to occur (+/- 95% CI) in C. maenas individuals (N=12) when injected with 0.1ml of different fractions. Only raw mucus and peak 1 & 2 produced any affects. Post-hoc LSD results are indicated by * (p<0.05) and ** (p>0.05).*

The mean times to convulsions (Fig.11.) varied greatly between the raw mucus and the fractioned peaks. The results of the one-way ANOVA indicated a significant difference ($F_{(2, 32)}$ =54.935, p<0.05). The post-hoc LSD tests highlighted where these significant differences occurred. There was no significance between the two fractioned peaks 1 and 3 (p=0.536), whilst there was a significant difference between the raw mucus and peaks 1 (p<0.05), and raw mucus and peak 3 (p<0.05). The results of mean time for twitching (Fig.12.) to occur highlighted slightly differing results. Peak 3 did not cause any affects, but the raw mucus and peak 1 and 2 elicited the twitching behaviour. The results of the one-way ANOVA indicated a significant difference between the fractions ($F_{(2, 32)}$ =36.353, p<0.05). The post-hoc LSD tests, showed a significance between all fractions; peaks 1 and 2 (p=0.036), raw mucus and peak 1 (p<0.05), raw mucus and peak 2 (p<0.05).

3.7 ***Lineus longissimus*** **RNA sequencing, N-terminal seq. and amino acid alignments**

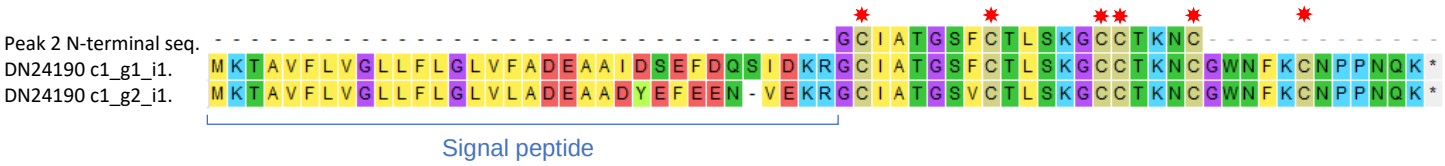

Fig.13*. N-terminal sequencing alignment of peak 2 isolated toxin against matched Trinity sequences from the transcriptomic data. Cysteine residue matches are highlighted by ✷. A predicted mass of 3.4kDa was estimated for the DN24190 alignment.*

N-terminal sequencing was run for isolated toxins from all three HPLC peaks. However, only one toxin produced viable results, peak 2 (Fig.12.). The N-terminal sequence was ran against the Trinity alignment database and matched with DN24190 alignment. The sequence preceding the matched toxin was identified as a signal peptide (SP) by utilising a SP predictor (http://www.cbs.dtu.dk/services/SignalP/). The N-terminal sequence alignment originally showed Aspartic acid (D) where the now Cysteine (C) residues are. Based on the matches of all other Amino Acids (AA) to the RNA sequence data, only the Cysteines did not match. Thus, these AA's were in fact Cysteine and this change to Aspartic acid (D) was due to sequencer analyses being known to have difficulties with Cysteine rich peptides. A peptide mass predictor (http://web.expasy.org/compute_pi/) was used to identify an estimated mass based on AA alignment. The mass of the DN24190 peptides were predicted at 3.4kDa once the signal peptide was cleaved.

***Table 5**. Toxin genes identified from L. longissimus RNA Trinity sequence alignments when searched in NCBI GenBank database.*

Trinity sequence alignment	GenBank identified toxins	Species	% matched identities	% Query cover	E value	GenBank Accession identifier
DN27342	Parbolysin isoform P7	*Parborlasia corrugatus*	80	24	1e-13	KT693320.1
	Parbolysin isoform P4		80	21	4e-10	KT693317.1
	Parbolysin isoform P6		79	21	2e-08	KT693319.1
	Parbolysin isoform P5		79	21	2e-08	KT693318.1
	Parbolysin isoform P2		88	10	3e-07	KT693315.1
	Parbolysin isoform P1		88	10	3e-07	KT693314.1
	Parbolysin isoform P3		86	9	2e-04	KT693316.1

Transcriptomic sequences that were cross referenced against GenBank only matched to Parbolysin isoforms from *Parborlasia corrugatus* (Table 5), which is a nemertean of the class Anopla, closely related to *L. longissimus*. However, when these sequences were converted to their amino acid alignment and re-run against a protein-protein search on GenBank, another match was identified, cytolysin A-III from *Cerebratulus lacteus* (Fig. 13.), another nemertean of the class Anopla. The AA sequence of Cytolysin A-III had a 69% identity match, whereas Parbolysin had a 66% identity match to DN27342.

