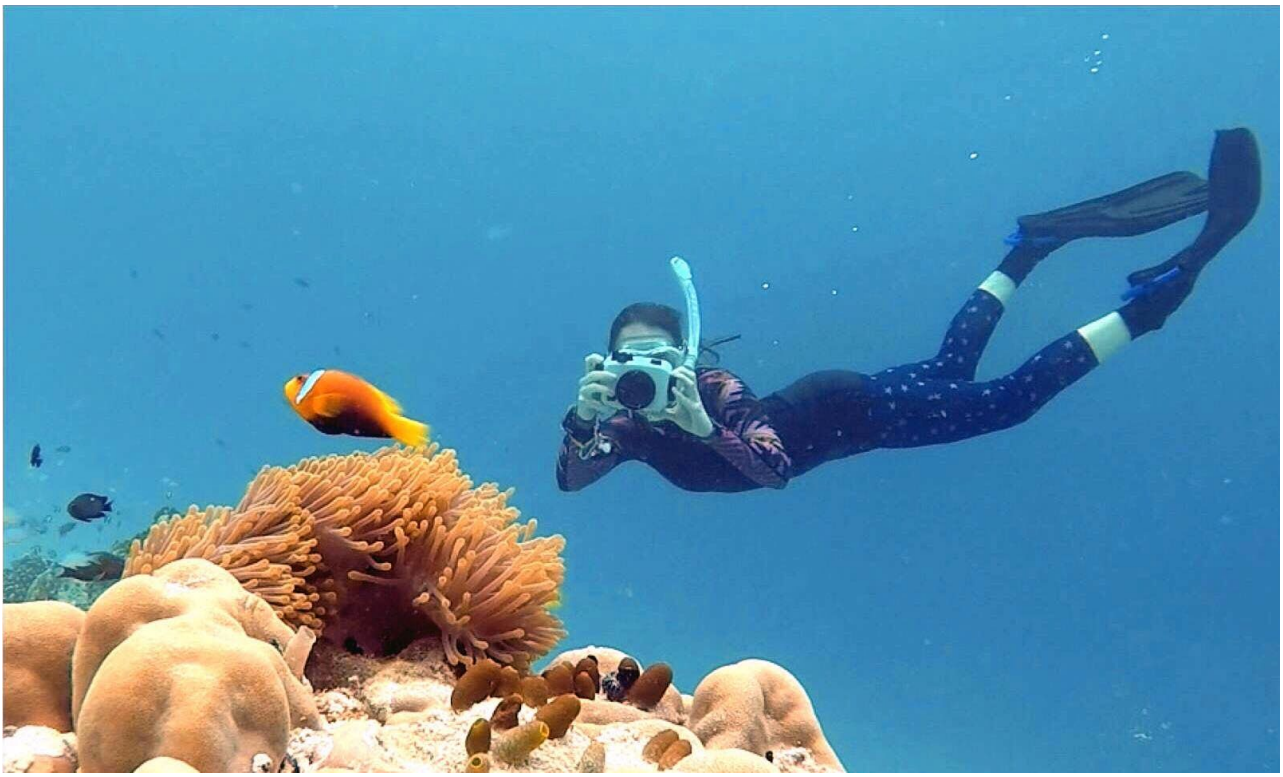
Behind anemone lines:
using omics to uncover the mechanisms involved in the iconic symbiosis between host sea anemones and anemonefish.



By

Cassie Mae Hoepner

BSc Hons (Mar Biol), Grad Cert Env Mgt

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ABSTRACT

Anemonefishes and host sea anemones have one of the most well-known and iconic symbiotic relationships. The association between anemonefishes and host sea anemones has existed for at least 12 million years, however, this symbiotic relationship is quite rare, with the 28 different species of anemonefish living symbiotically with only ten out of over 1700 species of sea anemones. Furthermore, it is clear that both host sea anemones and anemonefishes glean significant fitness advantages from their symbiosis, including improved lifespan and potential reproductive success for anemonefishes, and increased nutrients and protection from predators for host sea anemones, however, the mechanism that enables this relationship. i.e., anemonefishes resistance to their toxic host sea anemone, remains unclear. Current research has focused solely on anemonefishes and the potential for their unique mucus layer to provide a form of protection against the toxins in their host sea anemone venom, with very little consideration for the role the host sea anemone itself may play in the establishment of this symbiosis.

This thesis addresses this significant knowledge gap in our understanding of anemonefishes and host sea anemone symbiosis by exploring the relationship through the lens of the host sea anemone, via five research chapters. Through this body of research, I show that host sea anemones provide additional benefits to their anemonefish symbionts. Chapter 2 examined a previous hypothesis that the symbiotic relationship with toxic host sea anemones reduces the susceptibility of anemonefishes to ectoparasites. I found *A. ocellaris* living in symbiosis with a host sea anemone in the wild in Malaysia have a reduced ectoparasite load, and in an observational study in Malaysia and the Maldives there was no evidence that anemonefishes visited cleaning stations to remove ectoparasites, which provides further evidence that the sea anemone toxins may aid in protecting anemonefishes from ectoparasites. In Chapter 3 I quantified the nematocyte response of the host *Entacmaea quadricolor* to *A. percula* mucus. Acclimated and familiar *A. percula* trigger significantly

fewer nematocytes than unacclimated *A. percula*, however, there are still some nematocytes fired at *A. percula* mucus while in association with the sea anemone. In chapter 4, I analysed the lipid and glycan profile of *A. percula* mucus to reveal that no significant change in lipids composition occurred in mucus collected before, during and after association with an *E. quadricolor* host, during an eight-week experiment. For the first time this work demonstrates a change of glycan profile in *A. percula* mucus, however this change only occurred after three weeks of association with *E. quadricolor,* and within 24 hrs of removal from the *E. quadricolor* anemone the anemonefish mucus layer had largely reverted back to its original glycan profile. Such a delay in the acclimation of the anemonefish mucus layer is unexpected and further study is needed to uncover the initial mechanism used by anemonefishes that enables them to enter the venomous tentacles of host sea anemones while their mucus layer adapts at the glycan level. In chapter 5, I used a proteotranscriptomics approach to reveal a comprehensive profile of the tentacle transcriptome which results in 2,736 proteins being present in venom from the most popular host anemone *E. quadricolor.* This work revealed that while *E. quadricolor* tentacles express RNA transcripts for numerous and diverse toxins only 10% of these are encoded as proteins present in the venom and that the venom mostly consists of mostly non-toxin proteins. In the final chapter, chapter 6, I used differential expression analysis to examine the role *E. quadricolor* itself plays a role in the establishment of their symbiotic relationship with *A. percula*, by analysing transcript and protein data from samples collected from *E. quadricolor* before and after hosting with an anemonefish pair. Specifically, I found that neurotoxin tentacle transcripts and venom proteins responsible for membrane damage, pore formation, and paralysis were downregulated during hosting with anemonefish. I also found that both natural venom inhibitor tentacle transcripts and proteins rich in IG-like domains were upregulated in the presence of anemonefish.

Overall, this thesis demonstrates that host sea anemones play a much bigger role in the establishment and maintenance of their symbiosis with anemonefishes than previously thought, and by applying novel techniques to century-old questions, this thesis has redefined the research path to uncovering the mechanisms enabling the symbiotic relationship between host sea anemones and anemonefishes. Future research should consider the role of host sea anemone venom inhibitors as a resistance mechanism in symbiotic anemonefishes.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed

Date: 17/11/23

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Laboratory experiments were conducted on the traditional lands of the Kaurna people and in Naarm on the traditional lands of the Kulin Nation. Field experiments were conducted on Rawa Island, Malaysia and Naifaru, Maldives. I would like to acknowledge the importance of sea country in all locations research was conducted for this thesis.

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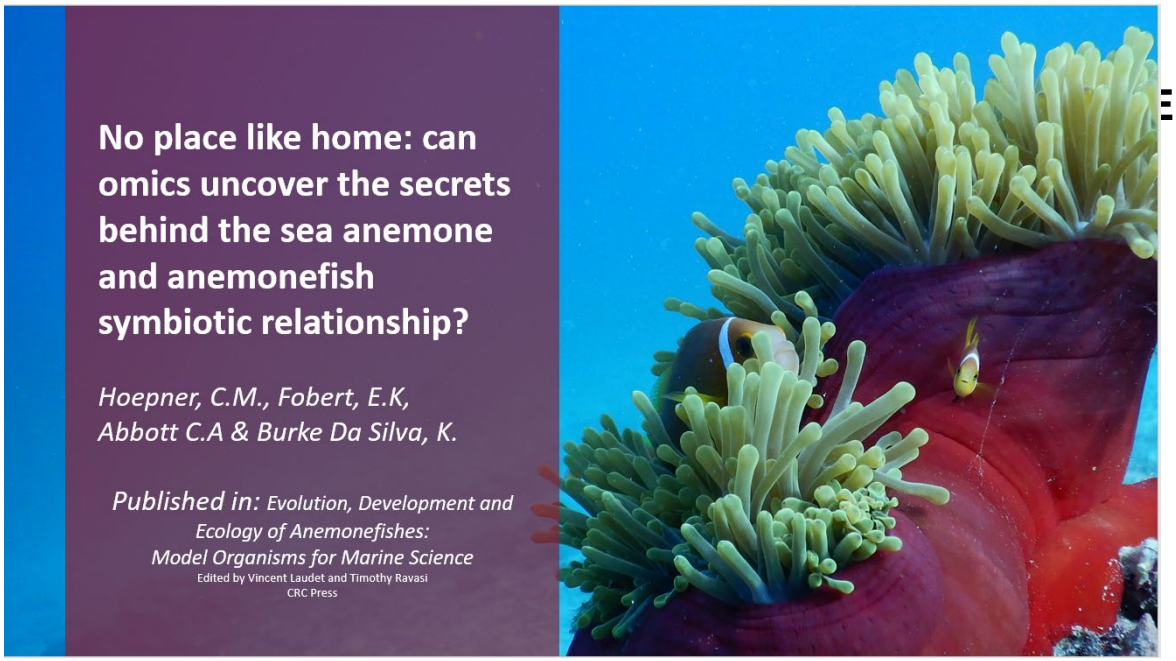

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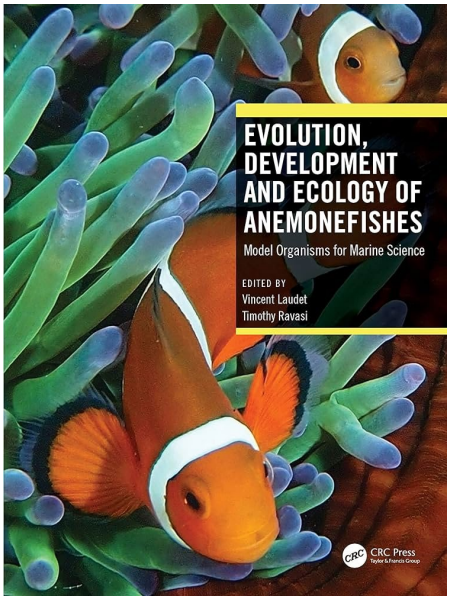

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1.1 Preface

Symbiosis is the coming together of two or more species, generally a smaller 'symbiont' and a larger 'host' where at least one species benefits from this relationship (Overstreet and Lotz 2016). The host organism acts as the provider of a resource, while the symbiont consumes this resource which is important to their life history. Symbiotic relationships can take a variety of forms: mutualistic, parasitic, predatory, competitive, or commensal (Dimijian 2000, Overstreet and Lotz 2016). Mutualisms are the most well-known form of symbiosis where both the host and the symbiont benefits from their relationship, commensalisms provide benefits to the symbiont and have no impact on the host, whereas parasitism negatively affects the host. Symbiotic relationships are the most widespread form of interspecies interaction, but assessing the costs and benefits of these relationships can be difficult and can switch with environmental changes (Leung and Poulin 2008). A key example of this is cleaner fish who form a mutualistic relationship with client fish who visit their cleaning stations to have ectoparasites removed (Arnal et al. 2001). However, cleaner fish can exploit this relationship by cheating their client fish, biting scales and mucus rather than removing ectoparasites (Bshary and Grutter 2002, Wismer et al. 2016) becoming just as parasitic to their clients as the ectoparasite they are supposed to be removing. Further, during detrimental environmental conditions, corals who host algal endosymbionts can expel their symbiont from their tissues (Brown 1997). Rises in sea surface temperatures impact the functioning of their internal algae leaving the symbiont without habitat while under thermal stress and the coral without a large proportion of their daily nutrition requirements (Curran and Barnard 2021). Research into these relationships often focus on the ecology and evolution of the symbiont with much more focus on how the symbiont benefits or forms this relationship rather than on the benefits that the host gains from the relationship.

Chemical defence is one of the most widespread antipredator strategies that has evolved across multiple taxa, including prokaryotes, plants and animals (Clucas 2010, Rowe 2010, Savitzky et al. 2012). Chemical defence can take one of two forms: (1) primary defence, that prevents predators from pursuing prey and (2) secondary defence, that comes into play once the prey has been attacked (Clucas 2010). Most commonly, a chemical defence involves a venom which contains toxic properties that can injure and kill predators or be used to acquire prey (Clucas 2010). Venom can be produced for a variety of purposes, but most contain an array of proteins, peptides and other complex molecules that act as neurotoxins, cytolysins and actinoporins (Frazao et al. 2012). Producing a chemical defence can be energetically costly, as energy is diverted away from growth and reproduction and used instead for chemical and protein synthesis (Halpin et al. 2008, Rowe 2010, Furstenberg-Hagg et al. 2014). Although costly, in terms of production, the benefits of chemical defences in terms of reduced predation, clearly function as an adaptive strategy. Relationships between venomous and non-venomous species generally function as predator-prey, where the predator evolves more toxic venom, and in return, the prey evolves resistance to the toxins (Holding et al. 2016, Arbuckle et al. 2017), referred to as a chemical arms race. Mutualistic relationships do exist between venomous species and non-venomous species, such as cnidarians and zooxanthellae or bacteria (Pontasch et al. 2013, Breusing et al. 2022), but rarely does it involve a vertebrate. The mutualistic symbiosis between anemonefishes and host sea anemones is a rare example of a venomous species and a non-venomous species both benefiting from association.

1.2 Publication

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19 No Place Like Home
Can Omics Uncover the Secret behind the Sea Anemone and Anemonefish Symbiotic Relationship?

Cassie M. Hoepner, Emily K. Fobert, Catherine A. Abbott, and Karen Burke da Silva

CONTENTS

19.1 INTRODUCTION

First recorded in 1868 (Collingwood 1868), anemonefish and anemones have one of the most well-known and iconic symbiotic relationships (Hobbs et al. 2012; Mebs 2009; Nedosyko et al. 2014). There are 28 different species of anemonefish that form associations with only ten species of host anemones (Fautin and Allen 1992). Although the association between anemonefish and sea anemones has existed for at least 12 million years (Marcionetti et al. 2019), this symbiotic relationship is quite rare, occurring in only ten out of over 1,200 species of anemones. Anemones also form a tripartite symbiosis with zooxanthellae that provide up to 85% of their daily nutrient budget (Lonnstedt and Frisch 2014). The symbiotic relationship with anemonefish has likely evolved three times amongst three unrelated anemone families (Thalassianthiade, Actinidae, Stichodactylidae) (Titus et al. 2019), with two genera contributing seven species (*Heteractis* – four species; *Stichodactyla* – three species) (Fautin 1991). In comparison, the anemonefish mutualism with anemones is thought to be present in the common ancestor of all anemonefish (Litsios et al. 2012). The evolution and diversification of anemonefish have benefited from their associations with host anemones, through increased rates of species diversification and morphological evolution in comparison to other coral reef fish without anemone associations (Litsios et al. 2012). The majority of anemonefish diversity is thought to have occurred in the last five million years, with 25 of the 28 species evolving during that time.

The mutualistic nature of the anemone and anemonefish symbiosis indicates that both organisms provide and receive a variety of benefits. For anemonefish, the toxic anemone provides a safe site for reproduction and protection from predation (Holbrook and Schmitt 2004). In return, anemonefish aid the growth, reproduction, and survival of anemones by providing nutrients (such as nitrogen and carbon) via faeces, increasing oxygenation by swimming amongst the tentacles, and actively defending their host anemone from various predators such as chaetodontid

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fishes and sea turtles (Godwin and Fautin 1992; Nedosyko et al. 2014; Frisch et al. 2016; Mariscal 1970a). This unique symbiosis has allowed anemonefish to develop a range of life-history traits that can be attributed to their close association with venomous host anemones. For example, evidence suggests that anemonefish have exceptionally long lifespans for a reef fish of their size, living up to 30-plus years (Buston and Garcia 2007), compared to five to ten years for other similarly sized reef fish (Sale 1980). Anemonefish are also unusually bold and aggressive for their size. For example, when approached, anemonefish will swim out of their anemone towards the threat, rather than retreating to safety (Godwin and Fautin 1992). These traits are not seen in closely related damselfish or other similar-sized reef fishes, thus providing anemonefish with a unique ecological advantage (Marcionetti et al. 2018).

Although the ecological success of both anemone and anemonefish is clearly enhanced due to the evolution of their symbiotic relationship, the mechanism enabling anemonefish resistance to anemone venom remains unclear. Exactly how anemonefish glean such significant fitness advantages that improve their lifespan and potential reproductive success is not yet fully understood; however, it is widely believed that anemonefish have a unique mucus layer covering their scales that is somehow involved in enabling the formation and existence of their symbiotic relationship with sea anemones. Despite decades of study, there are still many more questions that remain unanswered such as: how do anemonefish live unharmed amongst the anemone's tentacles? How did this symbiotic relationship first evolve? And how do anemonefish pick the best anemone host?

In this chapter we (1) present an overview of the symbiotic relationship between anemones and anemonefish, including the factors that influence host selection; (2) present current hypotheses and discuss the existing evidence within the literature with a particular focus on the advances omics techniques have provided; (3) explore anemone venom research and discuss how toxin resistance in other model systems can be applied to further our understanding of the anemonefish and anemone symbiosis; and (4) discuss how omics can be applied in the future to help answer the remaining questions surrounding this symbiotic relationship.

19.2 INFLUENCES ON ANEMONEFISH HOST SELECTION

The relationship between different anemonefish species and anemone host species follows a unique and organized pattern that is not yet fully understood, with new associations being discovered even now (Bennett-Smith et al. 2021) (Table 19.1). Anemonefish can be classified as host generalists; for example, Clark's anemonefish (*Amphiprion clarkii*), is the only anemonefish species to form associations with all ten species of host anemones. In contrast, there are nine species of anemonefish that are host specialists (*A. frenatus*, *A. chagosensis*, *A. pacificus*, *A. fuscocaudatus*, *A. latifasciatus*, *A. mccullochi*, *A. nigripes*, *A. sebae*, and *A. biaculeatus*), forming associations with only a single anemone species (Burke da Silva and Nedosyko 2016). Despite co-existing within the same geographic region, there are a large number of anemone hosts with which anemonefish species do not associate (Table 19.1). This pattern demonstrates that geographic range is not the factor that determines which anemonefish and anemone species associate (Fautin 1986). Other factors that may contribute to which species form associations could include: (1) anemone morphology, (2) anemone toxicity, and/or (3) intraspecific competition amongst anemonefish species.

19.2.1 Anemone Morphology

Anemone tentacle length may provide a selective advantage to anemonefish by concealing them from predators (Huebner et al. 2012). Anemone species with longer tentacles can provide a larger surface area for anemonefish to hide in and thus reduce the visibility of the anemonefish to predators (Huebner et al. 2012). Stevens and Merilaita (2009) hypothesized that anemonefish stripes act to break up the body shape, making it more difficult for predators to detect the anemonefish amongst the tentacles and thus enhancing the anemone's protective features at varying distances. The number of stripes on anemonefish was found to be correlated to the length of their host anemone's tentacle; anemonefish species with two to three stripes form relationships with anemone species that had longer tentacle morphology, compared to anemonefish species with one or no stripes (Merilaita and Kelley 2018) (Table 19.1). Merilaita and Kelley (2018) also found that anemonefish with fewer stripes formed associations with a smaller number of host anemone species compared to anemonefish species with more stripes.

Furthermore, the morphology of anemone tentacles may make a species attractive as hosts for anemonefish. For example, the beaded anemone (*Heteractis aurora*) and bubble-tip anemone (*Entacmaea quadricolor*) have unique tentacle shapes that increase the surface area in which the anemonefish can hide, with dense beaded or bulb-like tentacles (Figures 19.1a,b). The magnificent anemone (*Heteractis magnifica*) has the unique ability to enclose all its tentacles within its soft body by contracting inwards when disturbed (Figure 19.1c), providing increased protection to the anemonefish who can hide inside the anemone body during this dangerous time. As anemonefish rely on their anemone host for protection, anemonefish may favour hosts whose morphological traits offer them better shelter or protection from predators.

19.2.2 Anemone Toxicity

Host anemone species range in the potency of their venom, from low to high haemolytic and neurotoxic toxicities (Nedosyko et al. 2014). Interestingly, host anemones with higher haemolytic and neurotoxic toxicities have shorter tentacles (< 20 mm) compared with anemones with

TABLE 19.1
Matrix of Anemonefish and Host Sea Anemone Associations According to Toxicity

Amphiprion spp:	*Heteractis malu*	*Macrodactyla doreensis*	*Heteractis crispa*	*Heteractis aurora*	*Entacmaea quadricolor*	*Heteractis magnifica*	*Stichodactyla mertensii*	*Stichodactyla haddoni*	*Stichodactyla gigantea*	*Cryptodendrum adhaesivum*	Number of hosts	Number of stripes
clarkii	X	X	X	X	X	X	X	X	X	X	10	3
akindynos	Ø	Ø	X	X	X	X	X	X	X	Ø	7	2
chrysopterus	Ø	X	X	X	X	X	X	X	Ø	Ø	7	2
bicinctus			X	X	X	X	X	X	X	Ø	7	2
tricinctus	Ø		X	X	X	Ø	X	X	Ø	Ø	5	3
chrysogaster				X		X	X	X			4	3
perideraion	Ø	X	X	Ø	X	X	Ø	Ø	X	Ø	4	2
melanopus	Ø	Ø	X	Ø	X	X	Ø	Ø	X	Ø	4	1
allardi			Ø	X	X	Ø	X	Ø	Ø	Ø	3	2
ocellaris	Ø	Ø	Ø	Ø	Ø	X	X	Ø	X	Ø	3	3
percula	Ø	Ø	X	Ø	Ø	X		Ø	X	Ø	3	3
polymnus	Ø	X	X	Ø	Ø	Ø	Ø	X	Ø	Ø	3	3
omanensis			X	Ø	X	Ø	Ø	X	Ø	Ø	3	2
rubrocinctus	Ø		Ø	Ø	X	Ø	Ø	Ø	X	Ø	2	1
sandaracinos	Ø		X	Ø	Ø	Ø	X	Ø	Ø	Ø	2	1
akallopisos	Ø	Ø	Ø	Ø	Ø	X	X	Ø	Ø	Ø	2	1
barberi			X		X						2	1
ephippium	Ø		X	Ø	X	Ø	Ø	Ø	Ø	Ø	2	0
latezonatus			X		X			Ø	Ø		2	4
frenatus	Ø		Ø	Ø	X	Ø	Ø	Ø	Ø	Ø	1	1
chagosensis			Ø	Ø	X	Ø	Ø	Ø	Ø	Ø	1	2
pacificus	Ø	Ø	Ø	Ø	Ø	X	Ø	Ø	Ø	Ø	1	1
fuscocaudatus		Ø		Ø	Ø	Ø	X	Ø			1	3
latifasciatus					Ø	Ø	X	Ø			1	2
mccullochi			Ø		X			Ø	Ø		1	1
nigripes			Ø	Ø	Ø	X	Ø	Ø	Ø	Ø	1	1
sebae	Ø		Ø	Ø	Ø	Ø	Ø		Ø	Ø	1	2
biaculeatus	Ø	Ø	Ø	Ø	X (solitary)	Ø	Ø	Ø	Ø	Ø	1	3
Number of associates	1	4	14	7	17	12	12	9	8	1		
Tentacle length (mm)	40	175	75	50	100	100	20	10	10	5		
Toxicity rank	1	2	3	4	5	6	6	7	7	8		

Note: X indicates species that associate, Ø indicates species that are in the same marine province (Litsios et al. 2012), but do not associate. Association matrix updated from Burke da Silva and Nedosyko (2016) via Bennett-Smith et al. (2021), Allen et al. (2010), Allen et al. (2008), Fautin and Allen (1992), Hobbs et al. (2014), Scott et al. (2015); Pryor et al. (2022). Tentacle length and toxicity data from Merilaita and Kelley (2018), Nedosyko et al. (2014).