***Fig.14**. Sequence alignment of the Trininy DN27342 matched against Cytolysin A-III and Parbolysin isoform (accessions numbers P01527.1 & ALI86908.1 respectively). Amino acids are colour coded and sequence matches are highlighted in the same colour amino acid pairings. N.B. the signal peptide of DN27342 was edited out for visual representation. Estimated mass of DN27342 was 10.6kDa. Cysteine residue matches are highlighted by ✷.*

4. Discussion

4.1 ***Trachinus draco & Echiichthys vipera***

4.1.1 Spine morphology, aposematic colouration and ecological relevance

The full body images of *E. vipera* and *T. draco* (Fig. 3.) not only highlight important aspects of their morphological features, but they also give a valuable representation of their colouration

and patterning. The dark contrasting colouration of their dorsal spine coverings clearly stand out from the lighter colouration of the body. This contrasting colour would indicate an aposematic warning to predators of their venom (Cotts, 1940; Ruxton *et al*., 2004) when weever fish flare their dorsal spines (Lewis, 1976). This hypothesis is consistent with spine defenses throughout the animal kingdom often being conspicuous and contrasting in colour to the body (Inbar & Lev-Yadun, 2005). In addition to this, *E. vipera* has a caudal fin that exhibits a yellow and black banding pattern (Fig.3a). There have been many studies which show that yellow and black banding patterns provide substantial aposematic warning colouration (Schuler & Hesse, 1985; Kauppinen & Mappes, 2003). Also, there are studies that highlight the use of yellow and black banding as being an effective crypsis camouflage, perhaps through disruptive colouration blurring the body shape outline (Hoese *et al*., 2006). This colouration, in addition to their ability to alter their colour pattern to match the substrate (Lewis, 1976), provides both an effective aposematic defense against predators, in conjunction with a crypsis pattern to remain unseen by both predators and prey. A possible explanation as to why *T. draco* does not exhibit crypsis or further aposematic warnings is potentially due to their size. With a larger body size and/ or the evolution of venomous opercular spines (Fig. 5.), there becomes a reduction in potential predators. Therefore, an additional aposematic warning is not necessary. There does not seem to be much evidence on the predators of both *E. vipera* and *T. draco* adults, with no observed data to give indications of the types of potential predators. Only observed data for juveniles as prey has been recorded (Gibson & Robb, 1996). This could be due to juveniles not exhibiting the colouration of adults, what is known as, ontogenetic colour change (Booth, 1990). Also, ontogenetic variation in venom/ diet (Andrade & Abe, 1999) or lacking in a functionally developed venom apparatus altogether could play a role in this. Is this lack of observed predators in adulthood, due to their aposematic colouration in conjunction to venomous spines providing a substantial defensive arsenal? It remains uncertain, especially when research has shown that adult populations of *E. vipera* are overall increasing with no yearly decreasing trends in population sizes from 1974-2006 (Tulp, *et al.*, 2008).

It is clear that research regarding aposematism in marine systems is lacking, particularly in fish, thus further studies are needed to provide a basis for the hypotheses previously mentioned. With most of the available literature being on terrestrial vertebrates as birds, snakes and amphibians, we can only speculate on how aposematism in terrestrial systems might, if at all, apply to marine organisms in the same manner. A current gap within the literature on potential predators of both weever fish is also needed to be filled, especially in understanding the biological and ecological relevance of their warning colouration and defensive arsenal.

The literature surrounding the venom apparatus of both weever fish is sparse, with few detailed and photographic data. The dorsal spines of *E. vipera* and *T. draco* (Fig.4.) show a lack of a true venom gland, compared to other species as stonefish (Smith & Wheeler, 2006) that exhibit a distinct venom gland-like structure attached to their dorsal spines. The venom appears to be contained within a 'sac' like integumentary sheath (IS) structure surrounding the dorsal spines. The mechanism of action for venom release is when the spine is forced to push through and rupture the IS. The venom is then forced upwards along the anterolateral grooves (AG) on the outer surface of the spines via a change in pressure, and the AG act in a capillary like fashion to facilitate the flow of venom upwards. There was no presence of a hollow duct running through the centre of the spines (Fig. 6.), unlike venomous fangs of snakes. Previous studies have indicated that the venom is released from yellow to orange venomous tissue within the IS (Smith & Wheeler, 2006; Smith *et al*., 2016). However, during this investigation, no such tissue was observed during spine dissection, and the venom seemed to be a clear substance that was freely contained within the IS of both species. The orange colouration within the images (Fig. 4a & b) were a mottled pattern colouration on the outer surface of the IS. The reasoning as to why we ascertain that the structure of the venom apparatus does not contain a true venom gland, is that there seems to be two distinct features of a true venom gland; 1) the gland is usually not visible without dissection, as they are typically contained inside an organism connecting to the outer apparatus via a duct; 2) true venom glands are not usually disrupted or destroyed with the release of venom. In the case of *E. vipera*, once the IS was ruptured after milking, it took approx. 7-10 days for the IS/ VS to be repaired and re-cover the spines. No venom is produced until the IS has fully repaired.

Previous studies as Smith & Wheeler (2006) and Smith *et al*. (2016) adequately highlight different images of varying fish venom apparatus, however their distinction between venom gland structures is non-existent. For example, Smith & Wheeler (2006) show images of *Thalassophyrne amazonica, Ptarmus jubatus, Siganus stellatus* and *Synanceia verrucosa* dorsal venom spines. Although it is clear from the images that all species have distinctly different 'venom gland' apparatus, it is only *S. verrucosa* that has a structure that conforms to a true venom gland definition. Based on the confusion within the literature as to whether certain fish species contain true venom gland structures, it would be sensible for future studies to attempt to design a universal consensus on the nomenclature of venom structures, not only in fish but also one that can be applied across Animalia.

The opercular spines of *E. vipera* observed in this study, seem to contradict previous literature that suggesting they have venomous opercular spines with a distinct venom gland (Smith & Wheeler, 2006; Smith et al., 2016). However, in these studies there was a lack of macroscopic imaging, with only *T. araneas* opercular spines and venom gland being shown from the Trachinidae family. *E. vipera* opercular spines lack both an IS and AG that are consistent with venomous dorsal spines features (Fig. 5a & b). There was also no presence of a venom gland at the base of the spines or located within the operculum when dissected. *T. draco* opercular spines do however seem to have a VS and IS associated with them (Fig. 5c & d), which is consistent with previous claims (Smith & Wheeler, 2006; Smith et al., 2016). The spines also exhibit an AG, and there is a discrete difference between the surface structure of *E. vipera* and *T. draco* opercular spines (Fig.5.). Since closely related species as *T. araneas* also exhibit a venom gland on the opercular spine, it is possible that *E. vipera* has lost the venomous trait, likely due to the energetic demand of venomous spines, with evidence of other opercular venom losses in other fish lineages as *Thalassophryne spp*. (Smith & Wheeler, 2006; Smith et al., 2016). This energetic trade-off could also be linked to the aposematic warning colouration of *E. vipera,* with another venom system not being necessary. However, observing phylogenetic data of the Trachinidae family (Fig.15.), it appears that there is no evidence of opercular spines in both sister groups, Niphonidae and Percidae. Therefore, the single evolution of opercular spines in Trachinidae, with a later evolution of venomous opercular spines in the*Trachinus* genus is more likey to be parsimonious, than a loss of opercular venom in *Echiichthys*.

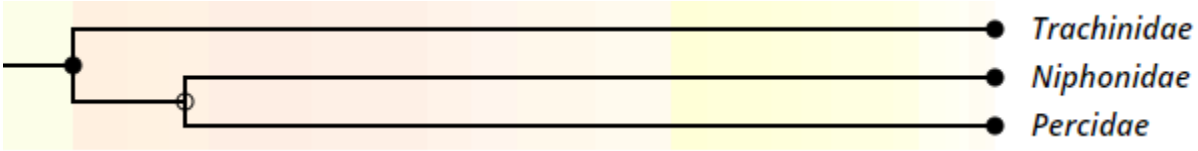

Fig.15*. Phylogenetic tree of the Trachinidae family and its sister groups of Niphonidae and Percidae, adapted from Meynard et al. (2012).*

4.1.2 Venom protein components

The SDS-PAGE results for *E. vipera* venom remained consistent with the previous literature. There was a band above 75kDa (Fig.7.), in which it would suggest that this is the 81kDa subunit of the 324kDa Trachinine. Thus, again it is clear that Trachinine breaks down very quickly from its 324kDa molecule into its 81kDa subunits, and that keeping this toxin functional remains difficult (Russel & Emery, 1960; Perriere *et al.,* 1988). There was also the 'non-migrating' HMW fraction that remained within the loading well of the SDS gel (Fig.7.). The fraction does not migrate

due to its aggregating properties with other proteins, and therefore cannot physically pass through the gel matrix. According to studies, this 'non-migrating' fraction is also a toxic component to the venom (Russel & Emery, 1960; Perriere *et al*., 1988), however without the isolation of this protein or any functional assays performed it is uncertain as to the exact role of the toxin in conjunction with *E. vipera* venom. Although the SDS-PAGE imaging is not to the highest of standards, there is clearly distinctive markers, by which indicated that both the venom extraction methods worked. The very faint banding of lanes 3 & 4 in comparison to 1 & 2, suggest that there is less venom extracted per individual when using the sponge technique (Almada *et al*., 2016), and that more individual milking would be required to match the protein concentration obtained from the spine removal method.