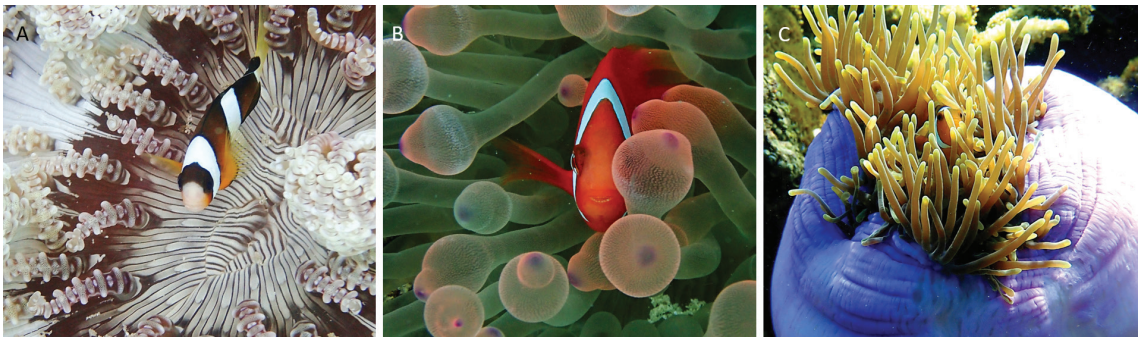

FIGURE 19.1 Various morphology of anemone hosts that aid in camouflaging anemonefish. A) Beaded tentacles of *Heteractis aurora*, B) bulb-like tentacles of *Entacmaea quadricolor*, C) retraction of tentacles by *Heteractis magnifica*. Images: Emily Fobert.

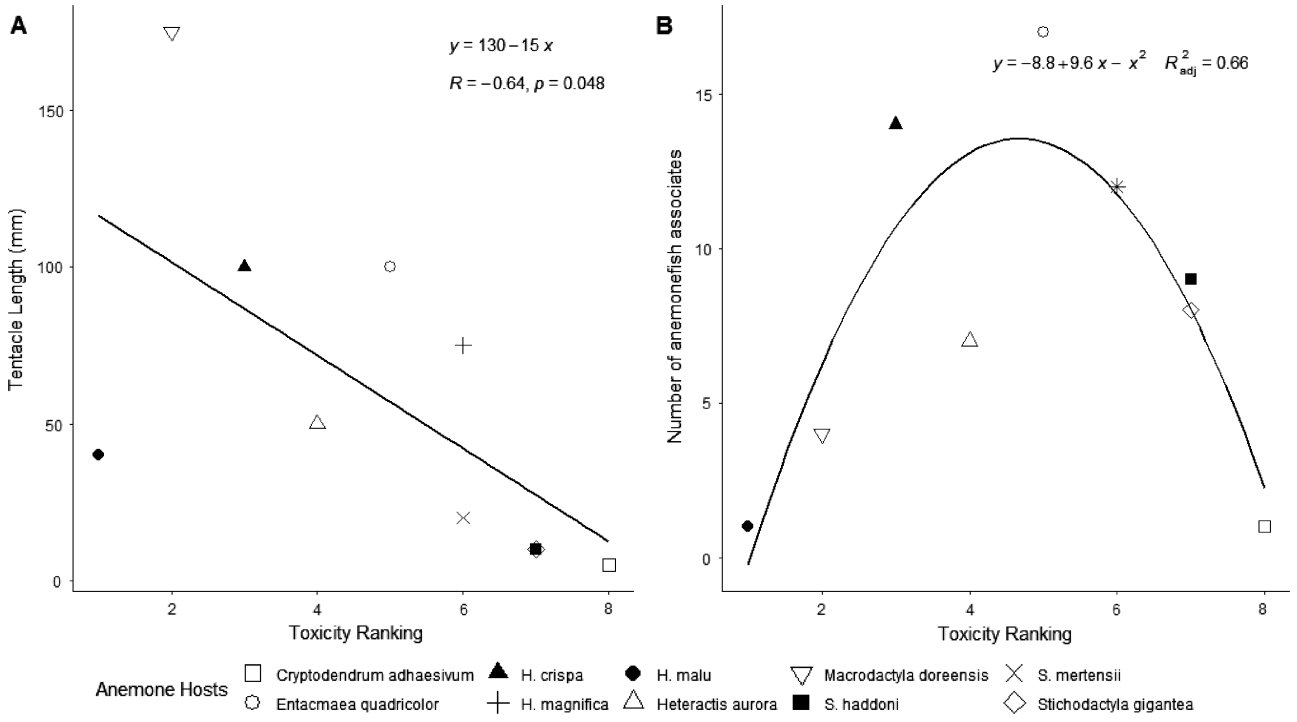

FIGURE 19.2 A) Negative relationship between anemone tentacle length (mm) and overall host anemone toxicity ranking (Merilaita and Kelley 2018). B) Relationship between number of anemonefish associates and overall host anemone toxicity ranking. Updated from Merilaita and Kelley (2018), Nedosyko et al. (2014).

mid-range or low toxicities (Figure 19.2a) (Merilaita and Kelley 2018). This creates a protective trade-off, where anemones with higher toxicity levels are potentially better able to protect their anemonefish through their venom and thus do not need to invest in increased tentacle length to provide shelter for the anemonefish. Less toxic anemone hosts may use a combination of a low toxicity venom and a longer tentacle length to provide better shelter for anemonefish, than low toxicity alone would. The corkscrew anemone (*Macrodactyla doreensis*) is a key example of this trade-off, having the second-lowest toxicity level but the longest tentacles of any host anemone (175 mm) (Fautin and Allen 1992).

A study by Nedosyko et al. (2014) found a relationship between host anemone haemolytic and neurotoxic toxicity and anemonefish preference (Figure 19.2b). Host anemones that fell into the mid-range toxicity had the highest number of anemonefish species as symbiotic partners. These results suggest toxicity may be an important factor in anemonefish host preference and that anemone toxicity and the fitness costs associated with withstanding toxin is an important aspect of anemonefish and anemone symbiosis. Forming an association with an anemone species that has low toxicity may provide a small fitness advantage to anemonefish by helping them to gain protection from predators; however, evolving resistance to an anemone species that has high toxicity may require large energetic costs, which could also have negative impacts on anemonefish fitness. Thus, anemone species with mid-range toxicity may provide the best protection

per energetic cost, and ultimately be preferred by more anemonefish species (Nedosyko et al. 2014). The anemone species *E. quadricolor*, which has a mid-range haemolytic and neurotoxic toxicity, forms associations with 16 of the 28 species of anemonefish, whereas the delicate anemone (*Heteractis malu*) with lowest toxicity and the pizza anemone (*Cryptodendrum adhaesivum*) with highest haemolytic and neurotoxic toxicity form associations with only a single anemonefish species (Fautin and Allen 1992). These association patterns provide support for the suggestion that toxicity plays a key role in the establishment and maintenance of symbiotic relationships between different anemone and anemonefish species (Nedosyko et al. 2014; Burke da Silva and Nedosyko 2016).

19.2.3 Interspecific Competition amongst Anemonefish Species

Interspecific competition for anemone host species can be an indicator of host quality or host preference by anemonefish. Fautin (1986) defined preferred hosts as those harbouring many anemonefish associates. Anemonefish are known to be aggressive, which is needed to maintain ownership of their anemones, as well as the social hierarchies within the anemone, to exclude or eliminate individuals from the anemone, or for larger more dominant species to obtain a preferred or occupied anemone (Burke da Silva and Nedosyko 2016; Buston 2003). Competitive exclusion between anemonefish species for preferred hosts is thought to be a key factor influencing which associations are found between anemone hosts and the different anemonefish species (Srinivasan 1999; Burke da Silva and Nedosyko 2016). The maroon clownfish (*Amphiprion biaculeatus*) is thought to be competitively dominant over all other anemonefish species (Srinivasan 1999) and is an anemone specialist only found in the anemone species *E. quadricolor*, the anemone in the mid-toxicity range. Similarly, other large anemonefish species such as *A. melanopus* are also generally found specializing in preferred mid-toxicity range host anemones, particularly when there is competition with other smaller anemonefish species on the same reef (Fautin 1986). As climate change continues to impact host quality and availability, it is likely that an increase in competitive exclusion by larger dominant anemonefish species may occur, leaving smaller anemonefish species vulnerable to predation (Saenz-Agudelo et al. 2011; Scott and Hoey 2017; Hoepner and Fobert 2022).

19.3 CURRENT HYPOTHESES AND OMICS APPLICATIONS TO UNCOVER THE MECHANISM BEHIND THE ANEMONE AND ANEMONEFISH SYMBIOSIS

Despite decades of research, the exact mechanism that enables anemonefish to live within the toxic environment of their host anemone has yet to be resolved. Several studies have found the mucus layer of anemonefish to be chemically different to that of other coral reef fish (Abdullah and Saad 2015; Balamurugan et al. 2015; Lubbock 1980), concluding that the anemonefish mucus layer may be the key to their protection. However, there are now new technologies available to help us investigate the mechanism(s) behind anemonefish resistance to anemone venom. Advancements in omics techniques such as genomics, transcriptomics, and proteomics will enable the exploration of this symbiotic relationship at a molecular level and may provide insights not previously attainable. In recent years, omics techniques have started to be used to tackle questions related to the symbiotic relationship between sea anemones and anemonefish, with a focus on metagenomics and genomics. Four main hypotheses have been proposed to explain how the anemonefish mucus layer can provide anemonefish with unique protection from the anemone venom. These hypotheses are summarized in Table 19.2, and each is discussed in the following with a focus on areas where omics technologies have currently been applied.

19.3.1 Hypothesis 1: Anemonefish Are Innately Protected from Anemone Venom

An early hypothesis was that anemonefish are born protected and therefore are innately immune to anemone venom (Elliot and Mariscal 1996; Miyagawa and Hidaka 1980). This research focused on the anemonefish species *A. clarkii*, which is able to form associations with all ten species of host anemones and can enter anemones with little or no acclimation time (Miyagawa and Hidaka 1980). Through multiple laboratory experiments, focusing on a number of different anemonefish species, it was noted that fish require an acclimation period in order to fully enter and remain within a host anemone (Balamurugan et al. 2015; Brooks and Mariscal 1984; Mebs 1994; Davenport and Norton 1958; Mariscal 1970a). This acclimation period can vary between anemonefish species, ranging from minutes to days before the fish can comfortably exist within the anemone (Balamurugan et al. 2015; pers obv; Miyagawa and Hidaka 1980). Anemonefish perform a range of specific behaviors – including touching anemone tentacles with their tail, biting the tentacle tips, and continuous fanning of tentacles with their pectoral fins – to acclimate and then enter the anemone (Balamurugan et al. 2015). Furthermore, anemonefish also lose their protection when isolated from their anemone host for more than 21 hours and are required to reacclimate (Mariscal 1970b). Overall, the experimental evidence clearly indicates that anemonefish require an acclimation period to form symbiosis with a host anemone. The acclimation period may activate the expression of novel genes that have been inherited from the one common anemonefish ancestor as the anemonefish species diversified (Litsios et al. 2012), allowing for the anemonefish to switch on their resistance to anemone venom.

TABLE 19.2
Previous Research into the Mechanism behind the Anemonefish Symbiosis with Anemones Fits into Four Main Hypotheses

	Hypothesis	Status	Reference	Methodology
1a	**Anemonefish are innately protected from anemone venom**	Rejected	Miyagawa and Hidaka 1980	Forced contact
			Elliot and Mariscal 1996	Forced contact
			Davenport and Norris 1958	Observation of behaviors
b	**Anemonefish gain protection through an acclimation period**	Supported	Mariscal 1970a, b	Observation of behaviors
			Brooks and Mariscal 1984	Acclimation time to surrogate anemones
			Mebs 1994	Ichthyotoxic activity
			Balamurugan et al. 2015	Observation of behaviors
2	**Anemonefish have a thicker mucus layer than other fish**	Insufficient evidence	Lubbock 1980	Nomarski optics (*A. clarkii*)
3	**Anemonefish mucus molecularly mimics the composition of anemone mucus**	Insufficient evidence	Schlichter 1976	Electrophoresis/radiolabelled mucus
			Elliot et al. 1994	Antibody assays
4	**Anemonefish mucus does not trigger firing of the anemone's nematocysts**	Insufficient evidence	Lubbok 1980	Nematocysts per cm^2
			Abdullah and Saad 2015	N-acetylneuraminic Acid Detection

19.3.2 Hypothesis 2: Anemonefish Have a Thicker Mucus Layer Than Other Fish

Another key hypothesis is that anemonefish have a thicker mucus layer than other coral reef fish species that cannot enter an anemone (Lubbock 1980). By having a thicker mucus layer, it is thought that the anemonefish are better able to withstand the sting of the anemone, or that the nematocysts – the firing cells that deliver the anemones' sting – are unable to penetrate the skin due to the mucus barrier. Lubbock (1980) showed that *A. clarkii* mucus was three to four times thicker than that of other coral reef fish species, but that there was no significant difference in mucus thickness when the anemonefish were associated with an anemone host (*S. haddoni* or *E. quadricolor*), compared to anemonefish separated from the anemone host for five months. As anemonefish are initially stung upon entering the anemone (Balamurugan et al. 2015; Brooks and Mariscal 1984; Mebs 1994; Davenport and Norton 1958; Mariscal 1970a) and the mucus thickness does not change with acclimation, it is unlikely that mucus thickness is the sole mechanism for anemonefish toxin resistance. Furthermore, only one of 28 species of anemonefish have been examined for mucus thickness; therefore it is currently unclear if all anemonefish species have thicker mucus layers than other coral reef fish.

19.3.3 Hypothesis 3: Anemonefish Mucus Molecularly Mimics the Composition of Anemone Mucus

One of the most popular hypotheses is that the anemonefish cover themselves in anemone mucus to molecularly disguise themselves and live undetected amongst the anemone's tentacles, referred to as molecular mimicry (Schlichter 1976; Elliot et al. 1994). It is proposed that the anemonefish cover their body in anemone mucus, thus inhibiting the firing of anemone nematocysts, via the same mechanism anemones use to recognize their own tentacles and prevent firing nematocysts at themselves. This is referred to as self-/non-self-recognition and anemone antigens (proteins or peptides) are thought to be involved in this self-recognition process (Elliot et al. 1994).

A study by (Elliot et al. 1994) found that anemonefish (*A. clarkii*) living within an anemone host (*H. crispa* and *S. haddoni*) had anemone antigens in their mucus, whereas these anemone antigens were not found in the mucus of *A. clarkii* that were separated from the anemone and only sharing an aquarium separated by a partition. Previously, Pantin (1942) found that anemones did not fire nematocysts at food sources covered in their own mucus, whereas it has been shown that anemones will fire nematocysts when presented with the mucus of another anemone species (Ertman and Davenport 1981). This evidence suggests that molecular mimicry likely plays a role in anemonefish protection from their host anemone.

There are three ways in which anemonefish may acquire anemone peptides or proteins in their mucus: (1) anemonefish may cover themselves with a coat of the anemone's mucus during brief contact with the anemone tentacles during the acclimation period; (2) some anemone surface antigens may be incorporated into the mucus coating of the anemonefish (Elliot et al. 1994); or (3) anemonefish produce their own proteins, molecularly similar to anemone proteins that they embed in their mucus layer when in contact with a host anemone. To date, there is no experimental evidence that discerns between these three possible mechanisms behind the molecular mimicry that allows the anemone to recognize the anemonefish as self, facilitating their symbiosis. However, metagenomics studies have found that

the microbiome of anemone and anemonefish mucus can converge during association (Pratte et al. 2018; Roux et al. 2019; Titus et al. 2020), demonstrating the potential for microbial proteins to be involved in molecular mimicry or the facilitation of the symbiotic relationship.

19.3.3.1 Omics Application: Metagenomics

Three recent studies have investigated the diversity of the mucus microbiome from anemonefish and their symbiotic sea anemone hosts (Pratte et al. 2018; Roux et al. 2019; Titus et al. 2020). All three studies found similar results, that the microbiomes of anemones and anemonefish were different when not in direct contact, and that direct microbial transfer or a shift in diversity occurs, making the microbiomes more similar when anemonefish and anemones are in contact. Specifically, Titus et al. (2020) found that the microbiomes of anemones (*C. adhaesivum*, *E. quadricolor*, *H. aurora*, *H. magnifica*, *and S. mertensii*) that were hosts to the same species of anemonefish (*A. nigripes* or *A. clarkii*) were more similar to each other than to that of anemones that were hosts to different species of anemonefish, or no fish at all. Pratte et al. (2018) also found that the microbiome of *A. clarkii* reverted back to a pre-association state after removal from the anemone *E. quadricolor.* The study by Roux et al. (2019) suggested that the convergence of microbiomes that occurred during anemone *H. magnifica* and the false clownfish (*A. ocellaris*) association could play a role in the establishment of their symbiosis. Bacteria in the mucus could allow for the transfer or processing of proteins and metabolites between the species, for example, to allow for the anemonefish to withstand the anemone's venom (Roux et al. 2019). This gives support to the hypothesis that anemonefish molecularly (or at least bacterially) mimic the anemone to disguise themselves amongst the anemone tentacles.

19.3.4 Hypothesis 4: Anemonefish Mucus Lacks the Trigger for Firing the Anemone's Nematocysts

The final key hypothesis is that the anemonefish mucus layer lacks the trigger for the anemone to fire nematocysts. Lubbock (1980) qualitatively observed the behavioral response of Haddon's anemone (*Stichodactyla haddoni*) to different mucus types on a glass rod (response categories: no response, poor response, strong response). *Amphiprion clarkii* mucus in contact with *S. haddoni* did not elicit a behavioral response (10/10) and *A. clarkii* mucus isolated from a host anemone also did not elicit a behavioral response (37/45), whereas mucus from closely related damselfishes elicited strong responses in all instances from *S. haddoni* – humbug damselfish (*Dascyllus aruanus*) (25/25), black-and-gold chromis (*Paraglyphidodon nigroris*) (5/5), and blue-green chromis (*Chromis caerulea*) (5/5). Lubbock (1980) also found that there was no difference between the number of nematocysts fired by the anemone at gelatine-covered coverslips in the presence or absence of anemonefish (10^4 capsules/mm^2) (Lubbock 1980), demonstrating that anemonefish presence does not impact the ability of the anemone to fire nematocysts at external stimuli. There is no study to date that has quantified the nematocyst firing response of a host anemone when presented with anemonefish mucus. However, the use of genomics has increased our understanding of the potential proteins utilized in the prevention of nematocyst discharge.

19.3.4.1 Omics Application: Genomics

A study by Marcionetti et al. (2019) identified the first candidate genes that may have evolved to grant anemonefish protection from anemone venom. This study utilized whole-genome assemblies from ten anemonefish species (*A. biaculeatus*, *A. ocellaris*, *A. perideraion*, *A. akallopisos*, *A. polymnus*, *A. sebae*, *A. melanopus*, *A. bicinctus*, *A. nigripes*, and *A. frenatus*) and applied molecular evolutionary analysis to uncover specific genes that were positively selected for during the evolution of symbiosis. Seventeen genes were identified as being under positive selection at the origin of anemonefish, which later switched to purifying selection. When advantageous traits evolve, they are usually positively selected for and then there is a switch to purifying selection to maintain these traits in descendants (Marcionetti et al. 2019).

Versican Core Protein was one of the genes identified and is particularly interesting due to its link to the anemone nematocyst firing mechanism. Nematocysts are highly specialized cells that distribute the anemone's venom by piercing the skin of predators or prey. The discharge of the nematocyst is controlled by chemosensory, mechanosensory, and endogenous pathways that respond to sensory stimulation (Anderson and Bouchard 2009). Anemones possess chemoreceptors for N-acetylneuraminic acid (Neu5Ac), a type of salic acid and a common carbohydrate side chain of glycoproteins found in fish mucus. Binding of the chemoreceptor to sugars in the mucus, specifically the acidic side chain of glycoproteins, triggers a multi-signal pathway that causes the nematocyst to fire (Anderson and Bouchard 2009; Ozacmak et al. 2001). Mucus from many coral reefs species has been shown to contain Neu5Ac; however, Neu5Ac has been found to be significantly lower in the mucus of *A. ocellaris* (Abdullah and Saad 2015). Abdullah and Saad (2015) found that *A. ocellaris* lacked Neu5Ac (1.6 mg/mL), in comparison to other non-symbiotic fishes such as the scissor-tailed sergeant (*Abudefduf sexfasciatus*) (50.4mg/mL) and moon wrasse (*Thalassoma lunare*) (71.9 mg/mL). Lubbock (1980) also showed that the mucus of *A. clarkii* was chemically different to other coral reef fish that are unable to enter host anemone species. The mucus of Clark's anemonefish (*A. clarkii*) mainly consisted of neutral glycoproteins, which could be produced by a lack of an acidic side chain on the N-acetylated sugars that is normally present in fish mucus glycoproteins (Abdullah and Saad 2015). Versican core protein found to be expressed in the epidermis of *A. ocellaris* is thought to potentially bind to N-acetylated sugars, masking their detection by anemone

chemoreceptors and thus failing to trigger nematocyst firing. Protein O-GlcNAse was also found to be positively selected for, and this protein has the potential to cleave the acidic side chain creating a neutral glycoprotein that does not stimulate the anemone chemoreceptors (i.e., does not trigger) (Marcionetti et al. 2019) providing support for the hypothesis that anemonefish mucus lacks the trigger for anemone nematocyst firing.

19.3.5 Hypotheses for Future Research

Of the hypotheses presented, only two hypotheses stand out as possible mechanistic explanations of anemonefish toxin resistance: firstly hypothesis (3), the anemonefish mucus molecularly mimics the composition of the anemone's mucus to inhibit nematocyst firing, and secondly hypothesis (4), the anemonefish mucus does not trigger the firing of anemone nematocysts. While these two concepts may seem similar, we are defining (1) inhibits firing: as mucus properties that bind to receptors preventing the nematocysts' firing (Elliot, Mariscal, and Roux 1994' Lubbock 1980) and (2) does not trigger: as mucus composition that lacks the trigger to stimulate the anemone's nematocyst firing. Ultimately, as the anemonefish need to perform acclimation behaviors in order to enter the anemone, there must be a change in the anemonefish's mucus at the molecular level. Moving forward in this chapter we will explore how lessons from venom research and toxin resistance in other species can be used as a model to better understand the anemone and anemonefish symbiosis and how omics have been used in these models and can inform future investigation into the potential mechanisms behind these hypotheses at the molecular level.

19.4 LESSONS FROM OTHER MODEL SYSTEMS

To uncover the mechanism(s) that anemonefish use to withstand the anemone's venom, we need to better understand the evolution of the anemone's venom itself. Anemone venom is a complex and diverse mixture of a variety of toxic components, including cytolysins (toxins that cause cell lysis), neurotoxins (toxins that damage or impair the nervous system), and phospholipases (enzymes which cause inflammation and pain) amongst many others (Anderluh and Macek 2002; Frazao et al. 2012; Madio et al. 2019). Furthermore, cnidarians (corals, anemones, and jellyfish) are the only organisms that do not have a centralized venom gland like other venomous organisms (e.g., snakes); instead, the venom is produced in tissues throughout their body via nematocytes and ectodermal gland cells (Madio et al. 2019). Nematocysts, which are found in the anemone tentacles, are highly specialized cells that venom is packaged into. Nematocysts consist of a capsule with an inverted tubule, which when triggered expels the tubule that disperses the venom by piercing the skin of predators or prey. The discharge of nematocysts is controlled by chemosensory, mechanosensory, and endogenous pathways that respond to external sensory stimulation (Anderson and Bouchard 2009). When predators or prey come into contact with the anemone, the anemone is able to chemically detect the response required and act accordingly. Ectodermal gland cells allow for the secretion of a larger volume of venomous mucus over the anemone, however, it is unclear if the venom composition of the mucus is the same or different to the venom packaged into the nematocysts (Madio et al. 2019), or if ectodermal gland cells are present in host anemones. While each component of the venom has a specific role, there are generally a few that contribute to the major lethality effect (Arbuckle et al. 2017). Potential symbiotic partners can benefit from this by attempting to evolve toxin resistance to the venom as a whole, rather than evolving resistance to each single component in the venom. This would enable partner species to selectively evolve resistance to the most lethal components or the most functionally similar elements, enabling multiple venom proteins to be treated as one for resistance purposes (Arbuckle et al. 2017).