Similarly, *T. draco* SDS-PAGE results (Fig.8.) remain consistent with the literature in that there is a banding just above 100kDa marker (Fig.8. – lanes 3 & 4), which would strongly suggest this is in fact the 105kDa Dracotoxin (Chhatwal & Dreyer, 1992a & b). However, as for the other bands, it is uncertain if these are toxins from the venom, or simply other proteins that have no toxic effect but aid in the facilitation and distribution of Dracotoxin. The venom from the opercular spines produced a banding just below 75kDa. Since no effort has been made to isolate or even examine the venom from *T. draco* opercular spines, this is the first reported case of the venom toxins molecular weight and we believe the protein to be 70-75kDa. Based on this evidence, and assuming that this banding is a toxic fraction, it would seem that the opercular venom contains different compounds to that of the dorsal spines. This would be a unique aspect, in the sense that it is rare for venomous animals to contain separately different venom apparatus with distinctly different toxins.

However, because there was no attempt to reduce the contamination in both *E. vipera* and *T. draco* SDS-PAGE, there is a possibility that some bands are associated with mucus than venom proteins (Baumann et al., 2014). Mucus samples were ran for a direct comparison to the venom gels, but no matching bands corresponded between them. Despite this, we still cannot be certain that all bands are toxin related. Yet, the closely matched bands to previous literature show a promising sign for simple fish venom characterisation for future studies.

The separation and identification of all toxic fractions is desperately desired in understanding the venom composition of *E. vipera* and *T. draco*. It is well documented that *E. vipera* venom contains a very high concentration of serotonin, even cited as having the highest concentration than any other animal venom. Thus, it would be sensible to attempt to provide evidence for these claims by utilising a simple serotonin ELISA assay in further studies. Additional

investigations should attempt HPLC and Mass Spectrometry techniques to better understand the constituents of the crude venom of both species, more specifically the opercular venom of *T. draco*. In conjunction, utilising bioassays can help to understand the ecological and biochemical purposes of the venom from their uniquely different venom apparatus. We are still uncertain if Dracotoxin has sub-units, similarly to that of all other Scorpaeniformes venom. Investigating this can aid further in understanding where *T. draco* and other Trachinidae species fit into the Scorpaeniformes family, regarding their toxin evolution.

4.1.3 RNA sequencing

Prior searches of *E. vipera* toxins on NCBI GenBank did not produce any matches. However, when the transcriptomic data was run through the GenBank database, there was a match of 97% gene identity with a 100% query cover to an *E. vipera* toxin known as Echiitoxin (Table 2 & 3). The possible reasons for this not being a target when searched without the sequence alignments could be due to the odd nomenclature of the toxin on Genbank or that the source of the sequence data is unpublished. Within current literature, the most widely cited and known toxin of *E. vipera* is identified as Trachinine (Perriere *et al*., 1988), however, the sequenced data on Genbank named it Echiitoxin (Table 2 & 3). This is either a mistake from the researchers not relating to the current nomenclature of this toxin, or that this in fact is an entirely new and uncharacterised toxin found within *E. vipera* venom. Though, this is unlikely, based on the predicted molecular weight of this protein being approximately 80kDa, suggesting that this is in fact the 81kDa subunit of Trachinine. Other authors have made a plea for a standardised nomenclature system of venom toxins (Mulley & Hargreaves, 2014), albeit on snake venom. But the uses of a standardised nomenclature of all venoms can be something which will disentangle the confusion when there are multiple research outputs on the same venom toxins.

The data reveals many identified toxins from all species that are within the family Scorpaeniformes. These toxins that have been matched with the dorsal RNA from *E. vipera* (Table 2), all have very high percent identity matches and percent query covers from sequence comparisons, as well as low E-values, which in conjunction suggest that the sequences are homologous. Percent matched identities range from 74-100%, whilst percent query cover ranges from 90-100%, with one species, *Hypodytes rubripinnis*, being an exception of 65%. The toxins matched are all α and β-subunits of venom toxins from a range of species within Scorpaeniformes (Chaung & Shiao, 2014). This further corroborates that the toxins from within Scorpaeniformes have all evolved from a single common ancestor and all the toxins are related with little variation, possibly being under the influence of negative selection (Chaung & Shiao, 2014). The data

suggests that the toxins of *E. vipera* also still hold true to Chaung & Shiao (2014) Scorpaeniformes ancestral gene hypothesis, due to the homologous sequences of other Scorpaeniform toxin genes, with regards to their E-values.

Another matched toxin molecule within the venom of *E. vipera*, was that of hyaluronidase from species of *P. volitans*, *P. antennata* and *S. verrucosa* (Table 2). This is a new find within the venom of *E. vipera*, as there is no previous identification of hyaluronidase being present within the venom (Russel & Emery, 1960; Perriere *et al*., 1988), it is mostly associated with *T. draco* venom (Chhatwal & Dreyer, 1992a & b). However, this could have easily been overlooked within previous research. Although sequence alignments have matched to hyaluronidase within other fish venoms, the percent query cover is relatively low, 57-63% , but with reliable homologous sequence matches with E-values of 0.0 (Table 2). This suggests a couple of possibilities, one being that the sequences matched are for a hyaluronidase-like molecule and that this gene has a high variability/ mutation rate and is prone to evolutionary changes between species. Another explanation could be that hyaluronidase is located throughout many tissues and this sequence could be from other cells that are associated with dorsal spines and not expressed within the venom. Until there has been actual isolation of this molecule from *E. vipera* crude venom, it is difficult to conclude on its presence and influence within the venom composition.

Opercular RNA (Table 3) also matched with Echiitoxin, with a much lower percent query cover (81%) than matched in the dorsal RNA sequences (100%) (Table 2). The opercular sequences (Table 3) also matched with a range of other Scorpaeniform α and β toxin sub-units, albeit, again with very low percent query covers (24-43%) in comparison to the dorsal sequences (90-100%) (Table 2). These matches seem contradictory, as *E. vipera* exhibits a lack of venom pertaining to the opercular spines (Fig. 5.). This low percent query cover in opercular sequences, could potentially indicate that in fact these toxin genes are expressed in a wide variety of body tissues and that a process of gene duplication and subfunctionalisation (Hargreaves *et al*., 2015) or neofunctionalisation (Fry *et al*., 2006) may occur with fish venom evolution. The topic of gene subfunctionalisation vs neofunctionalisation in the evolution of venom systems, particularly in snakes, is still a widely debated research area, and potential investigations into this regarding other systems as fish venom, could shed some more light on the origins and evolution of venom. Alternatively, it is possible that these sequences have a low percent query cover due to the opercular spines having lost the use of venom, and that these genes have become redundant and are no longer translated to produce venom toxins. These processes may also relate to the theory that piscine venoms evolved from ichthyocrinotoxins from the skin/ mucal secretions

(Cameron & Endean, 1973). If this theory holds true, these toxin genes may well be present in multiple different tissues throughout the body.

Based on the transcriptomic data, further research is needed to fully explore toxin genes of not only *E. vipera*, but other fish species. The transcriptomic analysis of RNA from multiple tissue samples of *E. vipera* would allow to understand better the expression of the venom toxins, and to test if in fact these toxin genes are expressed throughout multiple body tissues. Transcriptomic analysis of *T. draco* was not conducted due to a lack of funding. Therefore, future research should prioritise the RNA analysis of *T. draco* and be able to compare with closely related species as *E. vipera*, as well as Scorpaeniformes.

4.1.4 Phylogenetics of Scorpaeniformes

Phylogenetic analysis of Scorpaeniform toxins (Fig.9.) produced a tree that is largely consistent with Chuang & Shiao (2014), were there are distinct clades of α and β sub-unit divergences, with a *Pterois* sub-clade within the β cladding. The *Pterois* α toxins appear to be more closely related to the β toxins, than the α toxin clade. It is possible that they evolved from changes in gene expressions of β toxins. There are few slight discrepancies between the phylogeny produced here and Chuang & Shiao (2014), such as the placement of *Dendrochirus zebra* toxins. However, it is uncertain if these differences in placement are due to slightly differing phylogenetic methods used or if the addition of the two *E. vipera* toxin sequences, play a role in altering the family tree to this extent.

Although the different fish toxins contain sub-units which assemble together to form dimers or tetramers, it should be noted that it is the individual sub-units that are homologous, and the sequences refer to these individual toxin sub-units rather than when they are assembled together.

The *E. vipera* toxin sequence DN30045, that matched with Echiitoxin, has a bootstrapping estimation of 84%, which suggests that the two sequences are homologous. The tree places DN30045 in the α toxin clade with Echiitoxin, closely related to *Scorpaenopsis oxycephala* and *Sebastapistes strongia* α toxins. The second matched toxin sequence DN30331 seems to be more related to the β toxin clade, being placed within this distinct clading. But further investigation of DN30331 is needed regarding both its biochemical and genetic nature, as we can be certain it plays a toxic role within the venom of *E. vipera*.

The positioning of *E. vipera*, close to *Scorpaenopsis oxycephala* and *Sebastapistes strongia,* in this phylogeny (Fig. 9.), is unusual when compared to Scorpaeniform phylogenies

based on mtDNA (Smith *et al*, 2016). These relationships based on mtDNA, highlight that *E. vipera* is a very distant branching from any of the species in the phylogeny (Fig. 9.). Therefore, based on the data provided on the toxin gene relationships (Table 2 & 3; Fig.9.), it would seem that the toxins are homologous, adding more perspective to the idea that gene duplication may have played a role in toxin evolution of a scorpaeniform ancestor. Whilst over a large evolutionary time scale, small alterations of sub-units between species has lead to the small discrepincies in gene sequences. This would explain the high degree of homogeneity in toxin sequences but also the distantly related mtDNA phylogenies.