While it is yet not clear how anemonefish are able to live within the toxic environment of anemones, we can look to other species and the mechanisms of toxin resistance utilized for new research avenues to explore in the anemone and anemonefish system. Resistance to toxins has evolved on multiple occasions across a wide variety of phyla, from mammals to fish and insects (Arbuckle et al. 2017). There are three main mechanisms that have been put forward to broadly explain the evolution of toxin resistance (Holding et al. 2016; Arbuckle, Rodriguez de la Vega, and Casewell 2017):

(1) Venom inhibitors: inhibitor proteins can inhibit the function of major toxic proteins found in venom through direct interaction, and are often members of large/old gene families. Venom inhibitors have been identified in at least 30 mammal species from six orders. Toxin-neutralizing serum factors, such as α1B-glycoprotein found in opossums and mongoose can neutralize snake venom metalloendopeptidases (SVMPs) and phospholipases (Holding et al. 2016; Voss and Jansa 2012). Venom inhibitors can also allow species such as snakes, for example, to be resistant to their own venom (Bastos et al. 2016). We know that anemones have self-recognition abilities which prevent the firing of nematocysts when their tentacles touch (Elliot et al. 1994). Proteins may have potentially evolved in anemonefish that can be used to disrupt or prevent the firing of nematocysts thus working as venom inhibitors. Versican core protein (Marcionetti et al. 2019), may be an example of this as it is thought to bind to N-acetylated sugars, masking their detection by anemone chemoreceptors.
(2) Target alteration: toxic proteins found in venom bind to a receptor protein in a prey species to elicit a toxic action. Thus, a small number of amino acid mutations in the receptor protein found in the prey can change it such that the toxin can no longer bind, while the receptor protein still maintains its

original physiological function. Target alterations are often members of small gene families, or even encoded by single genes. In cobras, binding resistance to alpha neurotoxins from snakes is caused by single amino acid substitutions that lead to glycosylation of the target protein that then prevents the binding of the toxin (Takacs et al. 2001). The evolution of the protein O-GlcNAse gene and the expression of this protein in the anemonefish epidermis potentially allow for the cleavage of the acidic side chain on glycoproteins in the anemonefish mucus (Marcionetti et al. 2019). This may be an example of target alteration as the nematocyst firing would no longer be triggered by the now neutral glycoproteins.

(3) Repurposed toxins: is the binding of venom proteins to an untargeted receptor, blocking the effects of the venom components that cause pain or other lethal actions. These can also occur with just a single amino acid replacement (Arbuckle et al. 2017). Grasshopper mice, who eat and are often stung by scorpions, are the only known example of a species that has evolved the use of repurposed toxins. This response results in the binding of the toxin to a downstream sodium channel rather than the targeted sodium channel, resulting in numbness in the mice rather than pain (Rowe et al. 2013). Anemonefish go through an acclimation process to associate with the anemone, however, what exactly happens at the molecular level during this acclimation is currently unknown. Repurposing of toxins to untargeted receptors could be activated during this process resulting in anemonefish no longer feeling the sting of their anemone host.

In general, in predator/prey relationships, prey species often evolve a biochemical defence or resistance to a predator's venom, triggering an increase in venom toxicity by the predator. Prey resistance will then also increase, resulting in a coevolutionary chemical arms race (Brodie III and Brodie Jr. 1999). In contrast, in a symbiotic relationship, where the aim is to maintain mutualistic benefit, a balance between maintaining venom toxicity level but still enabling a symbiotic partner to interact is important. However, the toxicity must remain at a level that can continue to benefit the toxic species. The anemone and anemonefish mutualistic relationship requires a balance of toxin resistance and venom strength, rather than an arms race of increasing toxin and resistance levels. Research by Nedosyko et al. (2014) supports this concept as they showed that host anemones with mid-range toxicity had the highest number of anemonefish associates, demonstrating that there is a trade-off between producing a venom that is too venomous or not venomous enough and being able to host anemonefish.

In recent years progress in deciphering the mechanisms behind the anemonefish and anemone symbiosis has stalled, despite technological development. Just as we use anemonefish as a model species for other research applications, study into this symbiotic relationship may benefit from the application of concepts and knowledge from venom transcriptomic and proteomic studies (Sunagar et al. 2016; Madio, Undheim, and King 2017) and the study of evolution of toxin resistance in other species, particularly of prey to snake venoms (Gibbs et al. 2020).

19.5 FUTURE USE OF OMICS

While researchers have begun to use omics to investigate a mechanistic explanation for anemone and anemonefish symbiosis, there is a wide array of omics techniques that could still be applied, particularly focusing on the fish mucus layer and how it acts to protect the anemonefish from the anemone venom. Fish mucus is comprised of a combination of proteins, lipids, and glycoproteins, all of which can be analyzed via omics to test the two leading hypotheses for the mechanism(s) behind this symbiosis: (1) ypothesis 3: anemonefish mucus molecularly mimics the anemone's mucus and (2) hypothesis 4: anemonefish mucus prevents the nematocysts firing. For example, given the importance of glycoproteins for triggering nematocysts response in anemones (hypothesis 3), analyzing the mucus layer of anemonefish using glycomics could provide insight into the side chain structure of the glycoproteins present in the anemonefish mucus and would provide support for the genomic research by Marcionetti et al. (2019). Additionally, utilizing proteomics, proteins from the anemone mucus can be identified and matched to proteins in the anemonefish mucus after association, which could determine if the anemonefish molecularly mimic anemone mucus (hypothesis 3). The merging of mucus microbiomes between anemones and anemonefish during association suggests that it is possible that mucus molecular composition will also show similarities during symbiosis. Further proteomics and transcriptomics studies of both fish and anemones under controlled experimental conditions or in the wild could be used to look to see if proteins targeted by venom components are altered and/or whether toxins are able to bind to decoy receptors as discussed earlier as mechanisms of resistance to snake venoms, to explore the possibility of anemonefish deploying these strategies.

Previously, the research into anemone and anemonefish symbiosis has focused solely on the anemonefish and how they adapt to live in the toxic environment of their anemone host. As this is a mutualistic relationship where both anemonefish and anemone gain fitness benefits from their association, the anemones' role in the formation of this symbiosis should also be explored. A combined transcriptomic and proteomic approach is becoming more popular when studying venom as it allows for a holistic view of venom composition (Madio et al. 2017). Using this approach, Madio et al. (2017) discovered 12 new families of venom proteins and peptides in Haddon's anemone (*S. haddoni*). Currently, research into anemone venoms focuses on novel toxin identification for drug discovery and medical applications, rather

than an ecological focus (Hoepner et al. 2019); however, applying widely used techniques that explore drug discovery to an ecological-based venom question could allow for the exploration of the mechanism behind the anemone and anemonefish symbiosis from a perspective not yet explored. For example, the bubble-tip anemone (*E. quadricolor*) is the most popular host of anemonefish (Nedosyko et al. 2014), yet research into its venom composition is very limited. A combined transcriptomic and proteomic approach to investigate the venom composition of *E. quadricolor* will allow for the comparison of the venom to other anemone hosts as well as non-host anemones and could identify potential unique features of the venom that lend itself to symbiosis with anemonefish. Analysis of venom before and after forming associations with anemonefish could also uncover any changes in the anemone venom or production that could enable or enhance the association with anemonefish. Omics is a promising field for investigating how anemonefish mucus layer interacts with anemone venom at the molecular level and closely interrogating hypotheses posed for future research.

19.6 CONCLUSION

Despite decades of research, we are still exploring and discovering exactly how the anemonefish can withstand the venomous sting of their anemone hosts and live harmoniously for mutual benefit. Of the numerous hypotheses explored, there are two main frontrunners that could explain the mechanisms of anemonefish resistance to anemone venom: (1) hypothesis 3: the anemonefish mucus molecularly mimics the composition of the anemone's mucus to inhibit nematocyst firing and (2) hypothesis 4: the anemonefish mucus does not trigger the firing of anemone nematocysts. These hypotheses do have areas of overlap and it may be a combination of both mechanisms that results in overall protection. The application of omics techniques, such as transcriptomics, proteomics, and metabolomics, as well as learnings from other model systems to this ecological question, may provide the molecular insight needed to finally uncover the secrets behind the anemone and anemonefish symbiosis.

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1.3 Thesis Aims and Hypothesises

Although there is considerable research focused on the symbiotic relationship between anemonefishes and host sea anemones, Chapter 1 highlights a range of knowledge gaps and avenues for future research. Here I use a range of omics techniques applied to an iconic symbiotic relationship to address these gaps. In particular, my objectives were to test: 1) the susceptibility of anemonefishes to ectoparasites, 2) the effect of anemonefish presence on host sea anemone nematocyte discharge, 3) anemonefishes' mucus metabolite adaptation when in association with a host sea anemone, 4) transcriptome and proteome composition of venom from the most popular host sea anemone and, 5). alteration to the transcriptome and proteome of host sea anemone venom when in association with a symbiotic partner. These experiments were carried out via a combination of qualitative field experiments and manipulative aquarium-based experiments, outlined below.

1.3.1 Chapter 2: Home is where the parasites are not: anemonefishes have low ectoparasite infestation rates, despite not visiting cleaning stations.

For my first data chapter, I aimed to assess a previous hypothesis that the symbiotic relationship between anemonefishes and their host sea anemones reduces their susceptibility to ectoparasites. To test this, I undertook field experiments in the Maldives and Malaysia quantifying anemonefishes cleaning station attendance, anemonefishes and cleaner fish interaction and anemonefish ectoparasite load. It was hypothesised that despite anemonefishes not visiting cleaning stations to have ectoparasites removed, that their ectoparasite load will be low due with living within a venomous host.

1.3.2 Chapter 3: Friend, food, or foe: sea anemones discharge fewer nematocytes at familiar anemonefish

In this chapter, I aimed to quantify the nematocyte response of host sea anemones to their symbiotic partner anemonefish. Using a before-after-control-impact (BACI) design, the host sea anemone *Entacmaea quadricolor* was presented with mucus collected from three different sources: 1) a symbiotic anemonefish (*Amphiprion percula*), 2) a non-symbiotic but closely related damselfish (*Chromis viridis*), and 3) a sea anemone food source (prawn), before and with anemonefish association. It was hypothesised that acclimated anemonefish mucus would trigger less nematocytes from a host sea anemone than other mucus stimuli presented.

1.3.3 Chapter 4: The delayed adaptation of anemonefish mucus to association with a host sea anemone.

The third data chapter aimed to analyse the lipid and glycan profile of anemonefish mucus in an attempt to pinpoint the mechanism used by anemonefish to withstand the venomous sting of their host sea anemone. Using a before-after-control-impact (BACI) design and MALDI TOF/TOF analysis, the lipid and glycoprotein composition of anemonefish mucus was compared before, with and after anemonefish association. It was hypothesised that the mucus of the anemonefish will become less glycosylated when in association with a sea anemone host, to reduce recognition when in contact with their sea anemone host's chemical receptors.

1.3.4 Chapter 5: Proteotranscriptomics of the most popular host sea anemone *Entacmaea quadricolor* reveals not all toxin genes expressed are recruited into its venom arsenal.

This chapter aimed to create a comprehensive profile of the tentacle transcriptome and venom proteome of the most popular host sea anemone *Entacmaea quadricolor*. In order to uncover the mechanism used by anemonefish to persist within the venomous environment of their host sea anemone it is important to better understand the composition of this venom. This comprehensive venom profile can then be used to compare how the venom profile of *E. quadricolor* which has a middle range toxicity compares to the venom profile of other host sea anemones as well as non-host sea anemones and how venom may influence the ability to form associations with anemonefish.

1.3.5 Chapter 6: Behind anemone lines: association with anemonefish alters tentacle transcripts and venom proteins of the most popular host sea anemone, *Entacmaea quadricolor*.

In my final chapter, I aimed to determine if the host sea anemone itself played a role in the establishment of their symbiotic relationship with anemonefish, as to date this is an unexplored side of this symbiotic relationship. Using a combined transcriptomic and proteomic approach, the toxin profile of *Entacmaea quadricolor* was compared with and without anemonefish association. It was hypothesised that host sea anemone venom would be altered when in association with anemonefish, through the downregulation of toxin proteins and peptides.

Chapter 2:

Initial concept **CMH** KBDS, experimental design **CMH** EKF, fieldwork **CMH** EKF CAA PB**,** lab work OYMS, data analysis **CMH**, first draft **CMH**, editing **CMH** EKF CAA KBDS

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2.1 Introduction

Coral reef ecosystems are biodiversity hotspots, supporting more species per square meter than any other ecosystem (Sikkel et al. 2018). Parasites make up a large part of this biodiversity; about 40% globally (Binning et al. 2013, Sikkel et al. 2014, Sikkel et al. 2018). Parasites elicit a fitness cost to their hosts (Anderson and May 1981), as the parasite utilizes energy that the host would otherwise use for growth, reproduction, and survival (Barber et al. 2000). Coral reef fish infected with internal and/or external parasites exhibit compromised behavioural, morphological, and physiological traits (Binning et al. 2013), causing negative affects at the individual and the population level. The presence of ectoparasites (external parasites) can result in reduced body condition, decreased growth, increased metabolic rate, challenges to swimming performance, decreased reproduction (Finley and Forrester 2003, Waldie et al. 2011, Binning et al. 2013), and negative physiological outcomes that ultimately result in high fitness costs increasing mortality and reduced population size (Demaire et al. 2020).

The high cost of parasitism has resulted in numerous adaptations that coral reef fish have evolved to either avoid or reduce ectoparasite load once affected (Binning et al. 2017). For example, Grutter et al. (2011) found that parrotfish can excrete a mucus cocoon that they sleep within to reduce ectoparasite susceptibility at night. Other species, such as lionfish, have toxic dorsal, pelvic and anal spines used to protect themselves from predators, which could also contribute to low ectoparasite levels (Sikkel et al. 2014). Alternatively, mutualistic interactions have evolved between infected coral reef fish and species that 'clean them of ectoparasites' (Sikkel et al. 2004), such as cleaner wrasse (ex *Labroides dimidiatus*), cleaner gobies (ex *Elacatinus evelynae*) and cleaner shrimp (ex *Ancylomenes pedersoni*). These

interactions involve the infected individuals known as 'clients', visiting a designated 'cleaning station', where the clients will display to the 'cleaner' by spreading their fins and gills to indicate readiness to be cleaned (Arnal et al. 2001, Soares et al. 2011). Cleaner fish will also approach clients to initiate cleaning leading to mutually beneficial outcomes (Trivers 1971). Cleaner species gain access to predictable food sources in the form of the ectoparasites, and the clients benefit through ectoparasite removal (Trivers 1971, Cheney and Cote 2001). For cleaner wrasse, gnathiid isopod larvae (Gnathiidae), is the main component of their diet. The interaction, although valuable, doesn't come without costs for the clients, such as daily time budget when visiting a cleaning station, and the risk of cheating, where the cleaner removes skin, scales or mucus from the client instead of ectoparasites (Bshary & Grutter 2002). However, as shown by Trivers (1971) and Bshary and Grutter (2002) the cost of ectoparasite infestation is very high for clients, thus the time and risks required to attend cleaning stations outweighs the costs of ectoparasite infestation. Grutter (1995) also found that the frequency and duration of cleaning interactions was positively correlated with mean ectoparasite load, with some client fish seeking cleaning services more than 100 times per day.

Ectoparasites are highly successful and are almost ubiquitous in coral reef ecosystems. One group of fishes, however, that rarely appear in ectoparasite literature on coral reefs, are the anemonefishes, *(Amphiprion spp)* in the Pomacentridae family. In fact, the only published occurrence of ectoparasites found for anemonefishes was a study dating back to 1966 in the Seychelles (Bowman and Mariscal 1968), where 5% of *Amphiprion akallopisos* anemonefish were found infested with the isopod *Renocila herterozota*. Bowman and Mariscal (1968) noted, however that they did not observe any visible ectoparasite infestation in anemonefishes populations across ten different countries, indicating that this finding was a

rare occurrence. A recent study by Zhokhov et al. (2020) found endoparasites (internal parasites) within the gills, stomach and intestines of multiple anemonefish species in Vietnam, but no ectoparasites were present. This anomaly appears to be specific to the anemonefishes, as the presence of ectoparasites is common in closely related Pomacentrids, with numerous studies reporting ectoparasite infestation across a variety of damselfish species (e.g. *Stegastes diencaeus*, *Pomacentrus chrysurus, P. amboinensis, Abudefduf sexfasciatus, Dascyllus aruanus*; (Sikkel et al. 2000, Cheney and Cote 2003a, b, Gunter and Adlard 2008, Sun et al. 2012, Sikkel et al. 2018) and frequent observations of this group engaging in ectoparasite removal with cleaner fish (Sikkel et al. 2004).

Anemonefishes differ from other coral reef fish species through their close symbiotic relationship with host sea anemones (Collingwood 1868, Fautin 1986). Sea anemone host species and anemonefishes benefit from their 12-million-year-old relationship (Titus et al. 2019), where anemonefishes gain protection from predators (Fautin 1991, Burke da Silva and Nedosyko 2016), a safe place to lay eggs (Saenz-Agudelo et al. 2011), and long-life spans (Buston and Garcia 2007, Sahm et al. 2019). Host sea anemones, on the other hand, benefit from increased nutrients and oxygenation, protection from predators, and improved recovery after bleaching events when living symbiotically (Godwin and Fautin 1992, Szczebak et al. 2013, Pryor et al. 2020, Schligler et al. 2022). Sea anemones are amongst the most venomous animals on the planet (Frazao et al. 2012, Hoepner et al. 2019), incorporating stinging cells (nematocytes) and secreting a venomous mucus layer over their body (Anderluh and Macek 2002, Norton 2009). This chemical defence acts to protect sea anemones from predators and disease and aids in the capture of prey (Mebs 2009, Beckmann and Ozbek 2012, Nedosyko et

al. 2014), and by extension, anemonefishes are thought to also be protected as a result of this relationship (Wang et al. 2019).

. The susceptibility of anemonefishes held in captivity without sea anemones, to infestation of both internal and external parasites in aquaria supports this claim. Anemonefishes in captivity are susceptible to common protozoan and fungal aquarium diseases (Mariscal 1970b, Wahab et al. 2009, Vorbach 2016). In aquaculture facilities anemonefishes are rarely kept in the presence of sea anemones, which require more specialized conditions for maintenance. A study by Wang et al. (2019) demonstrated a dose and time dependent mortality of the ectoparasite *Cryptocaryon irritans theronts* (a pathogen of white spot disease common in aquarium species) in response to different volumes of *A. clarkii* mucus. The mucus caused cell membrane rupture, removal of cilia and cell content leakage to the theronts within 10 mins of presentation (at 0.3 mg protein mL^{-1}). This study suggests the potential for anemonefishes' mucus to reduce ectoparasite attachment and thus raises the question of mucus chemical composition and its role in prevention of ectoparasite attachment. Crespigny (1869), hypothesized that anemonefishes in the wild are not susceptible to parasites due to their association with sea anemones, and proposed that the constant rubbing of anemonefish across the venomous sea anemone tentacles helps remove ectoparasites and thus negates the need for visiting cleaner fish stations. However, more than a century and half later, this hypothesis has not been tested or explored *in situ*, and the only evidence that anemonefishes may be protected from ectoparasites remains the absence of *Amphiprion spp.* in the ectoparasite literature.

This study aims to provide empirical evidence to support the hypothesis that anemonefishes are less susceptible than other coral reef associated fishes to ectoparasite infections and to

provide further arguments for the protective benefit of symbiosis with venomous host sea anemones. First, we postulated that if anemonefishes were susceptible to ectoparasites, they would frequent cleaner fish cleaning stations for ectoparasite removal, particularly as sea anemones are often used as a visual cue for client fish to mark the location of cleaning stations (Huebner and Chadwick 2012, Gilpin and Chadwick 2020), indicating that anemonefishes are often within a few meters of cleaning stations. We tested this by 1) conducting a systematic literature review to identify published instances of anemonefishes interactions with cleaner fish at cleaning stations; and 2) we conducted *in situ* observations of both active cleaning stations and of anemonefishes in their host sea anemone to record interactions between anemonefishes and cleaner wrasse. Secondly, we collected field samples from a population of the anemonefish *Amphiprion ocellaris*, to directly assess ectoparasite load at a location where ectoparasites were known to infect other coral reef fish species.

2.2 Methods

2.2.1 Systematic literature review

A systematic review of the literature was conducted via Web of Science on the 29th of October 2021, to assess the frequency of occurrence of anemonefishes at cleaning stations and records of cleaning interactions with anemonefish. The search query TS= (("cleaning station*" OR "cleaner wrasse*") AND (client* OR fish*)) returned 211 results. Papers were initially reviewed by title and abstract, 122 of which were deemed relevant and included in the study. An article was included if it provided new empirical data on 1) fish species visiting cleaning stations, or 2) fish interactions with cleaner wrasse. Cleaner wrasse studies including cognitive behaviour, laboratory experiments, mimicry or aquaculture were excluded. Each of the 122 papers were reviewed at the full text level, and the species of cleaner fish, location and species of client fish visiting cleaning stations were extracted. All studies were specifically

reviewed for any mention of the presence of anemonefishes and, if present, cleaner fish interaction.

2.2.2 Anemonefishes and cleaner wrasse interactions

2.2.2.1 Study site and species

To collect empirical field data on the frequency of anemonefishes interactions with cleaner wrasse and visitation to cleaning stations, we observed bluestreak cleaner wrasse (*Labroides dimidiatus*) cleaning stations at three locations within Lhaviyani Atoll, Maldives – Naifaru (N), Kuredu (K) and Veyvah (V), (Fig 2.1A). Two species of anemonefishes were present at these sites: A. *clarkii* and *A. nigripes,* that were utilising three different species of host sea anemone; *Heteractis magnifica*, *Heteractis malu* and *Stichodactyla gigantia*. Transects were performed at each site to establish the density of cleaner wrasse by counting the number of cleaner wrasse encountered within 2.5m either side of three 25m transects per site, and all cleaner wrasse encountered within the transect were identified as adult or juvenile based on size and colouration.

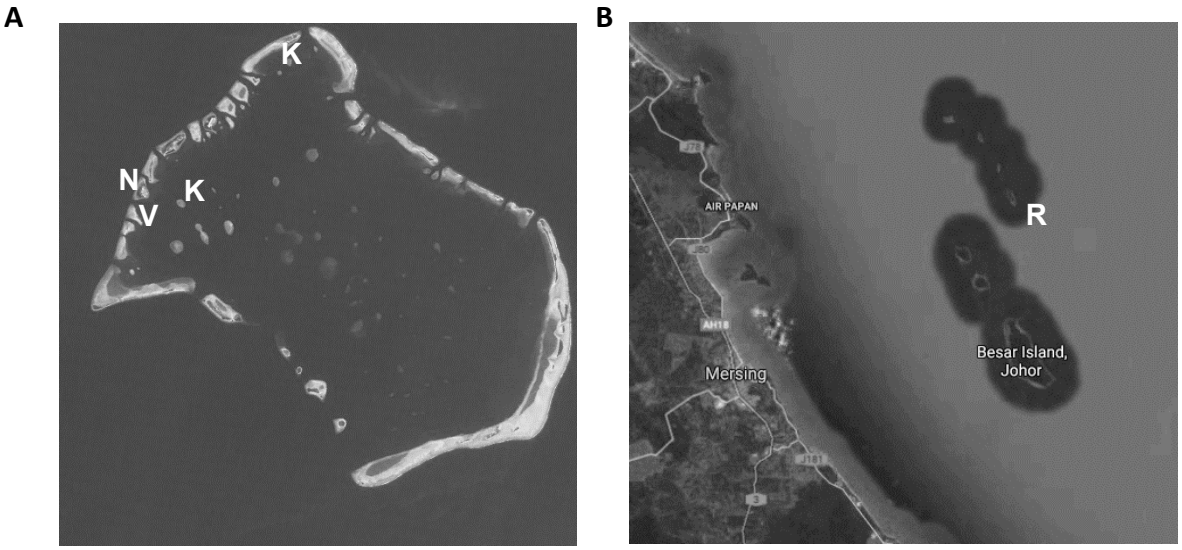


Figure 2.1: A) Cleaning station observation sites in the Maldives Lhaviyani Atoll, Maldives (N Naifaru, K Kuredu, V Veyvah). Observations at host anemone sites also occurred at Lhaviyani Atoll, Maldives (N Naifaru, KS Komandoo Sandbank, V Veyvah). B) Location in Malaysia where cleaning station sites were observed and ectoparasite load assessed, Rawa Island (R) Malaysia.

2.2.2.2 Anemonefishes visitations to cleaning stations

To assess the frequency of anemonefishes visits to cleaning stations, an observer on snorkel recorded the species of clients cleaned at a cleaning station for a period of 20-minutes. Nineteen cleaning stations across three locations in Lhaviyani Atoll, Maldives, were observed for a total of 6.33 hours: 4 hours at Naifaru (N) across 12 cleaning stations, 1 hour at Kuredu (K) across three cleaning stations, and 1.33 hours at Veyvah (V) across four cleaning stations (Fig. 2.1A). A cleaning interaction was defined as a client fish posing for a cleaner wrasse, allowing cleaning and the removal of ectoparasites to occur, and a cleaner wrasse engaging. A photograph was taken of each client fish that was cleaned, and these images were used to confirm identification of client species to the lowest taxonomic level possible. Host sea anemones were in the vicinity of all cleaning stations, with at least one sea anemone and anemonefish symbiont within 5m of each cleaning station observed.

2.2.2.3 Cleaner wrasse approaching anemonefishes

Anemonefishes are known to stay within close proximity (<2m) of their host sea anemone (Fautin 1991). For this reason, it is possible that anemonefishes are not often seen at cleaning stations because of the risks associated with the anemonefishes leaving the protection of their host sea anemone. To ensure cleaning interactions outside of cleaning stations were not missed, we undertook observations of anemonefish at their host sea anemone site to determine if cleaner wrasse visited the anemonefishes directly. On snorkel, 21 individual sea anemones inhabited by either one of two anemonefish species – A. *clarkii* or *A. nigripes* – were observed for 20-minutes (total = 7 hours of observations) at three different locations in the Maldives, (Naifaru (N), Veyvah (V) and Komandoo Sandbank (KS); Fig 1A). At each host sea anemone site, the size and species of host sea anemone and associated anemonefish species was recorded as was the number and sex of anemonefishes inhabiting the host sea

anemone. Cleaner wrasse-initiated interactions were defined as a cleaner wrasse approaching and attempting to engage an anemonefish in a similar manner that is used to engage and clean other fish species. Anemonefish initiated interactions were defined as an anemonefish approaching a cleaner wrasse to engage. Cleaning interactions were defined as an anemonefish posing for a cleaner wrasse allowing for cleaning and the removal of ectoparasites to occur coupled with a cleaner wrasse engaging. The density of cleaner wrasse within a 5m radius of a host sea anemone was also recorded. By swimming in circular transects, the number of cleaner wrasse at 0-1, 1-3 and 3-5m from the host sea anemone were counted and identified as adult or juvenile based on size and colouration.

2.2.3 Ectoparasite loads on anemonefish

2.2.3.1 Study site and species

We collected field samples from a population of *A. ocellaris* around Rawa Island, Malaysia, to directly assess ectoparasite load. The reef at Rawa Island (Fig 5.1B) is a shallow reef ledge with five species of anemonefishes: *A. ocellaris*, *A. frenatus*, *A. perideraion*, *A. clarkii* and *A. biaculeatus*, inhabiting four different host sea anemone species, *H. magnifica*, *Entacmaea quadricolor*, *H. crispa* and *S. gigantia*.

2.2.3.2 Establishing the presence of ectoparasites

As we were unable to capture any damselfish for ectoparasite infestation comparison, due to the complex coral matrix at Rawa Island, Malaysia, to confirm that ectoparasites were in fact present among coral reef fishes at our selected collection site, we repeated our cleaning station surveys to identify the species of client fish that visited the cleaning stations and interacted with the cleaner wrasse. We used cleaner wrasse-client interactions as an indicator of ectoparasite presence on the reef (Sikkel et al. 2004). Three cleaning stations on Rawa Island reef were observed for a total of ten hours. Methods were repeated following cleaning

station observations made in the Maldives. Host sea anemones were present at all cleaning station sites, with at least one host sea anemone hosting anemonefishes within 5m of each cleaning station observed.

2.2.3.3 Assessing ectoparasite load

Fifty-nine *A. ocellaris* were collected from Rawa Island reef on SCUBA and snorkel from a depth of 1-3m using handheld nets (approved by the Flinders University Animal Ethics Committee E479-19). Individual *A. ocellaris* fish were placed in clear plastic bags full of seawater and brought to the surface (methodology following Grutter (1995) and Sikkel et al. (2004)). Each fish was taken to a processing area 5m from shore and <50m from capture site and was transferred from the plastic bag into a 500mL container comprising seawater and Aqui-S solution (1 mL of 40x Aqui-S in 1L). Fish remained in the sedative solution for approximately 2-5 minutes until light sedation was achieved. Each fish was weighed, measured, and photographed, then placed in a 500mL formalin bath (1.25 mL of formalin in 1L fresh water) with aeration for 5 minutes. This process has been shown to successfully remove ectoparasites from coral reef fish species (Grutter 1995, Sikkel et al. 2004). Fish were placed into an aerated recovery bucket containing sea water for 15-20 minutes and observed until activity patterns indicated the fish were fully recovered from sedation. Each fish was returned to the host sea anemone from which they were collected. All liquid contents from the plastic bag, Aqui-S solution, and formalin solution were sieved through a 50 µm sieve to collect any invertebrates present. Samples were transferred into ethanol to preserve for identification. The samples were analysed at UCSI University KL using both stereo and compound (40x magnification) microscopes, and invertebrates (including any ectoparasites) were identified to the lowest taxonomic level possible.

2.2.4 Data Analysis

Statistical analyses were undertaken using the statistical software R (R Core Development Team 2013) using base R functions. A one-way ANOVA model was applied using the aov function and the TukeyHSD function was used to identify significance between groups. The correlation between ectoparasite number and the size and weight of anemonefish was calculated using the cor.test function from the package ggpubr. All statistical figures were created in R using the package “ggplot2” (Wickham 2016).

2.3 Results

2.3.1 Systematic literature review

One hundred and four publications from 1994 to 2021 were found that recorded cleaner fish interactions with client fish species. Thirty-one species of cleaner fish were found to interact with coral reef fish from 38 families. Not a single interaction was reported for any of the 31 cleaner fish species with any of the 28 species of anemonefish. Figure 2.2 provides an overview of the geographic range of anemonefishes compared to the location of studies examined. Of the 22 study locations, approximately 50% were from locations inside the distribution of anemonefishes.

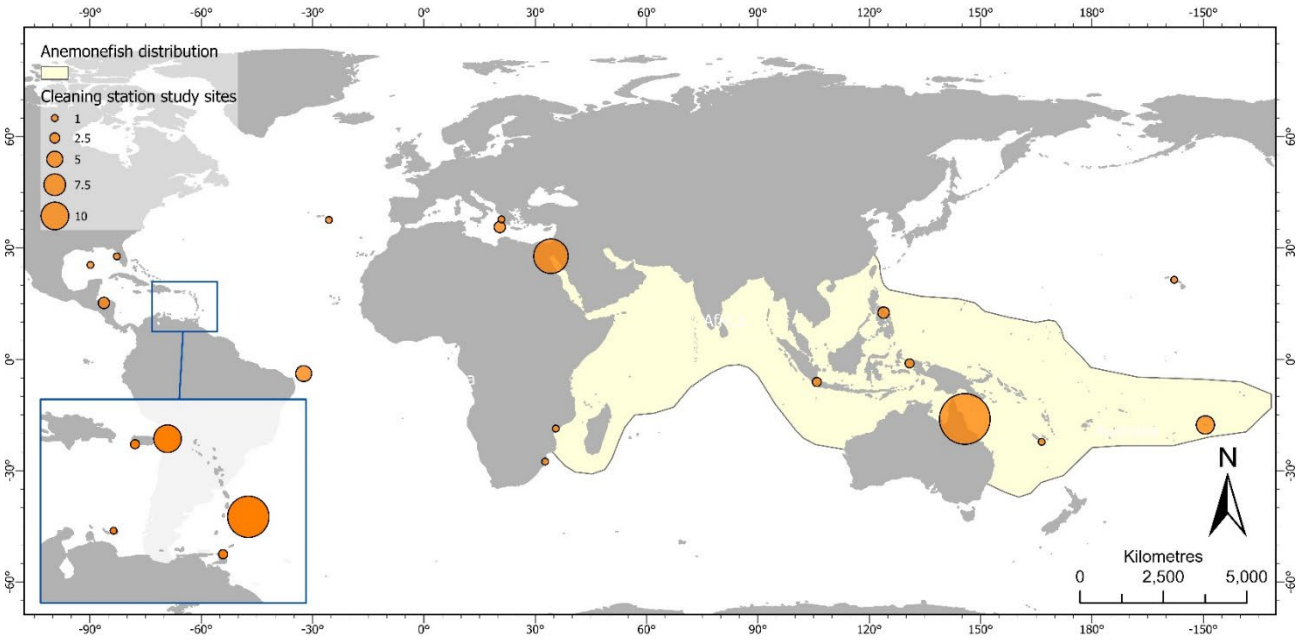


Figure 2.2: Overlap between study sites from the systematic literature review (orange dots) and anemonefishes distribution (yellow area).

2.3.2 Anemonefishes and cleaner wrasse interactions

2.3.2.1 Anemonefishes visitation to cleaning stations

The study sites in Lhaviyani Atoll, Maldives, had a mean density of 600 ± 80 cleaner wrasse, *L. dimidiatus,* per ha. In over 6 hours of observation, across three sites, we recorded 362 interactions between cleaner wrasse and client fish. Client fish were varied and identified to 14 different families and 47 species, with the highest number of species (17%) belonging to the Pomacentridae family (Fig 2.3). While many damselfish (Pomacentridae) were found attending and being cleaned at cleaning stations, not a single anemonefish visited or was cleaned at a cleaning station in the Lhaviyani Atoll, Maldives, despite all cleaning stations observed having at least one or more host sea anemones with anemonefishes within a 5m radius.

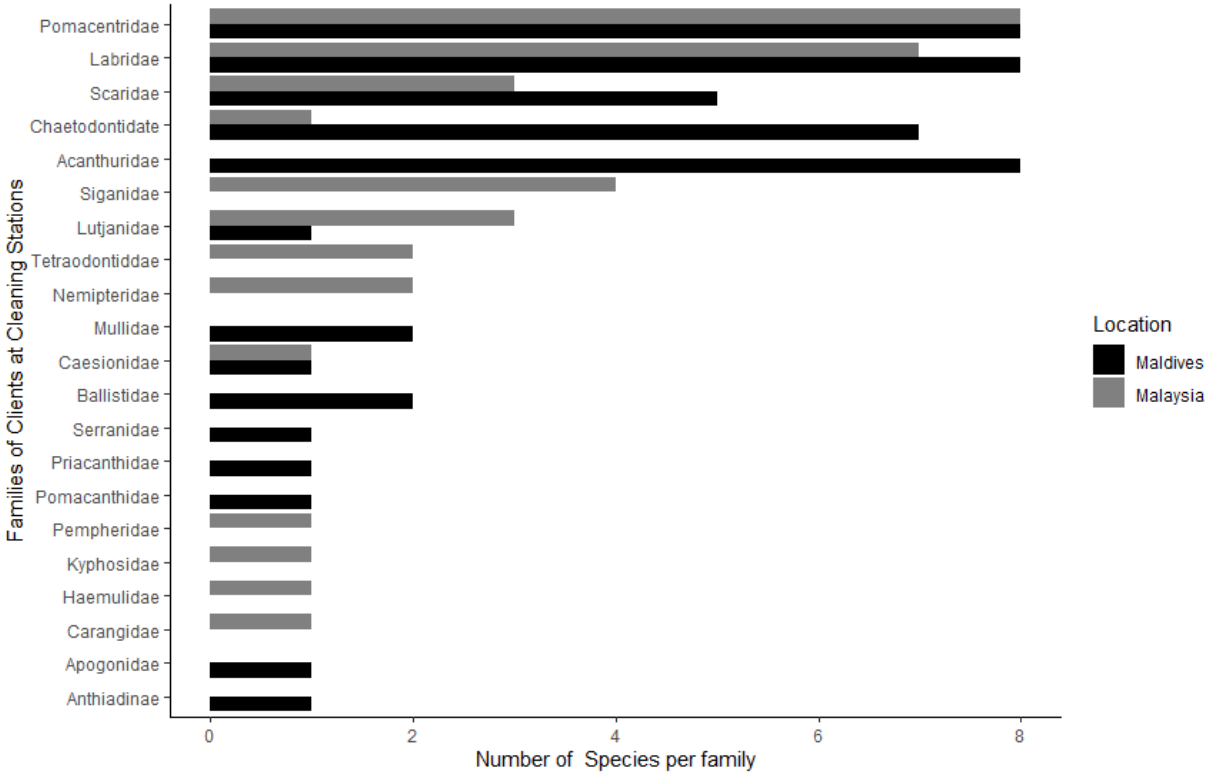


Figure 2.3: The number of fish species per family observed being cleaned by cleaner wrasse in Lhaviyani Atoll, Maldives, and Rawa Island, Malaysia, during cleaning station observations.

2.3.2.2 Cleaner wrasse approaching anemonefishes

The number of cleaner wrasse observed within a 5m radius of the target host sea anemone sites ranged from 1-11. No cleaning interactions were observed between cleaner wrasse and anemonefishes at 21 host sea anemone sites from over seven hours of observations (Table 2.1). Ten non-cleaning interactions (i.e. cleaning did not occur) were noted between anemonefish and cleaner wrasse, all of which were initiated by cleaner wrasse and all were immediately rejected by the anemonefishes (Fig 2.4A). All cleaner wrasse-anemonefish interactions observed were made towards the dominant female anemonefishes, never toward the breeding male or subordinate anemonefishes and all of the non-cleaning interactions were very short in duration (<2 seconds) and were initiated only by juvenile cleaner wrasse.

A) B)

Figure 2.4: A) Juvenile cleaner wrasse (*Labroides dimidiatus*) in close proximity to a host sea anemone (*Heteratis magnifica*) occasionally attempt to clean the dominant female anemonefish (*Amphiprion negripes*) with no success in Lh. Naifaru, Maldives. Image: Cassie Hoepner B) Cleaner wrasse (*Labroides dimidiatus*) cleans a client at cleaning station located next to host sea anemones hosting anemonefish at Rawa Island, Malaysia. Image: Emily Fobert.

Table 2.1: Summary of seven hours of observation at host sea anemone sites for cleaner wrasse and anemonefishes interactions.

Host anemone sites	**# of Anemonefishes observed**	**# of Cleaner Wrasse observed**	**Interactions** (cleaner wrasse initiated)	**Interactions** (anemonefishes initiated)	**Cleaning interactions**
21	124	104	10	0	0

2.3.3 Ectoparasite loads on anemonefish

2.3.3.1 Establishing the presence of ectoparasites

At the reef site Rawa Island, Malaysia, thirteen families and 35 species of clients were observed at cleaning stations, with a total of 1,295 interactions observed over a ten-hour period (Fig 3). This volume of interactions at cleaning stations confirms significant ectoparasite presence at Rawa Island, Malaysia (Sikkel et al. 2004). While 23% of species attending and being cleaned at cleaning stations were damselfish (Pomacentridae), not a single anemonefish species (Pomacentridae) visited or was cleaned at a cleaning station,

despite all cleaning stations having at least one host sea anemone and anemonefishes present within a 5m radius (Fig 2.4B).

2.3.3.2 Assessing ectoparasite load

Rawa Island, Malaysia had a mean *A. ocellaris* ectoparasite infestation rate of 0.6 (±1.1). The majority of *A. ocellaris* sampled (66.1%) had zero ectoparasites (x^2= 22.735, df =2, p < 1.62-14), with 34.9% of *A. ocellaris* infected with either one or more (range=1-7) ectoparasites from two species, *Gnathia sp.* (Praniza 3 life stage) and *Calanus sp.* (Figure 2.5a). Infected *A. ocellaris* had a mean ectoparasite infestation rate of 1.7 (±1.1), and only two female *A. ocellaris* had more than two ectoparasites; both were infected with both species of ectoparasites. Female *A. ocellaris* were significantly more likely to have ectoparasites than males or sub-adult juveniles (x^2= 5.051, df=2, p< 0.0296) (Fig 5.5b) with a weak correlation found between size and weight of *A. ocellaris* individuals and ectoparasite load (weight R=0.3, p< 0.01991, size R=0.27, p<0.03906) (Appendix S2.1).

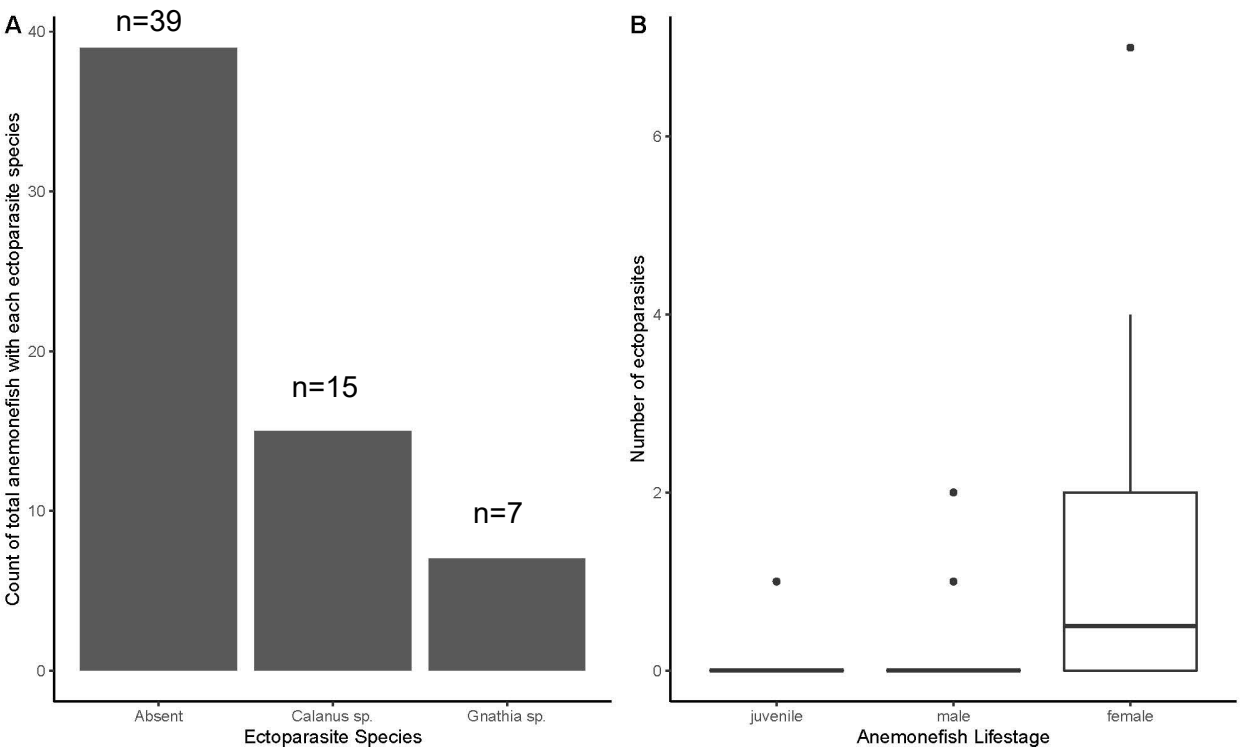


Figure 2.5: A) Count of total number of each ectoparasite species found. B) number of ectoparasites found on juvenile, male and female *A. ocellaris*.

2.4 Discussion

This study is the first to directly assess ectoparasite infestation of *A. ocellaris* anemonefish and to quantify anemonefishes interactions with cleaner wrasse. We found that most *A. ocellaris*, approximately two thirds, were not infested with any ectoparasites, and the remaining third had on average less than two ectoparasites. We also found zero cleaning interactions between cleaner wrasse and anemonefishes indicating that anemonefishes must rely on a different mechanism for ectoparasite removal.

A systematic review of the literature, in conjunction with 23 hours observing cleaning stations across multiple coral reef sites in two distinct coral reef habitats in Malaysia and the Maldives, revealed zero evidence of anemonefishes attending cleaner stations or being cleaned by cleaner fish. In the field we did not observe a single behaviour initiated by anemonefishes to be cleaned, and the only interactions observed *in situ* were initiated by juvenile cleaner

wrasse, and all were immediately rejected by the anemonefishes, suggesting that juvenile cleaner wrasse have not yet learned that anemonefishes are not valuable clients and in fact will chase and attempt to bite cleaner fish attempting to initiate contact (pers obs).

Studies investigating ectoparasite loads of closely related damselfish (e.g. *Microspathodon chrysurus, Pomacentrus amboinensis*) found that 60-100% of individuals were infested with a mean of 1-7 gnathiid isopods per individual (Sikkel et al. 2000, Sun et al. 2012), compared to only 34.9% of individuals infested with 1-2 ectoparasites per individual in this study. These findings indicate that Pomocentrids as a group are highly susceptible to ectoparasites, except for *A. ocellaris* in association with a sea anemone host. The venom of sea anemones likely plays a key role in the low ectoparasite load found, as other venomous fish species also appear to benefit from reduced ectoparasite loads. Lionfish (*Pterois volitans*) for example, have toxic dorsal, pelvic and anal spines (Kiriake and Shiomi 2011). A study by Sikkel et al. (2014) found that lionfish had fewer ectoparasites than ecologically similar species in both the lionfish's native and introduced range that do not possess a toxic defence (*Holocentrus rufus, H. plumierii H. flavolineatum, Lutjanus apodus, L. synagris, Epinephalus guttatus*). While lionfish did not have zero ectoparasites, the study found that they were not highly susceptible to the most common and generalist ectoparasites and they were rarely found at cleaning stations. A study by Munday et al. (2003), found that toxic skin secretions on coral gobies, (*Gobidon sp*.), did not reduce the rate of infestation by ectoparasites, but did influence the site of ectoparasite attachment. For example, coral gobies that produce a toxic mucus had ectoparasites only on their fins, whereas coral gobies without toxic mucus had ectoparasites attached throughout their body. While not eliminating ectoparasite infestation completely in

this case, toxin secretions prevented gnathiid attachment to the body, thereby reducing infestation load and minimizing associated costs of ectoparasites.