4.2 ***Lineus longissimus***

4.2.1 Toxin profile

Based on the peptide retention functions of SPE and the data observed from HPLC, it is clear the toxic components within the mucus are peptides. Therefore, we can conclude that the toxic mucus of *L. longissimus* does still adhere to the consensus that Anoplans employ toxins that are polypeptide in composition (Kem, 1976; Kem & Blumenthal, 1978; Kem, 1985). Considering this, speculation that *L. longissimus* produces TTX-like compounds via a symbiosis with bacterial *Vibrio spp*. is unlikely (McEvoy *et al*., 1998; Carroll *et al*., 2003), and the recent reevaluation of these studies (Strand *et al*., 2016) should be now considered, in that they were initially false-positive results based on scientific errors.

The results from HPLC (Fig.10.) indicate the presence of three major toxic fractions within the mucus of *L. longissimus*. This is further corroborated by the bioassays (Table 4), which indicate that the pooled fractions of 23-26 (peak 1), 27-29 (peak 2) and 30-33 (peak 3), all caused aspects of the neurotoxic affects observed from the raw mucus ethogram injections. It is also clear that it is these three major peaks only produce these affects, as the other pooled fractions of 2-22 and 34-42 did not cause any of the noted affects at all. The mass spectrometry searched the three main HPLC peaks for compounds. The data revealed that peak 1 contained a toxin with a mass of 6.4kDa, peak 2 of 3.2kDa and peak 3 had multiple mass hits of 9.9kDa, 6.6kDa and 4.1kDa. It is uncertain if the three masses from peak 3 all correspond to toxic peptides that work in unison or if one molecule is a toxin and the others are facilitating molecules for the toxin. These masses are consistent with previous literature highlighting that these toxin molecules are of low molecular weight and are small peptide molecules (Kem, 1976; Kem & Blumenthal, 1978; Kem, 1985).

It seems that the three toxin constituents all cause slightly different effects of convulsions, twitching and death (Table 4, Fig.11. & Fig.12.). The one-way ANOVA results indicate a significant difference between the raw mucus affects and the isolated fractions in both mean time of convulsions and twitching to occur. The ethogram results also show that peak 1 and 3 caused death, whilst peak 2 does not. Therefore, based on the analysis of these data sets, the individual toxic compounds seem to work synergistically to produce a significantly more potent effect than the compounds on their own. This is seen throughout many different venomous/ toxic organisms, by were the toxic constituents all work in tandem to produce a significantly more potent outcome (Vassilevski *et al.,* 2009; Mebs, 2000; Calvete *et al.,* 2009).

Although the recorded/ observed data suggests differing times to the noted behaviour e.g. convulsions, twitching etc. these times may not be as accurate due to human error or subjectivness in defining and the recorded timing of each categorical behaviour. The defining of such behaviours may differ between observers, and one individual's definition of a 'convulsion' or 'twitching' might be different. Another drawback within the data was the accuracy in the timing of such behaviours to occur. Again, subjectivness of each individual observer might be different. Thus, a better representation of this data should be combined from multiple sources and a consensus of the defining behaviours and timings of such, would allow for a more reliable data set.

Other problems with the bioassays arise when trying to understand the function of the toxins. It is still uncertain if the toxic mucus acts as poison/ toxungen or simply as an unpalatable deterrent to predators. By injecting the toxins into *C. maenas*, it is essentially being used as a venom, but this is not how *L. longissimus*' deploys its toxins. Therefore, tests such as LD_{50} would not be suitable in determining the acute toxicity, as it would not give an accurate representation of lethal dose in regard to its true utilisation and ecological function.

N-terminal sequencing of the fractionated peaks, produced only one viable alignment, that of peak 2 (Fig.13.). It is possible that the other peaks did not produce results due to the very high presence of Cysteine residues causing strong structural integrity of the peptides (Fig.13. & Fig.14.). This may cause cleavage problems during Edman degradation, resulting in unbroken AA chains. The sequence of peak 2 toxin was matched to a Trinity sequence DN24190. The identification of a signal peptide of DN24190 indicates that these peptides are secreted (Martoglio & Dobberstein, 1998). This should be expected since the toxins are secreted within the mucus, thus a signal peptide would be needed to be synthesised across the cell membrane. The high presence of Cysteine residues are likely an important aspect in the structure and function of the

peptides. These peptides could be Cysteine-rich secretory proteins (CRISPs), which have also found their uses in snake venoms, inhibiting smooth muscle contraction and cyclic nucleotide-gated ion channels (Yamazaki & Morita, 2004). These seem to coincide with many features in which *L. longissimus* toxins provide, such as, they are secreted, contain Cysteine-rich residues (Fig. 13. & 14.) and cause involuntary muscle contractions (Table 4).