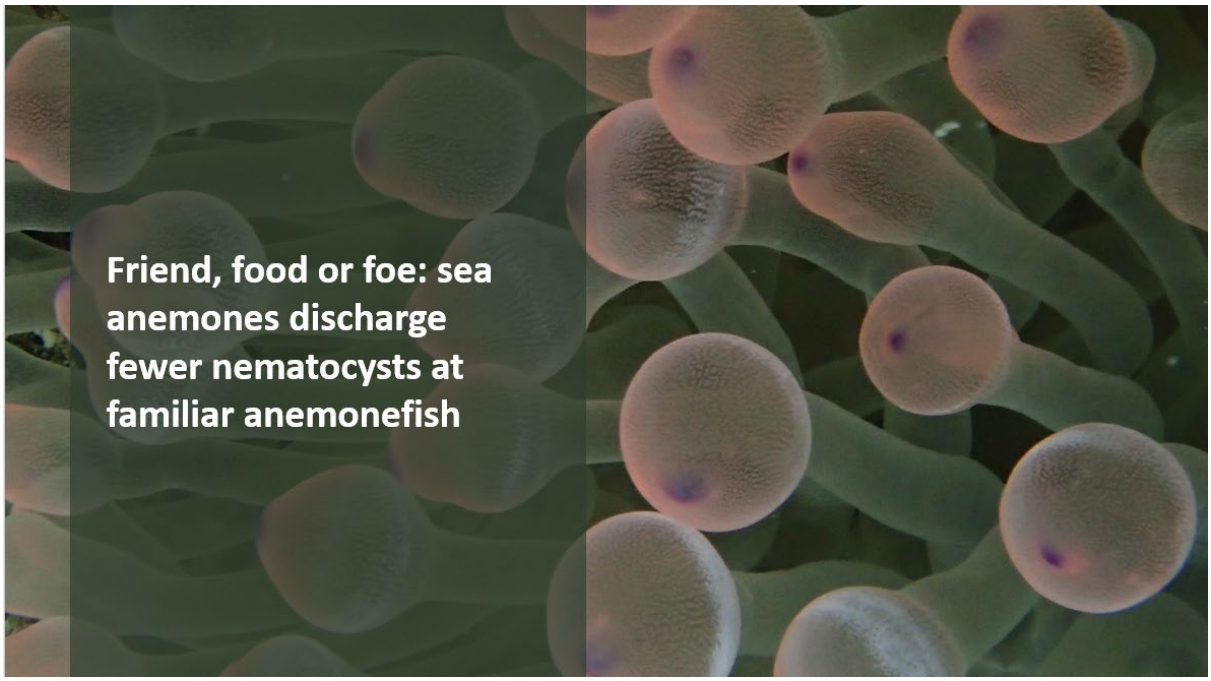
Twenty *A. ocellaris* individuals were found with ectoparasites in our study, of which 70% were large dominant females. The weak correlation between size and weight of *A. ocellaris* and ectoparasite susceptibility, may suggest a greater surface area for ectoparasite attachment (Appendix S2.2), or may indicate a behavioural difference in male and female anemonefishes. Dominant females spend more time outside of sea anemone hosts (e.g. Hoepner and Fobert 2022), which may increase their susceptibility to ectoparasites, or may affect the molecular makeup of their mucus layer. Further research is clearly needed to understand the mechanism(s) responsible for the low ectoparasite load found in *A. ocellaris* and if this is also seen in anemonefishes generally, as well as in smaller individuals who spend more time within their host sea anemone. We recommend that studies utilizing gnathiid cultures (e.g. Grutter et al. 2020) to explore the influence of sea anemone species on anemonefishes' ectoparasite infestation. Specifically, introducing anemonefishes that have been in association with a host sea anemone compared to anemonefishes not in association may provide insight into the probability of infestation and highlight the role of the host sea anemone symbiont in protecting anemonefishes from ectoparasites.

2.5 Conclusion

Despite the hypothesis posed over a century and a half ago, that host sea anemones provide anemonefishes with protection from ectoparasites, and the lack of anemonefishes recorded at cleaning stations in the literature, this is the first study to directly quantify ectoparasite load on an anemonefish species in the wild. This study illuminates that *A. ocellaris* anemonefish are in fact susceptible to ectoparasites, but the frequency of infection is lower

than that found in other coral reef fishes, particularly closely related damselfish (Sikkel et al. 2000, Sun et al. 2012). The finding that anemonefishes have ectoparasites but do not visit cleaning stations or allow cleaner wrasse to initiate cleaning even when in close proximity, suggests that anemonefishes rely on other mechanisms for ectoparasite removal. This study did not test mechanisms, but we propose that protection from ectoparasites is likely gained through the mutual and obligate relationship with their symbiotic and venomous partner, the sea anemone. Ectoparasite removal could be from either direct contact with the host sea anemone's venomous tentacles or through molecular changes to the mucus layer that makes anemonefishes less susceptible to ectoparasites, or potentially both.

Chapter 3:



Initial concept **CMH** KBDS EKF, experimental design **CMH** EKF, lab work **CMH** EKF CAA OP, data analysis **CMH** EKF, first draft **CMH**, editing **CMH** EKF CAA KBDS

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3.1 Introduction

Symbiotic relationships can take a variety of forms: mutualistic, parasitic, predatory, competitive, or commensal (Paracer and Ahmadjian 2000). Relationships between venomous and non-venomous species generally function as predator-prey, where the predator evolves more toxic venom, and in return, the prey evolves resistance to the toxins (Holding et al. 2016, Arbuckle et al. 2017). This type of relationship is well documented in the literature and is referred to as a chemical arms race (Brodie III and Brodie Jr. 1999). Most symbiotic relationships where venom is a factor are predatory, such as snakes and scorpions and their prey. The anemonefishes and host sea anemone symbiosis is a rare example of a mutualistic relationship where a venomous species and a non-venomous vertebrate species both benefit from the toxic relationship. It is more common to see a mutualistic relationship between a venomous species and a non-vertebrate, non-venomous species such as zooxanthellae or bacteria (Pontasch et al. 2013, Breusing et al. 2022). Venom is used by sea anemones for multiple purposes, ranging from defence to predation, and interspecific aggression (Anderluh and Macek 2002, Frazao et al. 2012). Yet, anemonefishes are able to enter host sea anemone after performing a range of behaviours (including: touching tentacles with the tail, biting the tentacles tips and continuous fanning of tentacles with their pectoral fins (Balamurugan et al. 2015)) to acclimate their mucus layer, which is thought to be the key to this symbiotic relationship (Mebs 2009). Despite this symbiotic relationship being well studied, there remains a lack of understanding regarding the mechanisms involved in anemonefishes resistance to sea anemone venom.

Sea anemone venom is a complex and diverse mixture of a variety of toxic components, including cytolysins (toxins that cause cell lysis), neurotoxins (toxins that damage or impair

the nervous system) and phospholipases (enzymes which cause inflammation and pain) amongst many others (Anderluh and Macek 2002, Frazao et al. 2012, Madio et al. 2019). Furthermore, cnidarians (corals, sea anemones and jellyfish) are the only organisms that do not have a centralized venom gland like other venomous organisms (e.g. snakes); instead the venom is produced in tissues throughout their body via cnidocytes and ectodermal gland cells (Madio et al. 2019). Compared to other venomous animals, relatively little is known about cnidarian venom production or delivery. Sea anemones utilise cells (cnidae), containing highly specialised and complex stinging organelles (cnidocytes) to distribute their venom and are deployed to capture prey and for defence purposes (Beckmann and Ozbek 2012). The class Anthozoa (which comprises all sea anemones) (Anderson and Bouchard 2009) has three types of cnidocytes: nematocysts, spirocysts, and ptychocysts. Nematocysts are the only cnidocyte that can inject venom, spirocysts are used to adhere to substrates and food, with ptychocysts involved in the construction of the tube that certain sea anemones dwell in. It is estimated that there are at least 30 different types of cnidarian nematocysts (David et al. 2008), with individual sea anemone species typically possessing between two to six different types (two of them exclusive to the class), found throughout various tissues (tentacle, filament, column, actinopharynx) (Krayesky et al. 2010, Jindrich 2011). The tentacles of sea anemones generally contain only spirocysts and two types of nematocysts: microbasic p-magistophore and basitrichous (Fautin 1981, Krayesky et al. 2010, Jindrich 2011).

The discharge of nematocytes are controlled by chemosensory, mechanosensory and neurological pathways that respond to external sensory stimulation (Anderson and Bouchard 2009). For example, when predators or prey make contact with a sea anemone's tentacles, it is able to chemically detect the stimulus and respond appropriately. Sea anemones possess

chemoreceptors that can detect N-acetylated sugars, which are chemically present in the mucus layer of fish (Abdullah and Saad 2015). When N-acetylated sugars, specifically the acidic side chain of the glycoprotein, binds to the sea anemone's chemoreceptor, a multiple signal pathway is triggered resulting in the firing of the sea anemone's nematocytes (Anderson and Bouchard 2009). It was previously thought that the firing of nematocytes was not controlled by the sea anemone, but Conklin and Mariscal (1976) showed that sea anemones can exhibit control over the firing of nematocytes and can adjust the number of nematocytes fired under experimental conditions. Previous studies have also demonstrated that sea anemones will reduce the number of nematocytes fired when satiated (Conklin and Mariscal 1976). It has been proposed that the production of venom and nematocytes can be energetically costly to produce (Fautin 2009, Sachkova et al. 2020, Kaposi et al. 2022), thus there could be fitness benefits, in the form of energy preservation, for sea anemones to exhibit some level of control over nematocyte firing.

While sea anemones utilise their nematocytes to capture prey and defend themselves from predators, their symbiotic partner, anemonefishes, are not only able to enter host sea anemones, but live unharmed amongst the host sea anemone's venomous tentacles for the duration of the anemonefishes' post-larval life (Mebs 1994, 2009). There are approximately 1170 sea anemone species (Rodríguez et al. 2022), yet only ten species from three families (*Thalassianthiade, Actinidae, Stichodactylidae*) form associations (as hosts) with one or more of the 28 species of anemonefishes (*Amphiprion*) (Fautin 1991, Burke da Silva and Nedosyko 2016, Tang et al. 2021). The host sea anemone provides a safe site for anemonefishes reproduction and protection from predation (Holbrook and Schmitt 2004), whereas the anemonefishes helps to increase the growth, reproduction, and defense of host sea

anemones, by providing nutrients from their faeces and increased oxygenation by swimming amongst the host sea anemones tentacles and chasing off potential sea anemone predators (Szczebak et al. 2013, Frisch et al. 2016, Schligler et al. 2022). As anemonefishes provide the benefit of predator defence, host sea anemones that form symbiotic relationships with anemonefishes could potentially reduce energy put toward chemical defence (nematocytes and venom production), thus a potential additional benefit to the relationship.

There has been much debate and research into the mechanisms that enable anemonefishes to survive in a venomous environment (Fautin 1991, Mebs 2009, Burke da Silva and Nedosyko 2016), but to date the exact mechanism remains unknown. One of the key hypotheses suggests that anemonefishes' mucus either inhibits or lacks the trigger for host sea anemone nematocytes to fire (Lubbock 1980, Abdullah and Saad 2015). Lubbock (1980) qualitatively observed the behavioural response of the host sea anemone, Haddon's anemone (*Stichodactyla haddoni*), when presented with mucus from different anemonefishes and damselfish species. When Clarke's anemonefish (*Amphiprion clarkii*) was both associated with Haddon's anemone, and not associated, the anemonefish's mucus (presented on a glass rod) did not elicit a strong behavioural response from the host sea anemone (0/10 and 8/45, respectively). In comparison, mucus from closely related damselfish (*Chromis caerulea, Dascyllus aruanus, Paraglyphidodon nigroris),* always elicited a strong behavioural result (35/35) from the sea anemone *S. haddoni*. In a more recent study, Abdullah and Saad (2015) chemically analysed anemonefish (*A. ocellaris*) mucus and found that it contained significantly lower concentrations of N-acetylated sugars (Neu5Ac) compared to the mucus of other coral reef fish (*Abudefduf sexfasciatus* and *Thalassoma lunare*). Neu5Ac is a salic acid side chain that has been shown to trigger the chemoreceptors that control sea anemone nematocyte

firing (Ozacmak et al. 2001, Abdullah and Saad 2015). However, no study to date has quantified host sea anemone nematocyte response to anemonefishes' mucus (as opposed to the qualitative work by Lubbock (1980)). Mucus protection from venom discharge is not unknown in the literature, with Greenwood et al. (2004) finding that mucus from nudibranchs inhibits nematocyst discharge from a number of prey sea anemone species but elicited nematocyst discharge from non-prey species. They also found that this protection does not enable nudibranchs to switch prey species and were unable to feed upon prey from a different geographic range.

In this study, we investigate the response of host sea anemone nematocytes to symbiotic and non-symbiotic coral reef fish. By building on the research by Lubbock (1980) and Abdullah and Saad (2015) we tested the hypothesis that anemonefishes' mucus lacks the trigger for stimulating sea anemone nematocyte firing, thus facilitating the anemonefishes and host sea anemone symbiosis. To test this hypothesis, we use a manipulative lab experiment to introduce mucus collected from 1) a symbiotic anemonefish species (*Amphiprion percula*), 2) a non-symbiotic damselfish species (*Chromis viridis*), and 3) a sea anemone food source (prawn), to trigger nematocyte firing by the sea anemone *E. quadricolor*. Following a before-after-control-impact (BACI) design, we test *E. quadricolor* nematocyte response to all three mucus sources during hosting and non-hosting periods to determine if anemonefish acclimation alters sea anemone nematocyte response to various stimuli. In a comprehensive review of host sea anemone taxonomy, Fautin (1981) found only three types of cnidocytes (spirocysts and two types of nematocysts: microbasic p-magistophore and basitrichous), to be in the various tissue types of all host sea anemones. Thus we also assessed which of these cnidocytes was deployed in response to different mucus stimuli.

3.2 Materials & Methods

3.2.1 Study species and experimental set-up

Ten Bubble tip anemones (*Entacmaea quadricolor*) (~5-7cm diameter) were obtained from an aquarium store in Adelaide South Australia (harvested from Western Australia), transported to the Animal House facility at Flinders University, and held in individual 30L tanks for a 2-week acclimation period (26.5 °C ± 0.7, salinity 37.5 ± 1.5, pH 7.91 ± 0.2). Sea anemones were fed a small piece of prawn every three to four days throughout the experimental period except in the 48 hours leading up to each sampling event. Each tank had a Fluval Aquatic Marine Nano 3.0 lights (2500 lux on a 12:12 L:D light cycle). This experiment was approved by the Flinders University Animal Ethics Committee (E470-18). Mucus from three different sources was used to stimulate sea anemone nematocytes firing: a symbiotic fish, *Amphiprion percula,* a non-symbiotic fish species, *Chromis viridis* - a common species of damselfish on coral reefs throughout the Indo-Pacific, and a common aquarium food source, frozen prawn. Ten pairs of *A. percula* (female and male) (n=20) obtained from Cairns Marine Aquarium fish distributor in Queensland were transported to Adelaide via air and housed in 30L tanks that contained a terracotta pot (a sea anemone surrogate) for a 2-week acclimation period prior to the experiment. Recirculating tanks (30L) holding the anemonefish pair were attached to a separate sump system to the anemones (27 °C ± 0.6, salinity 36.5 ± 1.5, pH 8.01 ± 0.2). Twelve Blue Green Chromis damselfish (*C. viridis*), also obtained from an aquarium store in Adelaide, South Australia, were transported to the Animal House facility at Flinders University and were held in an isolated 200L holding tank for a 2-week acclimation period, then separated into individual 30L recirculating tanks attached to a separate sump system to the anemonefish, for the experimental period. *A. percula* and *C. viridis* were fed twice daily with commercial pellets (Hikari Marine S) and mysid shrimp.

3.2.2 Sample Preparation

Glass microscope slides were used to collect a mucus sample from each of the three sources. Each glass slide was marked with a glass pen at 2.5cm from the bottom of the slide to create a 2.5 cm^2 area for sampling (following methodology used by Pantin 1942, Conklin and Mariscal 1976, Mauch 1998, Greenwood et al. 2004, Todaro and Watson 2012). *A. percula* and *C. viridis* were individually collected in a hand-net, and mucus was collected by gently scraping the sampling area of the glass slide along the side of the fish from operculum to tail; one slide was used for each side of the fish (approved by the Flinders University Animal Ethics Committee E470-18). Light scraping was used to avoid the collection of epidermal skin cells. For the prawn mucus samples, a small fleshy piece of defrosted prawn was rubbed onto the slide sampling area. Although the fish mucus is designed to exist in an aqueous environment and could be clearly seen clinging to the sampling area, the slides were left to dry to prevent mucus washing from the slide. Imagining from SEM clearly shows full mucus cover on all three slide types, providing a 2-dimensional surface to introduce to the anemone. We tested our method for introducing a slide to sea anemones by placing a blank slide (n=10) within the sea anemone tentacles. Zero nematocytes were triggered by the sea anemones in all ten replicates, consequently we did not include blank controls at each of the subsequent timepoints.

3.2.3 Sampling design

Nematocyte response was measured across a nine-week period (Fig 3.1). In week 1, each sea anemone (n=10) was randomly assigned to one of the three mucus treatment types (anemonefish, damselfish, prawn) and two slides with the corresponding mucus were introduced to the sea anemone to induce nematocyte firing (Conklin and Mariscal 1976, Hoepner et al. 2019). One slide was introduced to the sea anemone at a time, mucus side facing down into the tentacles for a period of five seconds. The second slide was introduced

to tentacles on the opposite side of the sea anemone to ensure for greater coverage. This process was repeated in weeks 2 and 3, alternating the treatment slide introduced so that each sea anemone was tested against all three treatments across the three weeks. Following the 'before' association period, a pair of *A. percula* (female and male) were introduced into each tank with a sea anemone and left for three weeks to establish symbiosis. All anemonefish entered the sea anemone within 48 hours after introduction and remained living within the tentacles for the 3-week period, illustrating full association. The three treatment types were repeated over a further three weeks, at week 7, 8 and 9 for the 'after' association period. In this study hereafter, references to the BACI design will be referred to as the 'non-hosting period' and 'hosting period' to reflect the nature of the symbiosis. The anemonefish mucus used to elicit nematocyte firing was collected only from the female (larger) anemonefish (n=10) living in the same tank with a sea anemone host, thus the sea anemones were responding to 'familiar' anemonefish mucus. The same anemonefish was also presented to the same sea anemone as the non-hosting period.

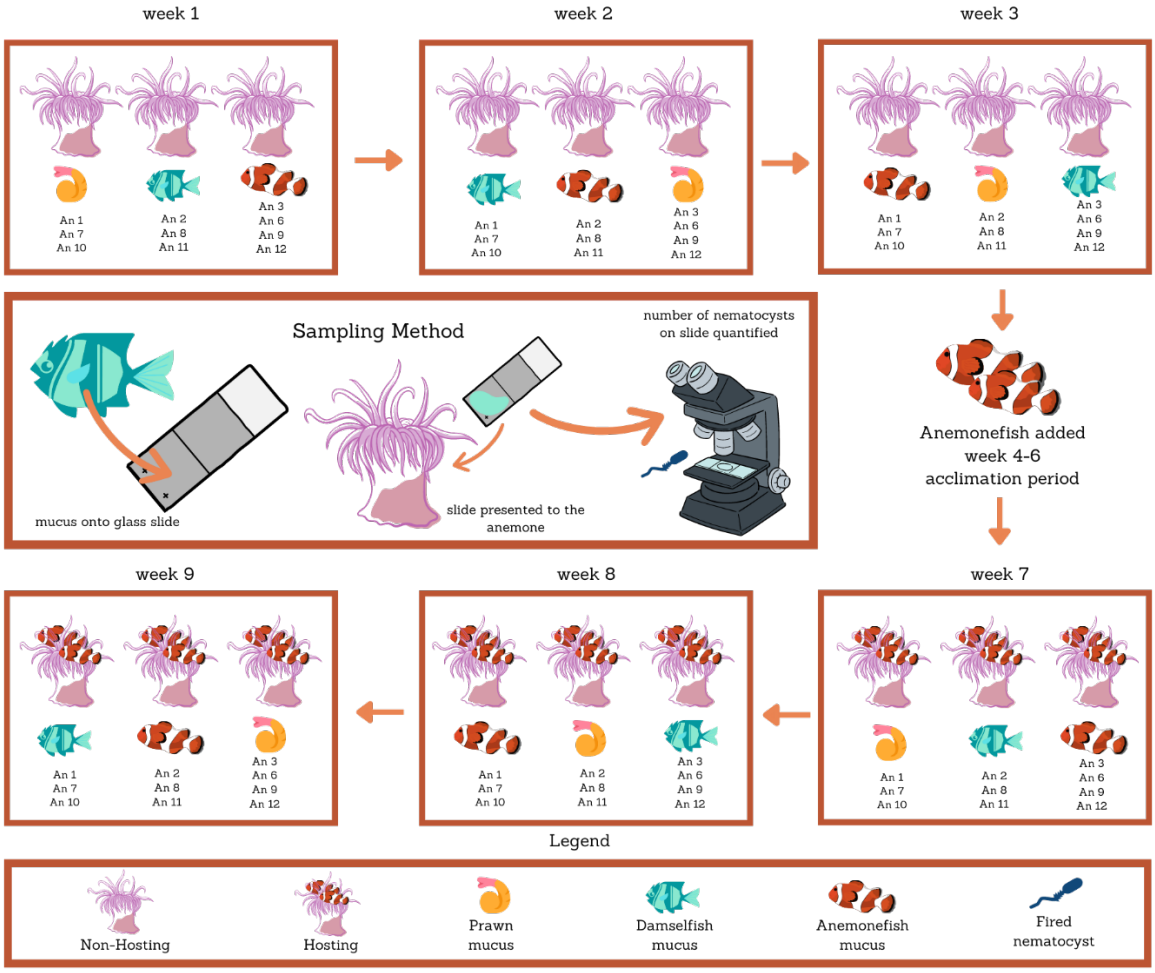

Figure 3.1: Experimental design showing mucus types introduced to *Entacmaea quadricolor* anemones (n=10) across the nine-week experimental period to assess nematocyte discharge during hosting and non-hosting periods. Each mucus type (anemonefish, damselfish, prawn) was presented to each anemone over the three-week non-hosting period. A pair of *Amphiprion percula* anemonefish were then added for a three week acclimation period. Mucus sampling was then repeated over three weeks for the hosting period, with 'familiar' anemonefish mucus introduced to the corresponding anemone.

3.2.4 Nematocyte Counts

After a slide was introduced to a sea anemone, it was stained using methylene blue (0.5g in 100mL H_2O) and allowed to air-dry. Fired nematocytes capsules were counted under a Zeiss compound microscope at 400x magnification. All nematocytes were counted within the marked 2.5 cm^2 area of the glass slide by moving at 1mm intervals back and forth across the slide. As two slides of each treatment were introduced to a sea anemone at each time interval,

the number of capsules fired was calculated as the mean number of nematocytes across the two slides. Sea anemone behaviour was observed during the slide introduction, and in instances where the sea anemone retracted its tentacles during sampling or did not open out on the sampling day (*A. percula* non-hositng =1, *C. viridis* non-hosting =1, Prawn hosting =2), we excluded the data as this did not represent a typical predator/defence response. In all instances, slides that were excluded based on sea anemone behaviour contained <10 nematocytes. When referring to nematocytes, we defined 'fired' as a nematocyte that has been fired from the tentacle of the sea anemone, 'unfired' as a nematocyte that was still intact within the sea anemone tentacle e.g. Fig 3.3A (used to ID the nematocyte type present), 'discharged' as a nematocyte that had been fired and that had expelled its internal contents (thread etc.) and 'undischarged' as a nematocyte that has been fired but has not expelled its internal contents.

3.2.5 Data Analysis

All statistical analyses were undertaken using the statistical software R using base R functions (R Core Development R Core Development Team 2013), except where otherwise stated. All statistical figures were created in R using the package "ggplot2" (Wickham 2016). To assess how nematocyte firing differed between treatments, we used a linear mixed effects (LME) model using the 'lmer' function in the R package 'lme4' (Bates et al. 2014) with mean capsules as the response variable, and mucus treatment (anemonefish, damselfish, prawn) and experimental period (before association, with association) included as fixed factors in the model. Additionally, individual sea anemone ID was included as a random factor to account for non-independence among repeat observations, and sampling week was included as a random factor to account for any effects of time on nematocyte firing. Significant differences between pairs of group means were determined using Bonferroni post hoc analysis.

3.2.6 Nematocyte Identification

Nematocyte type was identified using a combination of Light Microscopy and Scanning Electron Microscopy (SEM) images and the nematocyte nomenclature guide by Östman (2000) as a key. Light Microscopy was used to image unfired nematocytes from three *E. quadricolor* tentacles using a Leica DMi1 at 400x magnification. Scanning Electron Microscopy (SEM) was used to image fired nematocytes from the glass slides and was performed by Flinders Microscopy and Microanalysis using a FEI Inspect F50 Scanning Electron Microscope with an EDAX Octane Pro detector (electron beam energy 5kV and working distance 10mm). Slides were prepared using a dual target sputter coater to deposit a 20nm layer of gold on top of a 5nm layer of chromium. A 1mm2 area of each slide was analysed by stitching together 100 SEM images in GIMP 2.10.22. Three slides were imaged from the non-hosting period (anemonefish, prawn, and damselfish mucus), and one slide was imaged from the hosting period (anemonefish mucus). Only the anemonefish sample was imaged during the hosting period as this was the only species that showed significant change over the experimental periods. Ten discharged capsules from each slide (n=40) were measured using ImageJ (Abramoff and Magalhães 2003, Schneider et al. 2012).