4.2.2 RNA sequencing

Whelan *et al*. (2014) attempted to find transcriptome sequence similarities to known toxins of nemerteans, one of these included an *L. longissimus*. This study highlighted that there were 4 toxins produced by *L. longissimus*; Neo-VTX α-subunit (stonefish toxin), plancitoxin-1 (Echinoderm toxin), SE-cephalotoxin (Cephalopod toxin) and cytotoxin A-III (nemertean toxin). However, this study did not reveal the actual percentage alignment matches of these toxin genes, only highlight if they shared similarities. Therefore, we are uncertain how analogous these genes actually are. The transcriptomic data we have produced did not match with Neo-VTX α-subunit, plancitoxin-1, SE-cephalotoxin. In combination to this, our HPLC results indicate the presence of only 3 toxic proteins (Fig. 10.), not 4 as suggested by Whelan *et al*. (2014). Given the diversity and desired functionality of toxins throughout the animal kingdom, combined with the notion that they can potentially evolve from non-toxic proteins (Fry *et al*., 2009; Hargreaves *et al*., 2014), it is no surprise that toxin genes can be matched to a wide scope of other, possibly unrelated toxins.

Our Trinity transcriptomes only contained a single match alignment to Parbolysin isoform toxins from another nemertean, *Parborlasia corrugatus* on the GenBank database (Table 5). The matched percent identities were quite low (9-24%), indicating very sparse alignment matches. However, due to these being isoforms it is entirely likely that these toxin molecules exhibit a wide variability in both their AA alignments and/ or RNA transcripts. The Trinity sequence was further converted into its coding AA alignment. The AA alignments produced a greater percent Identity match of 61-66% with Parbolysin isoforms, further adding that the RNA sequences of the isoforms have great variability. The AA alignment produced a further match to a Cytolysin A-III toxin of the nemertean, *Cerebratulus lacteus,* with a match of 69% identity. This match was also found in previous literature that annotated *L. longissimus* RNA transcripts (Whelan *et al*., 2014). Based on these results, it is almost certain that the *L. longissimus* toxin in question is most likely a cytolytic toxin. Berne *et al*. (2003) also found similarities in AA alignment of *P. corrugatus* and *C. lacteus*, thus it is interesting that *L. longissimus* can also be added to this group. This suggests that

cytolytic toxins in nemerteans seem to share striking similarities, and this could further indicate that these toxins have been passed from a common ancestor.

There are two possible reasons why no toxin matches, other than the cytolytic peptides, were identified, either *L. longissimus* toxins are unique to that species and not seen in any other organism, or no similar toxin sequences (both AA and DNA) have yet been identified and added to the GenBank database. The latter seems the most likely.

4.2.3 *Lineus longissimus* toxin conclusions

Based on the corroboration of all data achieved on *L. longissimus* toxins, we can conclude that peak 2 toxin is DN24190 from the transcriptome and it also acts as a neurotoxin (Fig.11., Fig. 13. & Table 4). Given the predicted mass similarity of DN24190 (Fig. 13.) of 3.4kDa (after signal peptide has been cleaved) to that of the toxin mass identified from HPLC peak 2 as 3.2kDa, there is a high certainty that these two peptides are the same. This peptide potentially could be similar to the Cerebratulus B-toxins neurotoxins (Kem 1976 & 1985). Parbolysin and Cytolysin A-III from other nemerteans are cytolytic toxins that causes cell lysis. Due to the similarities in AA sequence of these toxins to the matched DN27342 sequences (Table 5 & Fig.14.), it should be assumed that either peak 1 or 3 contain this cytolytic toxin due to the behavioral effects noted (Table 4). According to the literature, the mass of Parbolysin is 10.3kDa (Berne, *et al.*, 2003). Thus, we can expect that *L. longissimus*' cytolytic toxin would be of a similar molecular weight. The DN27342 alignment (Fig.14.) was run on a molecular weight predictor, which indicated an estimation of 10.6kDa. This coincides with the mass of Parbolysin, and therefore we can assume they are analogous in cytolytic function. The only closely matched peak mass to DN27342 was that of the 9.9kDa peptide of peak 3. Therefore, based on the miniscule differences in mass we can assume that peak 3 contains DN27342 which is a cytolytic peptide. The small difference in mass could be due to the loss of the signal peptide or breaking of Cysteine residues. That just leaves peak 1 toxin remaining unknown. Yet, it is even possible that peaks 1 & 3 both contain cytolytic peptides, as their desired biological effects are very similar (Table 4).

Further research into *L. longissimus* toxins is still needed however. Identifying the remaining elusive toxin and the identity of the other molecules of peak 3 is key for future studies. As well as studying the biochemical nature to the toxins, there is also a need in understanding the ecological function of them. It remains unknown if the toxins act as a deterrent in the surrounding water to warn off predators or by making themselves unpalatable. A simple experiment would be to use a choice flume test for *C. maenas,* with *L. longissimus* mucus running

down one of the corridors. This can determine if the natural predator finds the water saturated in mucus unfavorable or not. More research into these two aspects will allow for a better understanding on the evolution of nemertean toxins, which is already an untouched area.

4.3 **The future of marine toxinology: Where do we go from here?**

With the rise in research status of toxinology, there have been huge efforts to catalogue and characterise many natural toxins from varying sources throughout the animal kingdom. This has led to the creation of many successful venom-based drug therapies (Clark, 1996; Vetter *et al*., 2011), whilst there have also been massive efforts focusing on toxins with therapeutic potential (Romano & Tatonetti, 2015). Some venom based drugs have already been created from toxins of marine species. Prialt (Ziconotide) was the first drug that was developed from a neurotoxic venom. It was created using the toxin peptide x-conotoxin M-VII-A, from the cone snail, *Conus magnus*. The drug is an analgesic based compound to treat varying forms of chronic pain (McIntosh *et al.,* 1982; Clark, 1996). There are still ongoing efforts to use other marine toxins as pharmaceuticals, attempting to treat certain autoimmune diseases as multiple sclerosis using anemone toxins (Beeton *et al*., 2011; Chi *et al.,* 2012) and even cognitive diseases as Alzheimer's and Schizophrenia using nemertean toxins (Mayer *et al*., 2010; Zawieja *et al*., 2012). More recently, research has shown the pharmacological potential of *E. vipera* venom (Fezai *et al.,* 2016). This study highlighted the apoptotic and cell cycle arrest properties of *E. vipera* crude venom when subject to colon cancer carcinomas. With this, the authors plead for a better identification and characterisation of more fish venoms, including that of *E. vipera*. So, even the pharmacological potential of marine toxins, although very promising, are still neglected.

Little is known about marine toxins and the mechanisms by which most of these toxins affect biological systems. This makes it difficult to design any effective treatment against them. While marine envenomations and poisonings are not usually as fatal or as much a concern to the public as snakebites, the threat is still high, with as many as 1800 per annum reports of marine envenomations/ poisonings in the USA alone (Balhara & Stolbach, 2014). As in the case with HWI treatments being a debated therapy, a similar case has been highlighted with cnidarian stings, with such treatments as HWI, ice packs, vinegar and urine all being cited in various literature as effective treatments (Wilcox & Yanagihara, 2016; Yanagihara *et al*., 2016). But little efforts have been made to conclude on the efficacy of such treatments, and in some instances, could mean the difference between life and death.

The evolutionary relationship of toxins throughout major lineages is also an area in which research is lacking, with the most highly regarded studies being conducted on snakes (Fry *et al*., 2006; Hargreaves *et al*., 2015). Understanding the evolution of toxins can highlight not only key aspects of predator-prey interactions, but also the evolution of complex molecules and their biological activity. More recent evidence has suggested that that venoms have evolved convergently in fish lineages multiple times. There may be a lot more species of venomous fish than previously thought, with an estimate of >1200 species, compared to the previous evaluation of ~200 (Smith & Wheeler, 2006; Smith *et al.,* 2016). Thus, there could be countless other toxic marine taxa each equipped with a suite of unique and uncharacterised toxin compounds, that could be used for pharmaceuticals and bioassays. Moreover, new broad scale macroevolutionary patterns of biodiversity in relation to venomous and toxic organisms are being studied (Harris & Arbuckle, 2016). These large evolutionary studies are highlighting the ecological diversity and effects that evolving a toxic arsenal can have on lineages and groups of organisms. These large-scale studies combined with actual genetic data can potentially piece together, the challenging task of understanding toxin evolution throughout Animalia. Since marine organisms are thought to have evolved toxins first (Rantala *et al*., 2004; Casewell *et al*., 2013; Dittman *et al*., 2013), it would be a logical notion that understanding older toxic linages within the marine environment, would potentially allow for a better understanding of toxin diversity within terrestrial species.

4.4 **Conclusion**

It is clear marine toxin research is neglected, however, with a push in the right direction this field can open new and exciting avenues for toxinology, and push the boundaries of modern science. From this simple study, we have highlighted new and interesting facets of marine toxins from the three-species studied, even though there was very small and limited funding for this project. This clearly highlights that although the data we have generated still needs to be delved into in more depth, it is a fundamental start in pushing marine toxinology in the right direction. With greater funding and access to more advanced proteomic and transcriptomic methods, this research area can join the forefront of toxinology. The potential for a pharmacological goldmine of active compound, combined with the unknown evolutionary facets of the toxins, should now be a clear indication to drive researchers in exploring this field further and with scrutinising detail.

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