3.3 Results

3.3.1 Quantitative analysis of nematocyte firing in response various stimuli.

The interaction term between mucus treatment and experimental period was significant (x^2= 7.96; df =2, p <0.0187) (Fig 3.2, Table 3.1), indicating that the number of nematocyte capsules fired by a sea anemone was dependent on both stimuli presented and whether the sea anemone was hosting an anemonefish. Specifically, the experimental period affect, showed that the mean number of capsules fired was significantly lower in response to familiar anemonefish mucus (mean diff =510.3, df =22.3, p<0.0185). Showing that while *A. percula*

was in association with the sea anemone host there was significantly less nematocytes fired compared to the non-hosting period, when sea anemones had not been in association with *A. percula*. There were no significant differences in nematocyte firing for the *C. viridis* and prawn mucus treatments between the hosting and non-hosting periods. Before sea anemone association (non-hosting), the two different fish species triggered relatively high numbers of nematocytes, with the symbiotic *A. percula* mucus triggering 820.5 ± 470.8 nematocytes and the non-symbiont, *C. vidris* mucus triggered 1151.7 ± 385.4 nematocytes. The food source – prawn mucus, triggered a lower number of nematocytes (275.3 ± 370.4). However, when the anemonefish were present within the sea anemone host (hosting), *A. percula* mucus triggered significantly fewer nematocytes (320.6 ± 253.9) whilst both the non-symbiont *C. vidris* and the prawn mucus triggered roughly the same number of nematocytes as during the before association period (897.5 ± 258.8 and 369.1 ± 402.1 respectively).

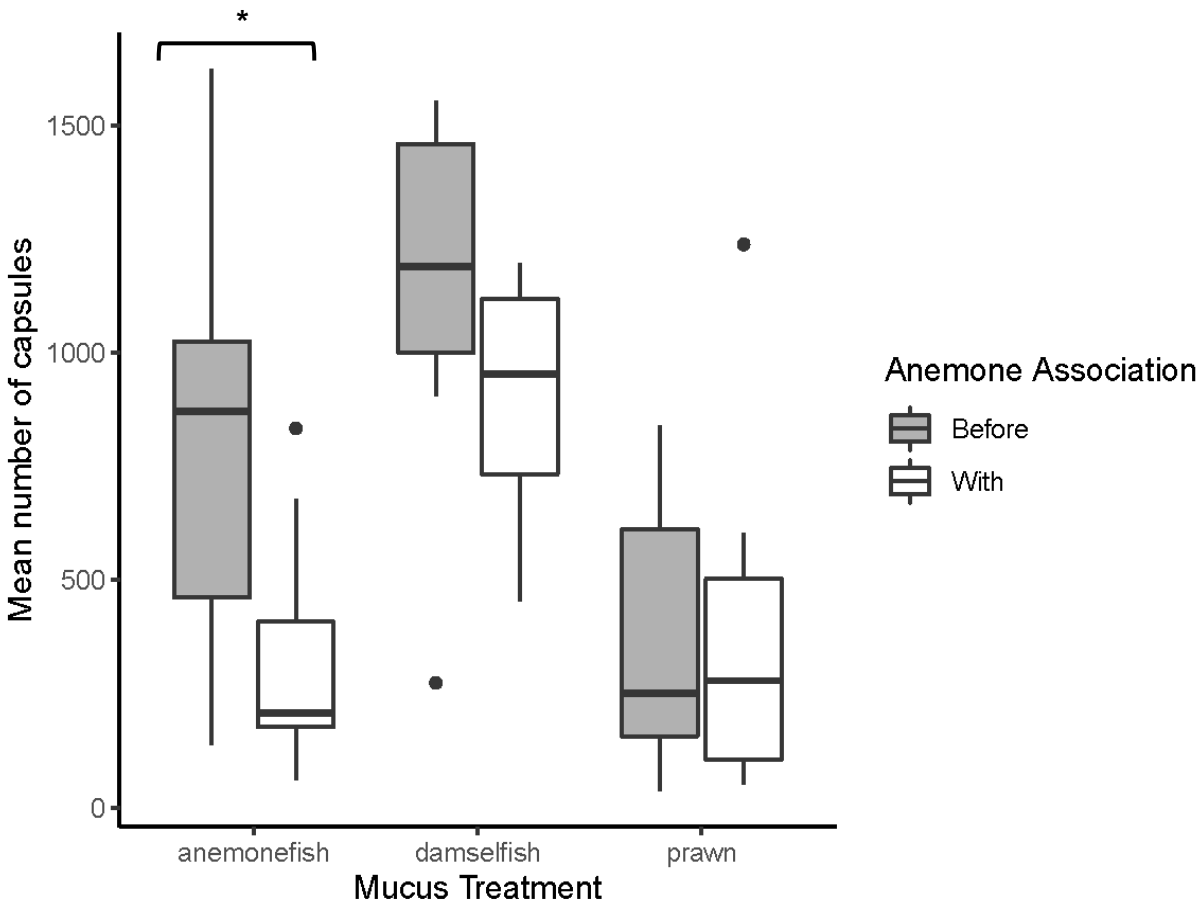

Figure 3.2: Mean number of nematocyte capsules fired by *Entacmaea quadricolor* during experimental periods (hosting and non-hosting), and mucus treatments (symbiotic *Amphiprion percula* (anemonefish), non-symbiotic *Chromis viridis* (damselfish), food source (prawn)). Boxplot shows the median and interquartile ranges. Significant differences between mean number of capsules are shown by an asterix (*). A single asterix (*) represents a significance level of $p<0.01$. Dots (·) represent outliers.

Table 3.1: Output from linear mixed effects model testing for differences in *Entacmaea quadricolor* nematocyte response between experimental periods (hosting and non-hosting), and mucus treatments (*A. percula*, *C. viridis*, prawn). P-values derived from Wald chi-square test with type II sums of squares. Significance is indicated in bold.

Random effects			
Term	*Variance*	*SD*	
Anemone ID	0	0	
Week (intercept)	18,625	136.5	
Residual	87,898	296.5	
Fixed effects			
Term	χ^2	*df*	*P*
Mucus treatment	**46.44**	**2**	**8.255e-11**
Experimental period	3.23	1	0.07248
Mucus treatment X experimental period	**7.96**	**2**	**0.01872**
Post Hoc – Bonferroni			
Term	*Mean diff*	*df*	*P*
***A. percula* non-hosting/hosting**	**510.3**	**22.3**	**0.0185**
***C. viridis* non-hosting/hosting**	233.3	22.3	0.2574
Prawn non-hosting/hosting	-26.9	23.5	0.8961
Non-hosting *A. percula* / *C. viridis*	-294.9	47.1	0.0950
Non-hosting *A. percula* /prawn	**461**	**47.1**	**0.0048**
Non-hosting *C. viridis* /prawn	**756**	**47.1**	**<0.0001**
Hosting *A. percula* /C. viridis	**-571.9**	**47.1**	**0.0004**
Hosting *A. percula* /prawn	-76.1	47.7	0.8545
Hosting *C. viridis* /prawn	**495.8**	**47.7**	**0.0030**

3.4.2 Morphological characterization of cnidocyte type in *Entacmaea quadricolor*More than 75,000 nematocyte capsules were observed and quantified in this experiment and only a single type of nematocyte was identified, regardless of mucus stimuli presented. Nematocyte identity was confirmed using a combination of Light Microscopy (Fig 4.3 A-B) and Scanning Electron Microscopy (Fig 3.3 C-F). All nematocytes were identified as basitrichous (Fautin 1981, England 1991, Östman 2000, Reft 2012) (Figure 3.3 A). The capsule (C) size was on average 21.6 µm long and 6.2 µm in width and capsules were found to be both undischarged (UC) and discharged (DC) (Figure 3.3 D), with only 18% of capsules observed under SEM undischarged (Table S3.1). During nematocyte firing, small apical flaps (AF) at the bottom of the capsule are opened (Figure 3.3 E). Apical flaps are unique to Anthozoa (Rodriguez et al. 2014) and are opened during the shaft and tubule exiting the capsule. The spines (S) at the base of the capsule (C) cover an area of about 15µm x 1 µm with each spine around 0.5-1.5 µm length (Figure 3.3 E). The tubule (T) is a long string-like tail from the capsule with spirals of small barbs 0.1-0.5µm along the length (TS) (Figure 3.3 C, F). The tubule distributes the venom into the tissue using its barbs to anchor in place (Godknecht and Tardent 1988). Spirocysts were not quantified across all slides due to poor contrast under light microscopy (Krayesky et al. 2010) however, they were able to be observed under SEM (Appendix S3.2). Scanning Electron microscopy highlighted that spirocysts were present on all slides; however, counts within a $1mm^2$ area showed that there were only two spirocysts on the anemonefish before association slide and one spirocysts on the anemonefish with association slide, compared to seven spirocysts identified on the prawn slide and 16 spirocysts on the damselfish slide (Appendix S3.1).

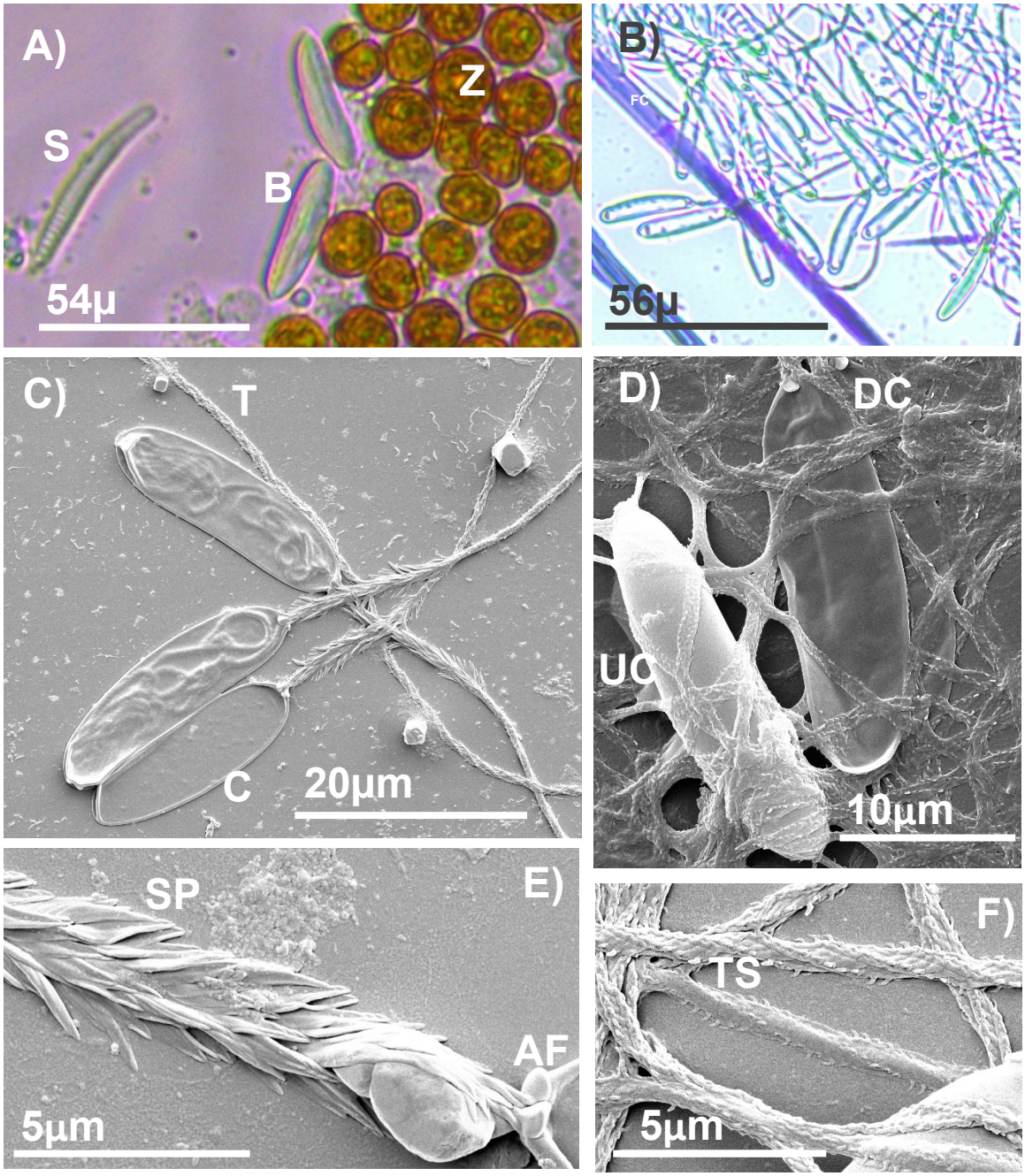

Figure 3.3: Light Microscopy and Scanning Electron Microscopy of basitrichous nematocytes from Entacmaea quadricolor. Image A) shows Light Microscopy of unfired nematocytes (basitrichous), spirocysts and zooxanthellae inside the tentacle. Image B) shows Light Microscopy of fired nematocytes (basitrichous) at 400x magnification. Image (C) shows SEM of fired basitrichous. Image D) shows SEM of fired capsules discharged and undischarged. Image E) shows SEM of the apical flaps which are open as the capsule is fired and spines at the base of the capsule. Image F) shows SEM of tubule spines which aid in anchoring to tissue.

3.4 Discussion

For decades, it has been hypothesized that anemonefishes are able to live within the stinging tentacles of host sea anemone species because the chemical composition of their mucus layer inhibits the firing of sea anemone nematocytes (Lubbock 1980, Abdullah and Saad 2015). This study provides the first empirical evidence that anemonefish not acclimated to their host sea anemone are very susceptible to nematocyte firing, whereas mucus from an acclimated anemonefish does not illicit the same response, but rather a significantly reduced number of nematocytes fired. We also found that the anemone species *E. quadricolor* fired only a single type of nematocyst – basitrichous (Oliveira et al. 2012) through activation of the tentacles regardless of different stimuli presented. We also identified a small number of fired spirocysts under SEM that were also observed unfired in the tentacles of *E. quadricolor* by Fautin (1981) and Reft (2012).

Prior to acclimation (non-hosting) the sea anemone nematocyte response to the anemonefish stimuli was similar to the response of a close non-symbiotic relative of anemonefish, the damselfish (*C. viridis*), where mucus from both elicited a high level of nematocyte firing. However, after a three-week acclimation period with a host sea anemone, where the anemonefish performs a range of behaviours that enable them to enter, become familiar to, and live within the host sea anemone (Balamurugan et al. 2015), the number of nematocytes fired at the familiar anemonefish mucus decreased significantly, while nematocyte firing remained high in response to the mucus from the non-symbiotic fish species *C. viridis*. This finding disputes results from Lubbock (1980), who found no significant difference between the observed behavioural (qualitative) response of the host sea anemone *S. haddoni* when presented with acclimated (familiar) and unacclimated mucus from the anemonefish species

A. clarkii. Our study indicates that qualitative observation cannot accurately predict nematocyte response to stimuli and that quantitative methods are required.

The reduction in nematocyte firing suggests that mucus of familiar associated anemonefish does not illicit a typical nematocyte firing response from a host sea anemone, such as that directed at non-symbiotic associated fish species, e.g., the damselfish. After the acclimation period, the host sea anemone no longer recognizes the anemonefish mucus as that of a prey or predator species, and consequently it significantly reduces its nematocyte response. The change in nematocyte firing may be due to a change in the biochemical composition of the anemonefish mucus when anemonefish are in association with a host sea anemone, masking recognition by the sea anemone as suggested by Elliot et al. (1994). Lubbock (1980) found that the mucus of the anemonefish *A. clarkii* was chemically different to *D. aruanus*, a closely related damselfish that is unable to enter the sea anemone, without getting stung. Interestingly, Abdullah and Saad (2015) found that *A. clarkii* had significantly lower levels of N-acetylneuraminic acid (Neu5Ac) in its mucus compared to other coral reef fish species (*Abudefduf sexfasciatus*, and *Thalassoma lunare*). Neu5Ac is the most common type of salic acid, a carbohydrate side chain of glycoproteins found in fish mucus that is a known trigger for sea anemone nematocyte firing. Todaro and Watson (2012) found that activating chemoreceptors for N-acetylated sugars resulted in nematocyte discharge at maximal forces, so all prey items, including small prey items that contained this chemical stimulus, will promote firing. On the other hand, large animals that make accidental contact with an anemone, but do not contain N-acetylated sugars, do not elicit maximal discharge of nematocytes (Todaro and Watson 2012). The removal of a Neu5Ac side chain, as seen in *A. clarkii* mucus (Abdullah and Saad 2015), is likely involved in protecting anemonefish from sea

anemone venom, as neutral glycoproteins are thought not to trigger sea anemone nematocyte firing. Marcionetti et al. (2019) used genomic analysis to identify key proteins in the anemonefishes' epidermis that were positively selected for during anemonefish evolution. Versican core protein was positively selected for and is thought to bind to N-acetylated sugars potentially masking detection of anemonefish by sea anemone chemoreceptors. The gene for the protein, O-GlcNAse, was also found to be positively selected for and has the potential to cleave the sialic acid side chain creating neutral glycoproteins (as seen by Abdullah and Saad (2015)). The evolution of these proteins in anemonefishes may have enhanced the establishment of the symbiosis between host sea anemones and anemonefishes and support the reduction of nematocytes as seen in this study.

Anemonefish presence, had no impact on host sea anemone nematocyte response to other stimuli presented, indicating that host sea anemones do not lose their ability to fire nematocytes when hosting anemonefish. These results are also supported by Lubbock (1980) who found no difference between the number of nematocytes fired by the host sea anemone at gelatine covered coverslips in the presence or absence of anemonefish (10^4 capsules/mm^2) (Lubbock 1980). Prawn mucus triggered a lower-level nematocyte response throughout this experiment, regardless of anemonefish presence or absence. This relatively low number of nematocytes was not predicted in response to a prey item but could potentially be explained by the fact that the host sea anemones were fed frozen prawn regularly while in captivity, prior to and during the experiment, and thus they may have recognized that a lower energy investment was required to capture this non-living food source. Another possibility may be the removal of the prawn shell prior to purchase, thus a potential chemical cue that triggers

host sea anemone nematocyte may be lacking. Mucus from *C. viridis*, however, triggered higsth numbers of nematocyte firing throughout the experiment, regardless of anemonefish presence or absence. As a non-symbiotic fish species, *C. viridis* are recognized by sea anemones as a predation threat, triggering higher numbers of nematocytes to be discharged.

3.5 Conclusion

This study has provided a new piece in the puzzle of sea anemone and anemonefishes symbiosis. Acclimated and familiar anemonefish trigger significantly fewer nematocytes than unacclimated anemonefish, indicating that a change occurs in the anemonefish mucus layer during the acclimation process. However, as there are still some nematocytes fired at anemonefish mucus while in association, there is likely additional mechanisms used by the anemonefish to gain and maintain resistance to sea anemone venom. As anemonefish and sea anemones have lived in association for the last 12 million years (Litsios et al. 2012), a realistic timeline exists for coevolutionary responses to both venom production and development of resistance mechanisms. Triggering fewer nematocytes maybe an important adaptation that has enabled anemonefishes to enter a host sea anemone, providing opportunity to activate other additional protection mechanisms. Future studies should explore the molecular changes in the anemonefishes' mucus layer during the acclimation process, particularly with the role that glycoproteins play in the establishment and maintenance of the symbiotic relationship. Finally, assessing the nematocyte response of other host sea anemone species to other anemonefish species mucus may help determine if the reduction in nematocytes fired occurs across all 28 species of anemonefishes when they are acclimated to their sea anemone host.

Chapter 4:

Initial concept **CMH** KBDS CAA EKF, experimental design **CMH** EKF, lab work **CMH** EKF CAA DR, data analysis **CMH** EKF, first draft **CMH**, editing **CMH** EKF CAA KBDS DR

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4.1 Introduction

The main surface of exchange between a fish and its surrounding environment is via their external mucus layer (Reverter et al. 2018). A mucus layer can serve many functions including; osmoregulation, intra/inter species communication, protection from toxins, and disease resistance (Shephard 1994, Reverter et al. 2018). Mucus is composed of metabolites, amino acids and immune molecules, and vary in composition with endogenous and exogenous factors (Reverter et al. 2018). Metabolites, which include glycoproteins and lipids are the end products of cellular regulatory processes and can indicate response of biological systems to genetic or environmental changes (Fiehn 2002). Lipids help maintain the internal structure of mucus and contribute to mucus viscosity (Lewis 1970), but there is a lack of information on the role of fish mucus lipids on cell signalling and inflammation response (Reverter et al. 2017). Glycoproteins, such as O-glycosylated proteins (mucins), make up a large proportion of fish mucus and are involved in a variety of functions including immune response, anti-microbial functions and alarm signalling (Reverter et al. 2018). Glycans are the sugar side chains attached to the protein and contribute to the barrier function of mucins and can protect glycoproteins from cleavage by proteases (Varki 2016).

Metabolomics is the high throughput study of metabolites provides a powerful tool to analyse chemical diversity and changes in the natural environment (Kusano et al. 2015). Metabolomics can distinguish individual signals which can be used for many purposes, from disease biomarkers to indicators of short-term environmental changes in ecology or even in chemotaxonomy studies (Wolfender et al. 2009). The metabolites of fishes are largely unexplored with only four studies (Ekman et al. 2015, Reverter et al. 2017, Ivanova et al. 2018, Heim et al. 2023) and one review (Reverter et al. 2018) currently published, with much

uncertainty remaining, including assigning identification and functionality to metabolites detected. Ekman et al. (2015), used LC-MS/MS for the first time to investigate skin mucus metabolites in Fathead Minnow; detecting 204 distinct metabolites and 72 metabolites that with significant association with sex. Reverter et al. (2017), found that phylogeny and butterflyfish species influenced the gill mucus metabolome of eight butterflyfish species but that geographic site and reef habitat did not. Ivanova et al. (2018), used metabolic tecniques to develop a workflow to test fish skin mucus in farmed salmon to develop and improve upon non-invasive sampling techniques, finiding differences in variance level between absorbion and scraping methods. Finally, Heim et al. (2023) compared the mucus metabolomes of anemonefishes and damselfishes, identifing differences in lipid composition that may allow for hosting with sea anemones.

Since it was first discovered in 1868, the symbiosis between host sea anemones and anemonefishes has fascinated scientists and the public alike (Collingwood 1868). Twenty-eight species of anemonefishes associate with only ten different species of host sea anemones (Fautin and Allen 1992, Madio et al. 2019), out of the approximately 1170 sea anemone species (Rodríguez et al. 2022). Sea anemones use their venom for a variety of functions including defence and the acquisition of prey. Sea anemones are able to distribute their venom via two different methods; (1) firing of nematocysts, where the venom is encased inside a capsule allowing for penetration and (2) secretion of a venomous mucus over the external surface of their body (Madio et al. 2019). Despite decades of study, the mechanism enabling anemonefishes to live within the venomous tentacles of their host sea anemone remains unresolved (Mariscal 1966, Mebs 2009, Burke da Silva and Nedosyko 2016). Numerous studies have explored the mechanism and each study has concluded that the

anemonefishes' mucus layer is involved in the protection process, but exactly how it functions is still being debated (Davenport and Norton 1958, Mariscal 1970a, Lubbock 1979, Russell 1982, Brooks and Mariscal 1984, Elliot et al. 1994, Mebs 1994, Elliot and Mariscal 1996). Initially, researchers thought anemonefishes were innately resistant to host sea anemone venom; but Mariscal (1970a) and Balamurugan et al. (2015) found that anemonefishes (*Amphiprion xanthurus* and *Amphiprion sebae*) are initially stung but are protected after a period of acclimation. The acclimation process involves the anemonefishes performing a range of unique behaviours, including touching tentacles with their tail, biting the tentacles tips and continuous fanning of tentacles with their pectoral fins (Balamurugan et al. 2015). After extended periods away from a host sea anemone, anemonefishes need to re-perform their acclimation behaviours to re-establish their invulnerability to host sea anemone venom and thus rejection of the innate protection hypothesis is clear (Fautin 1991, Mebs 1994, Hoepner et al. 2022). Furthermore, proteins have been found to differ in the mucus layer of acclimated and unacclimated anemonefishes and different proteins are also found when anemonefishes live in different species of sea anemone hosts (Schlichter 1976, Fautin 1991). Balamurugan et al. (2015) found that the mucus layer of the anemonefish *Amphiprion sebae* has a unique protein band with a size of 79.6 kda after acclimation in the sea anemone *Stichodactyla haddoni* which was not present before acclimation. Schlichter (1976) also found that the amino acids serine and glycine, increased in the mucus layer after acclimation of *Amphiprion clarkii* to *Heteractis crispa*.

Since this discovery, two main hypothesis have emerged (Hoepner et al. 2022): (1) Schlichter (1976), proposed that anemonefishes acquire substances from their host sea anemone that enables them to survive within the sea anemone's tentacles. Elliot et al. (1994), defined this

as the mucus layer acting to molecularly mimic the host sea anemone, preventing the detection of the anemonefishes amongst the host sea anemone's tentacles. (2) Lubbock (1980), suggests an alternate hypothesis where the mucus layer of the anemonefishes modulates the levels of the stimulatory compounds that trigger the nematocysts of the host sea anemone, which are present in the mucus layer of non-symbiotic fishes. Ozacmak et al. (2001) found that N-acetylneuraminic acid is one of the stimulatory compounds that triggers sea anemone nematocyst discharge. With research by Abdullah and Saad (2015) supporting Lubbock (1980)'s alternate hypothesis, as they found that *Amphiprion ocellaris* had far less N-acetylneuraminic acid (Neu5Ac, sialic acid) only 1.6mg/mL, compared to non-symbiotic fishes, *Abudefduf sexfasciatus* (Scissor-tailed Sergeant) that had 50.4mg/mL and *Thalassoma lunare* (Moon Wrasse) containing 71.9 mg/mL.

There is evidence that the anemonefishes' mucus layer changes when in association with host sea anemones and that acclimation behaviours are required to initiate this change (Balamurugan et al. 2015). As discussed, the two main hypotheses are clear, either the mucus layer of the anemonefishes changes chemically or chemicals from the host sea anemone are incorporated into the mucus layer of the anemonefishes. However, what is not clear is the mechanisms behind each of these hypothesises. What elements of the mucus change chemically? What components of host sea anemone mucus allow for molecular mimicry and how are they acquired and maintained in the mucus of anemonefishes? Many questions remain unanswered for how this symbiotic relationship occurs; beyond the simple thought that the anemonefishes' mucus layer is the key. Therefore, this study aims to reveal the lipid and glycan profile of anemonefish mucus via metabolomics, if the anemonefish mucus plays a key role in the establishment of the relationship with host sea anemones, we would expect

to see alteration to mucus composition when anemonefishes are in association with a host. We performed a before-after-control-impact (BACI) experiment and Matrix-assisted laser desorption/ionization-time of flight (MALDI TOF/TOF) analysis to profile the lipid and glycan composition of mucus from *A. percula* in association with host sea anemone, *Entacmaea quadricolor,* the most sought-after host by anemonefishes.

4.2 Materials and Methods

4.2.1 Study species and experimental set-up

Twelve pairs of anemonefish (n=24) *(Amphiprion percula)* were purchased from Cairns Marine and transported to the Animal house facility at Flinders University, South Australia. All fish were acclimated to their new environment for three weeks and each pair was housed in 30L tanks that contained a terracotta pot that acts as a sea anemone surrogate. *A. percula* were fed daily with commercial pellets (Hikari Marine S) and mysid shrimp. Six *E. quadricolor* sea anemones, obtained from a local aquarium store in Adelaide (harvested in Cairns), were transported to the Animal House facility at Flinders University and held in a 200L holding tank (27 °C ± 0.6, salinity 36.5 ± 1.5, pH 8.01 ± 0.2) for a 2-week acclimation period with an Aqua One MariGlo LED 90 light (500 lux on a 12:12 L:D light cycle). Sea anemones were fed weekly with a small piece of prawn. Each tank had a Fluval Aquatic Marine Nano 3.0 lights (2500 lux on a 12:12 L:D light cycle). Six pairs of *A. percula* were randomly assigned to either the control or treatment groups. Control and treatment tanks were on a separate, recirculating water system (control: 27 °C ± 0.6, salinity 36.5 ± 1.5, pH 8.01 ± 0.2, treatment: 27 °C ± 0.6, salinity 36.5 ± 1.5, pH 8.01 ± 0.2).

4.2.2 **Sampling design**

Mucus from *A. percula* was sampled weekly, for a period of 8 weeks from both fish in association with *E. quadricolor* sea anemones (hosted treatment) and fish without a sea anemone (non-hosted control) (approved by the Flinders University Animal Ethics Committee E470-18) (Fig 4.1). Mucus from all 12 *A. percula* pairs was sampled at week 0 for an initial mucus sample. One week later an *E. quadricolor* host sea anemone with a tile for attachment was added to each of the six treatment tanks and the terracotta pot was removed from these tanks. *A. percula* mucus was sampled again for both the treatment and control groups 48 hours after *E. quadricolor* host sea anemones were added to the tank and again after 1, 2 and 3 weeks. One-week later all *E. quadricolor* host sea anemones were removed from the treatment groups and the terracotta pots re-added. *A. percula* mucus was re-sampled for both the control and treatment groups 24 hours followed by 1, 2 and 3 weeks after *E. quadricolor* removal. Throughout the entire 8-week experiment *A. percula* in the control group were kept without a host sea anemone.

4.2.3 **Sample preparation**

Glass slides were prepared prior to sampling by dipping the slide into molecular grade 100% ethanol (Sigma-Aldrich 200-578-6) for 10 seconds for sterilisation and placed into a 50mL falcon tube to air dry. *A. percula* were gently collected in a net for sampling. Mucus was collected by gently scraping the sterilized glass slide along the body of the fish, from operculum to tail, on both the left and right side of the body. Care was taken to avoid the collection of epidermal skin cells. The slides were then transferred into a 50mL tube and immediately frozen at -80°C until processing.

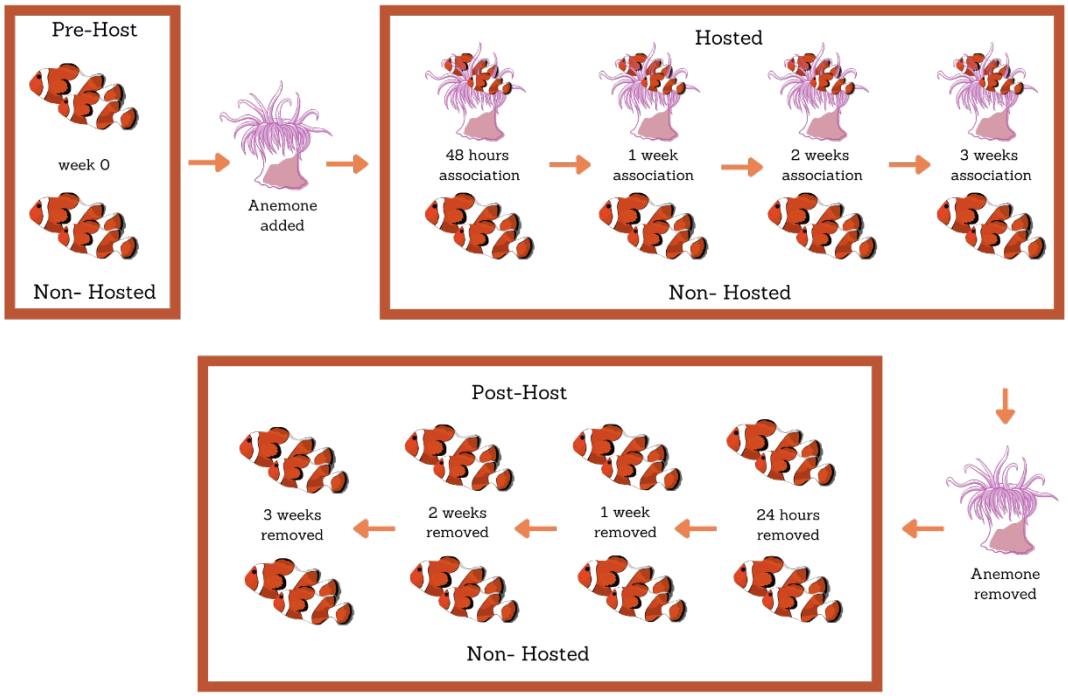


Figure 4.1 Mucus collecting procedure for *Amphiprion percula* anemonefish with and without *Entacmaea quadricolor* sea anemone presence over the 8-week experimental period, for hosted (n=12) and non-hosted (n=12) groups.

4.2.4 **MALDI TOF/TOF analysis**

The samples underwent metabolomic analysis at the Melbourne Centre for Nanofabrication, with support from Monash University and Dr David Rudd. To prepare the samples for analysis a chloroform/methanol phase partition was performed separating each sample into a lipid and aqueous phase, which was stored at -80°C before use (Fig 4.2). The aqueous phase contained soluble proteins largely glycoproteins an N-glycan digest was used to separate the glycan side chain for analysis.

For the lipid phase, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) (Bruker Germany) measurements were performed to identify changing peaks. 2x 4.5µl of each sample, was pipetted onto a novel nanofabricated surface (Minhas et al. 2020) for MALDI analysis. A total of 5000 hits was used to create a spectrum for each sample. Using FlexAnalysis (Bruker, Germany) each spectrum was

background subtracted and had the signal to noise ratio set to Centroid 7. Samples were then peak binned using MetaboAnalyst (www.metaboanalyst.ca) (Pang et al. 2020) with a mass tolerance of 1m/z and retention time tolerance of 30 seconds. Samples were median normalised, log-transformed and centre scaled.

For the aqueous phase, samples were thawed and pooled (n=3-4 per pool) into nine duplicate timepoint groups (n=18) for each of the treatment groups (Wk 0; 48h, Wk 1, 2 and 3 association with host; 24 hrs, 1, 2 and 3 weeks removed from host) and pooled into three time intervals (Wk 0, 48h and Wk1 association; Wk2 to 3 association, 24 hrs removed; 1,2 and 3 weeks removed) for the no treatment control group (n=7-11). Due to COVID-19 delays these samples were stored at -80°C for 18 months before analysis could take place. Amicon® Ultra 0.5mL 3K spin columns (Merck KGaA, Darmstadt, Germany) were used to concentrate the pooled samples before the N-glycan digest. The pooled samples were loaded onto the Amicon® spin column and spun at 14,000 g for 20 mins. All proteins/peptides < 30 aa were discarded in the flow through. Concentrated proteins >3000 Da were washed with 400 µl UltraPure™ DNase/RNAse-Free Distilled Water (Invitrogen™, 10977015) and spun for 14,000g for 25 mins. Approximately 40 µl of retentate was recovered for each sample.

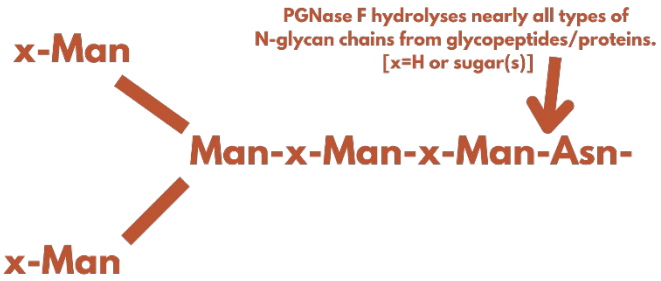


Figure 4.2 Cleavage site on glycoproteins used to separate glycan sugar side chain from protein for glycan analysis using PGNase F.

Peptidase N-Gylcosidase F (PGNase F) was used to cleave the innermost Glc NAc and asparagine residues of the mannose, hybrid and complex oligosaccharides from the N-linked glycoproteins in our sample (Fig 4.2). Approximately 35 µl of the concentrated pooled sample was added to 10 µl of 1 x Phosphate Buffered Saline (PBS) pH 7.4 (Gibco™,70011044), 50 µl of UltraPure™ water and 250 units recombinant PGNASe F (Glycerol-free) (New England Biolabs, Ipswich, MA, USA) in a spin column and incubated overnight in a beadbath at 37°C. Following glycan digestion, the Amicon® Ultra 0.5mL 3-K spin columns were utilised again this time to remove the PGNase F and the proteins which had been removed of glycans, and the glycans assumed to be < 3K were collected in the flow-through following centrifugation at 14,000g for 30 mins. The samples were freeze dried down to 5µl to further concentrate samples for mass spectrometry. Two µl of each sample was mixed with 2µl of matrix (10mg/ml 2,5 Dihydroxybenzoic Acid (Fluka Analytical), in 50% Acetonitrile, 50% H_2O) and 1ul was pipetted onto FlexiMass-DS disposable targets for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) (MALDI-7090, Shimadzu). A total of 5000 hits was used to create a spectrum for each pooled glycan sample. Using MMass (Strohalm et al. 2008) each spectrum was background subtracted and spectrum smoothed. Samples were then peak binned using MetaboAnalyst (www.metaboanalyst.ca) (Pang et al. 2020) with a mass tolerance of 1 m/z and retention time tolerance of 30 seconds. Samples were median normalised, log-transformed and centre -scaled. Accuracy ±0.05da.

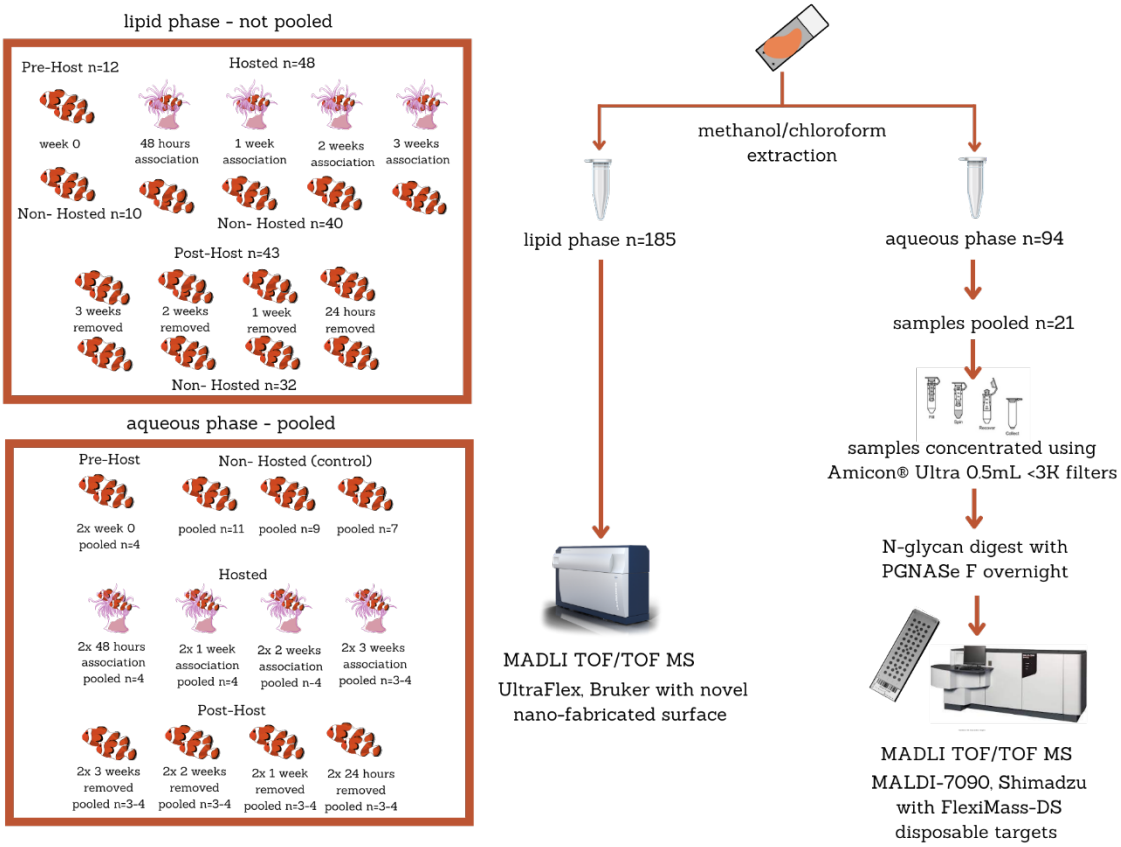


Figure 4.3 Workflow for MALDI TOF/TOF analysis

4.2.5 **Data Analysis**

Distance matrices were generated using vegdist in the R package “vegan” with a bray dissimilarity index (Dixon 2003). Principal Coordinate Analysis (PCoA) was performed using the R package “ape” (http://ape-package.ird.fr/). POST HOC PERMANOVA was performed to test for significance using pairwise.adonis2 and SIMPER was used to identify ions driving the dissimilarity using simper both from the “vegan” R package. Graphs were created in R used the package “ggplot2” (Wickham 2016). Line graph of average sequence profile of glycans was created in excel.

4.3 Results

4.3.1 Lipids

Using MALDI TOF/TOF we detected 65 unique lipid features (mass between 212.275 and 701.6635 Da) from the *A. percula* mucus samples. Principal Coordinate Analysis (PCoA) was used to visualise shifts in the lipid composition of *A. percula* mucus when associating with *E. quadricolorv*. Figure 4.4A shows that association with *E. quadricolor* does not affect the overall lipid composition of *A. percula* mucus, as there is no separation between the lipid profiles when *A. percula* are being hosted or not-hosted with *E. quadricolor* (Appendix S4.2). We also found that the lipid composition of the control *A. percula* group had varied across the eight-week experiment (Fig 4.4B) (Appendix S4.1).

4.3.2 Glycans

Using MALDI TOF/TOF we detected 37 unique glycan features (mass between 437.41 and 1373.39 Da) across the *A. percula* mucus samples. A Principal Coordinate Analysis (PCoA) . showed that association with *E. quadricolor* significantly alters the glycan composition of *A. percula* mucus (Fig 4.5A). After three weeks of association with *E. quadricolor* the glycan profile of *A. percula* mucus sits apart from all other samples ($p<0.043$) (Fig 4.5B) (Appendix S4.3). This is the only timepoint that shows change during association with *E. quadricolor* (Fig 4.6), as after removal from a host the glycan profile reverts to its original state ($p<0.012$). There were seven glycans whose percentage dissimilarity drove the significance observed (Fig 4.6B). Three of these glycans increased with hosting and four decreased with hosting (Figure 4.6D).

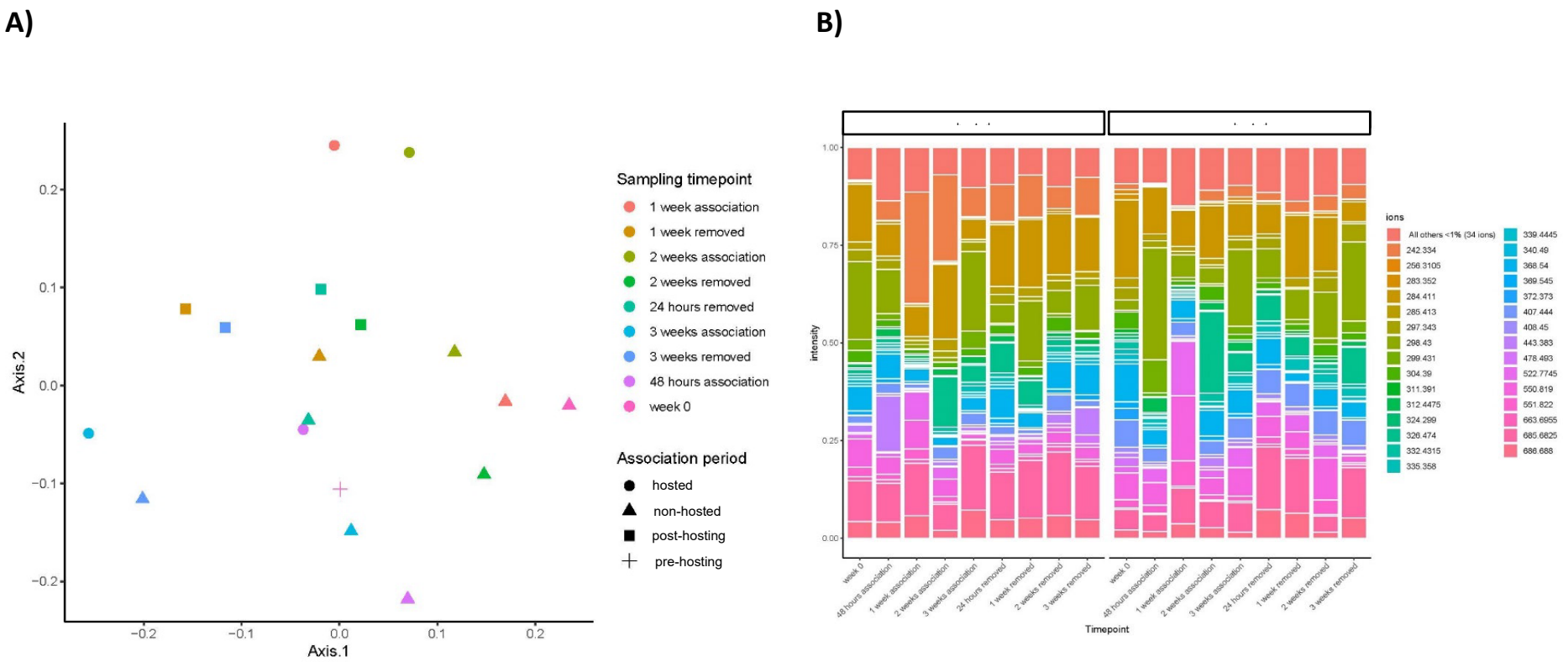
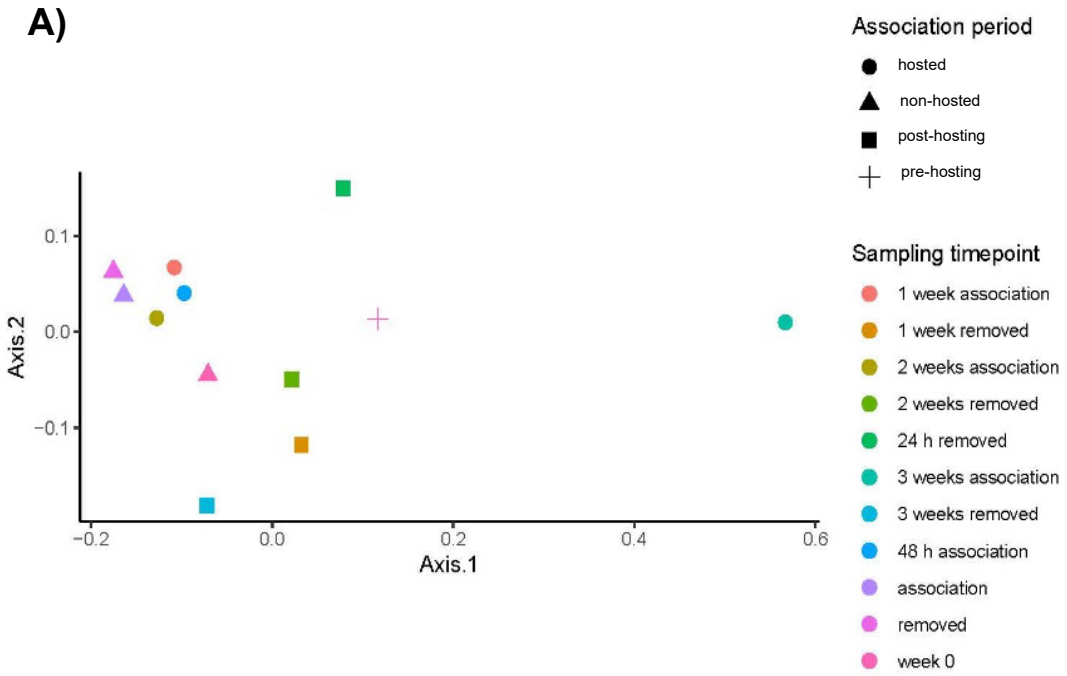
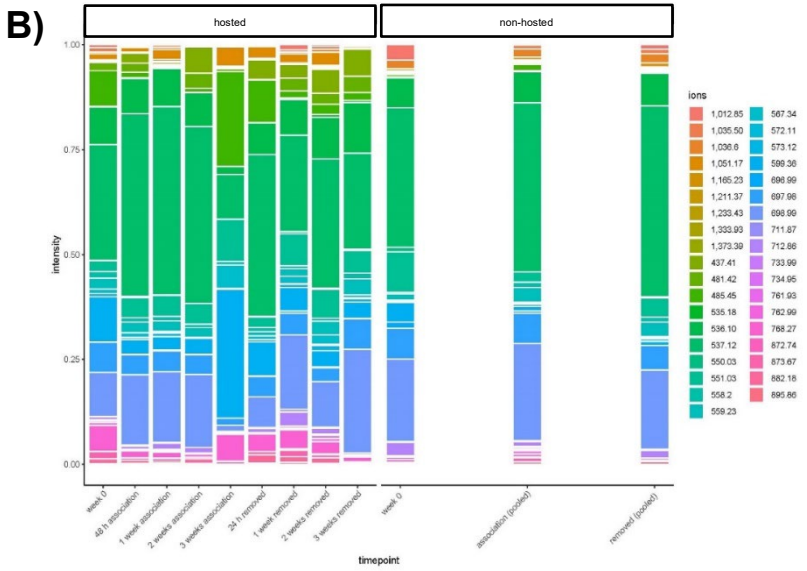


Figure 4.4 *Amphiprion percula* mucus lipid profile across hosted and non-hosted periods with *E. quadricolor*
A) PCoA
B) stacked bar graph showing lipid composition of mucus by association timepoint.
Each timepoint is the average of 5-9 samples.

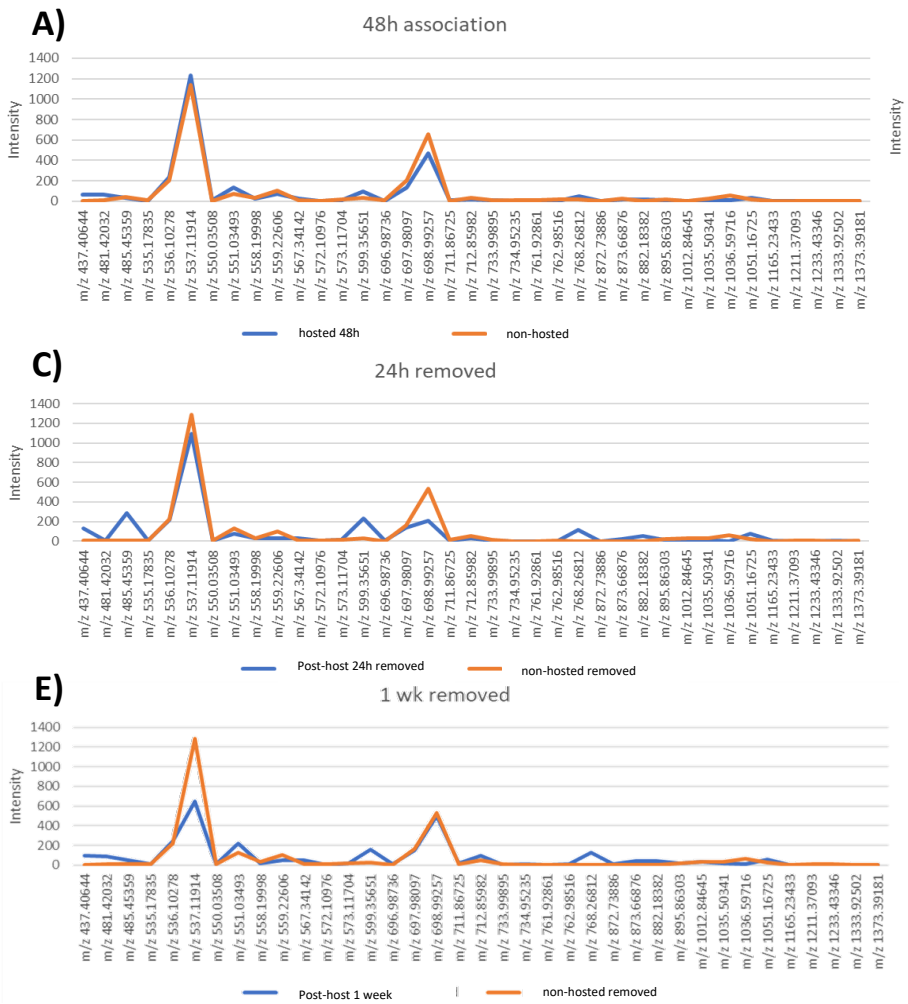




C)

	Df	SumOfSqs	R2	F	Pr(>F)
Post-hosting vs Control					
Symbiosis	1	0.07688	0.07938	0.9934	0.422
Symbiosis:Timepoint	3	0.19516	0.20149	0.8405	0.562
Residual	9	0.69656	0.71914		
Total	13	0.96860	1.00000		
Post-hosting vs Hosting					
Symbiosis	1	0.05796	0.04533	1.5246	0.238
Symbiosis:Timepoint	**6**	**0.91643**	**0.71679**	**4.0178**	**0.012**
Residual	8	0.30414	0.23787		
Total	15	1.27851	1.00000		
Control vs Hosting					
Symbiosis	1	0.07135	0.05426	1.2292	0.264
Symbiosis:Timepoint	**3**	**0.72127**	**0.54848**	**4.1419**	**0.043**
Residual	9	0.52241	0.39726		
Total	13	1.31503	1.00000		

Figure 4.5 *Amphiprion percula* mucus glycan profile across hosted and non-hosted periods with *E. quadricolor*
A) PCoA B) stacked bar graph showing glycan composition of mucus by association timepoint. Hosted timepoints are the average of two pooled samples each with 3-4 individual fish mucus samples. Non-hosted timepoints are pooled samples each with 7-11 samples. C) POST HOC PERMANOVA of glycan composition between association periods and timepoints (**Bold** indicates significance)



D)

	m/z	Change	Cumulative dissimilarity (%)
Control vs Hosting	485.454	+	55.5%
	537.119	-	19.8%
	551.035	+	62%
	599.357	-	33.2%
	697.981	-	77.1%
	698.993	-	45.9%
	768.268	+	66.6%
Post-Hosting vs Hosting	437.406	-	63.4%
	485.454	+	56.8%
	537.119	-	22.1%
	551.035	+	50.1%
	599.357	-	43.3%
	697.981	-	72%
	698.993	-	35.8%
	768.268	+	68.3%

Figure 4.6 Glycans from *A. percula* mucus are altered with three weeks association with *E. quadricolor*. A) glycan profile 48 hours after association with *E. quadricolor*. B) glycan profile after 3 weeks of association with *E. quadricolor*. C) glycan profile 24 hours after *E. quadricolor* removal. D) Table of glycans significantly altered after 3 weeks of *E. quadricolor* association + increase when hosted, - decrease when hosted with dissimilarity percentage from SIMPER. E) glycan profile 1 week after *E. quadricolor* removal. Blue is the mean treatment group and orange the mean control group. Mean treatment group is the average of two pooled samples each with 3-4 *A. percula* mucus samples. Mean control group is the average of three pooled samples each with 7-11 *A. percula* mucus samples.

4.4 Discussion

This preliminary research utilised a metabolomic approach to explore shifts in the lipid and glycoprotein composition of *A. percula* mucus when the fish was in association with the host sea anemone *E. quadricolor*. Hypotheses examining host sea anemone and anemonefishes symbiosis have focused on the anemonefishes' mucus layers for decades, with a key hypothesis suggesting that anemonefishes' mucus lacks the stimulatory compounds to trigger the nematocysts of sea anemone hosts (Lubbock 1980). We found that the lipid composition of *A. percula* mucus does not change when in association with *E. quadricolor* but the glycan composition of *A. percula* mucus changes significantly after three-weeks of association with *E. quadricolor*. This delay in adaptation reveals there must be other mechanisms that allows for the initial entry of anemonefishes into this venomous environment.

Heim et al. (2023) compared the lipid profile of anemonefishes' and damselfishes' mucus and found that the sphingolipid class of ceramides was a specific feature of anemonefishes' lipid mucus composition. Heim et al. (2023) suggested monitoring changes in ceramide content of anemonefishes' mucus when in association with a host sea anemone to determine whether anemonefishes' lipid content is affected by association. Our study does not support Heim et al. (2023)'s hypothesis as we found no changes to the lipid mucus composition of *A. percula* when in association with *E. quadricolor*. We did find that lipids in *A. percula* mucus flux overtime, however, this was found in both the hosted (treatment) and non-hosted (control) samples. Lipids perform important barrier functions in mucus and therefore may provide the initial defence needed for anemonefishes to enter the host sea anemone and perform acclimation

behaviours, as indicated by the differences in lipid composition between anemonefishes' and damselfishes' mucus (Heim et al. 2023).

We found seven key glycans in the mucus whose dissimilarity drove the significance when *A. percula* was in association with *E. quadricolor*. Three of these glycans increased when hosted and four decreasing when hosted. As there is a distinct lack of data on the metabolome of marine fishes (Reverter et al. 2017, Reverter et al. 2018, Heim et al. 2023), let alone glycan composition, we were unable to identify the types of glycans represented by these altered glycoproteins. The evolution of proteins containing these sugar groups (e.g. the 11 glycans we observed to change with association), or proteins with the ability to modify sugar profile (discussed below Marcionetti et al. (2019)) in anemonefishes may have enhanced the establishment and maintenance of symbiosis between host sea anemones and anemonefishes.

Glycosidases are carbohydrases-enzymes that catalyse the hydrolysis of glycosidic bonds in complex sugars releasing lower molecular weight end products. Post translational modifications (PTMs) frequently occur in N- and O-linked glycosylated proteins, with even small changes altering the function of a protein (Varki 2016). Sialic acids are nine-carbon sugars that can be either O- or N-glycosylated at the cell surface of mucins (Visser et al. 2021). There is large variability in the structural diversity and biological function of sialic acid, however, analysis of these glycans is challenging as there is very little known about their biosynthesis and function (Visser et al. 2021). One of the most prevalent sialic acid derivatives is N- acetylneuraminic acid (Neu5Ac). Recent studies have indicated that the glycan content of anemonefishes mucus may be involved in the reduction seen in nematocyst firing (chapter 3). Abdullah and Saad (2015) analysed the chemical

composition of anemonefish (*A. ocellaris*) mucus and found that it contained significantly lower concentrations of Neu5Ac compared to the mucus of other coral reef fish species (*Abudefduf sexfasciatus* and *Thalassoma lunare*). Neu5Ac is a salic acid side chain that has been shown to trigger the chemoreceptors that control sea anemone nematocyte firing (Ozacmak et al. 2001, Abdullah and Saad 2015). Marcionetti et al. (2019) used genomic analysis to identify key proteins in anemonefishes' epidermis that were positively selected for during anemonefishes evolution. They found that versican core proteins that are thought to bind N-acetylated sugars were positively selected for and thus may be used to mask detection of anemonefishes by sea anemone chemoreceptors. The gene for the protein, O-GlcNAse, was also found to be positively selected and has the potential to cleave the sialic acid side chain creating neutral glycoproteins (as seen by Abdullah and Saad (2015)). The evolution of these proteins in anemonefishes may have enhanced the establishment and maintenance of symbiosis between host sea anemones and anemonefishes.

Anemonefishes can generally acclimate to their host sea anemone in less than 24-48 hours (Mariscal 1970c, Balamurugan et al. 2015, pers obv), and this is the point at which we would expect to see changes in the anemonefishes' mucus composition. However, this is not what was found in this study. It took three weeks of association with *c* before clear changes in the glycan composition of the *A. percula* mucus were seen. Balamurugan et al. (2015) found that anemonefish secrete an intracellular mucous lining in the hypodermal region, that is not seen in *Terapon jarbua* (a fish species that does not associate with sea anemones). Therefore, perhaps it is not the external mucus layer that is the key to the initial formation of this symbiotic relationship, but rather that the internal mucus acts as

a barrier to sea anemone nematocysts until the external mucus layer has the opportunity to adapt to the new environment through the glycan changes, we observed in this study.

Mariscal (1970c) found that after 20 hours isolated from a host sea anemone, anemonefishes were stung (n=21) upon reintroduction to the host. We found that within 24 hours of *E. quadricolor* removal, the glycan profile of *A. percula* mucus largely returned to its original state, completely matching the control (non-hosted) profile at one week removed (next sampled timepoint). The loss of these changes in the glycan profile within 24 hours of removal from a host sea anemone, provides further support that anemonefishes are not innately protected from host sea anemone venom as previously thought (Hoepner et al. 2022) but indeed adjust their glycan profile when in association with a host sea anemone.

4.5 Conclusion

This study examined the lipid and glycan profile of *A. percula* mucus when in association with *E. quadricolor*. We found that the lipid profile of *A. percula* mucus is not influenced by association with *E. quadricolor*, instead remaining in a state of flux. *A. percula* glycans, however, were influenced by symbiosis with *E. quadricolor* e but only after three weeks of association. Such a delay in the acclimation of the mucus layer is unexpected and further study is needed to uncover the initial mechanism used by anemonefishes that enables them to enter the venomous tentacles of host sea anemones while their mucus layer adapts at the glycan level. Further, we were unable to identify the mucus glycans that were altered during symbiosis. Future studies to identify the glycans in anemonefishes' mucus and the proteins they are on, will provide further insight into the protective capabilities glycan presence or absence are able to provide to anemonefishes and if the Neu5Ac (salic acid) content of mucus is lowered when anemonefishes are in association with host sea anemones.

Chapter 5:

Initial concept **CMH** CAA KBDS, experimental design **CMH** CAA, lab work **CMH** CAA, data analysis **CMH,** ZS, CAA, first draft **CMH**, editing **CMH** EKF CAA KBDS

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5.1 Introduction

Cnidarians are a venomous phylum, with all species relying on toxins for defence and food acquisition (Madio et al. 2019). Sea anemones, like other cnidarians utilise nematocytes and mucus secretions over their body to distribute their venom, which contains a complex mixture of small molecules, peptides and proteins, known as toxins (Prentis et al. 2018). While the venom of sea anemones has begun to be characterised, the knowledge base on anemone toxins is still significantly behind species such as snakes and spiders; with very little studies utilising genomic or proteomic methods (Prentis et al. 2018, Surm et al. 2019). Sea anemone genomic and transcriptomic studies suggest that venom contains proteins from numerous toxin families which are structurally and functionally diverse and are mostly under purifying selection (Surm et al., 2019; Surm & Moran, 2021). Using a transcriptomics approach, Smith et al. (2023) determined that the venom phenotype of the sea anemone *Nematostella vectensis* (non-host) may change quickly with expression of a single dominant toxin family enabling ecological specialization in species. Expression of dominant toxins may consequently enable wide-spread ecological functions and thus may act convergently amongst animals with similar niches or behaviours. Dutertre et al. (2014) combined venom duct transcriptomics and proteomics to find that cone snails are able to rapidly produce and release two distinctive venom types depending on the need that arises. Similarly using transcriptomics only, sea anemones have been shown to have distinctive toxin gene expression profiles in different tissue types (Macrander et al. 2016, Ashwood et al. 2021, Ashwood et al. 2022), as they do not have a centralised venom gland.

There are approximately 1170 sea anemone species (Rodríguez et al. 2022), yet only ten specific species of sea anemones from three unrelated anemone families

(*Thalassianthiade, Actinidae, Stichodactylidae*) (Titus et al. 2019), form associations (as hosts) with one or more of the 28 species of anemonefishes (*Amphiprion*) (Fautin 1991, Burke da Silva and Nedosyko 2016, Tang et al. 2021). The host sea anemone provides a safe site for anemonefishes reproduction and protection from predation (Holbrook and Schmitt 2004), whereas the anemonefishes help to increase the growth, reproduction, and defence of host sea anemones, by providing nutrients from their faeces and increased oxygenation by swimming amongst the host sea anemones tentacles and chasing off potential sea anemone predators (Szczebak et al. 2013, Frisch et al. 2016, Schligler et al. 2022). The venom of host sea anemones is understudied with only a few unique proteins/peptide sequences (available in sequence databases) compared to those available for both non-host sea anemones and other venomous species (7,579 toxin sequences reported in Tox-Prot of which 285 toxin sequences are from Actiniaria, as of Mar 2023 (Jungo et al. 2012)). There are only 51 toxin sequences reported in Tox-Prot (as of Mar 2023 (Jungo et al. 2012)), from seven of the ten host sea anemone species indicating little knowledge availability on the venom arsenal of host sea anemones. Nedosyko et al. (2014) showed that there is variation in toxicity across the ten-host sea anemones; with host sea anemones with a middle range toxicity forming more anemonefish associations than those host sea anemones with a high or low toxicity. However, it is unclear how toxic host sea anemones are compared to non-host sea anemones or the influence of symbiosis on host sea anemone venom production.

The study of sea anemone venom has begun to use a combined transcriptomic and proteomic approach, also known as proteotranscriptomics (Madio et al. 2017, Liao et al. 2019, Ramirez-Carreto et al. 2019, Levin and Butter 2022). Using proteotranscriptomics

provides a more holistic overview of venom complexity enabling the detection of novel proteins (Madio et al. 2017). Three recent studies have utilised transcriptomics focusing on host sea anemones who form associations with anemonefishes (Barua et al. 2022, Delgado et al. 2022, Kashimoto et al. 2022). Delgado et al. (2022), examined the toxin expression profiles of five host sea anemones and a closely related non-host, utilising existing transcriptomes from NCBI generated from different sea anemone tissues (outer and inner tentacles, column, exocodic and endocodic tentacles etc). Delgado et al. (2022) inferred that haemostatic and haemorrhagic toxin gene expression is a dominant feature of host sea anemones. Barua et al. (2022) & Kashimoto et al. (2022), created new transcriptome datasets of host anemones from Okinawa, Japan; to explore nematocyte expressed genes, phylogeny and co-expression in the evolution of sea anemones hosting anemonefish. Kashimoto et al. (2022) noticed that nematocyte gene expression is generally uniform across host sea anemones, indicating that symbiosis is likely related to small gene or expression changes (Marcionetti et al. 2019). Barua et al. (2022) observed that association with *Symbiodiniaceae* and anemonefishes significantly affect gene expression in host sea anemones, particularly in relation to nutrient exchange and metabolism. However, there has only been a single study on host sea anemone venom that has used a combined transcriptomic and proteomics approach (Madio et al. 2017). Madio et al. (2017) observed that there is a disparity between toxins expressed in the tentacle transcriptome compared to those recovered in the venom proteome illustrating the importance of more direct proteomic investigations in this area, in order to fully understand venom diversity and functionality.

The sea anemone species*, Entacmaea quadricolor,* forms the most associations with anemonefish species, 17 of 28 (Hoepner et al. 2022). Nedosyko et al. (2014) found that *E. quadricolor* was of mid-range toxicity compared to other host sea anemones and together with its unique bulb-like tentacles provides optimal conditions for anemonefishes. There are only three *E. quadricolor* toxin protein sequences reported in ToxProt (as of Mar 2023 (Jungo et al. 2012)) making it impossible to ascertain a complete picture of toxin characteristics and their evolutionary implications. In order to understand how anemonefishes can withstand their venomous host environment it is important to develop an in-depth profile of the host sea anemone venom to which the anemonefishes must develop resistance to. In this study we expand upon the previous work of Barua et al. (2022), Delgado et al. (2022), Kashimoto et al. (2022) & Madio et al. (2017) to uncover the full toxin protein arsenal of *E. quadricolor* venom using a proteotranscriptomic approach.

5.2 Materials & Methods

The results from these methods will be presented across chapters 5 and 6. Chapter 5 will explore the tentacle transcriptome and the venom proteome of *E. quadricolor*, whereas chapter 6 will focus on the differential expression of the *E. quadricolor* transcripts and proteins when in association with an anemonefish symbiont.

5.2.1 Study species and experimental set-up

Six bubble tip anemones (*Entacmaea quadricolor*) (~5-7cm diameter) were obtained from an aquarium store in Adelaide, South Australia (harvested from Western Australia) and transported to the Animal House facility at Flinders University and held in individual 30L tanks for a 2-week acclimation period (26.5 °C ± 0.7, salinity 37.5 ± 1.5, pH 7.91 ± 0.2). *E.*

quadricolor were fed a small piece of prawn every three to four days throughout the experimental period except in the 48 hours leading up to each venom sampling event. Each tank had a Fluval Aquatic Marine Nano 3.0 lights (2500 lux on a 12:12 L:D light cycle). Six pairs of anemonefish (n=12) *(Amphiprion percula)* were housed in 30L tanks containing a terracotta pot that acts as an anemone surrogate when a sea anemone was absent. Recirculating tanks (30L) holding the *A. percula* pairs were attached to a sump system that was separate from the anemones (27 °C ± 0.6, salinity 36.5 ± 1.5, pH 8.01 ± 0.2). *A. percula* were fed twice daily with commercial pellets (Hikari Marine S) and mysid shrimp.

5.2.2 Sea anemone venom and tentacle collection

The *E. quadricolor* seaanemones were starved for 48 hours prior to tenacle and venom sampling. Three tentacle samples were cut from each *E. quadricolor* individual(n=6) during the non-hosting period (Fig 1), by stretching out the tentacle with sterile tweezers and slicing the tentacle at the base with a disposable scalpel. The tentacles were immediately place in 400ul of RNA*later* (Sigma Aldrich Missouri, United States) and stored at -80°C. Each sea anemone was subsequently milked for venom, as described by Sencic and Macek (1990) & Hoepner et al. (2019), and the venom was freeze dried then stored at -80°C. An additional three tentacles were collected 72 hours post venom milking (Madio et al. 2017) for making RNA. A pair of *A. percula* were added to each tank after tentacle removal for a three week acclimation period (Fig 5.1). Tentacle and venom sampling was then repeated for the hosting timepoint.

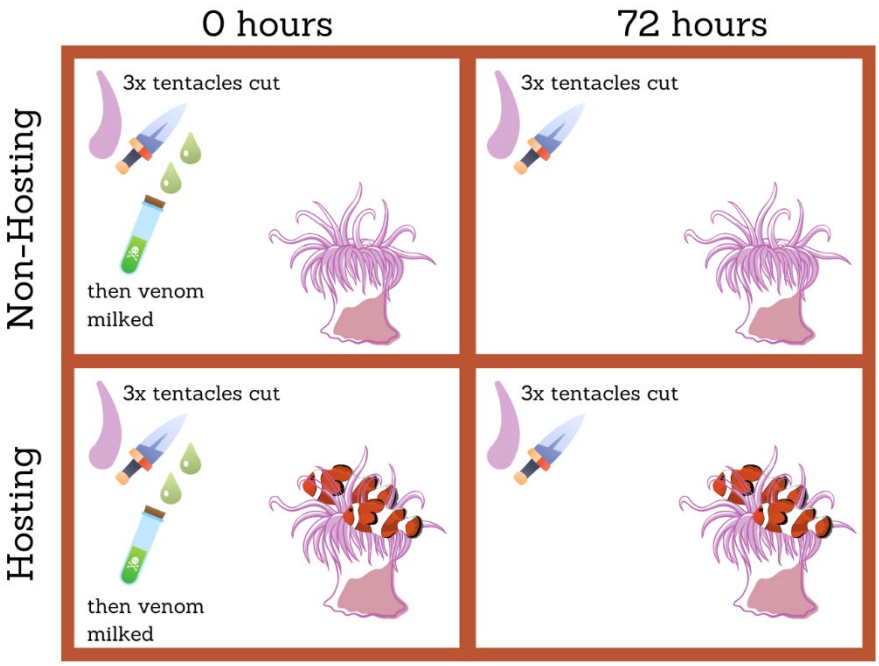


Figure 5.1 Sampling process for collecting *Entacmaea quadricolor* tentacles and venom for transcriptomic and proteomic analysis respectively. n=6

5.2.3 Transcriptomics

5.2.3.1 RNA Isolation and library preparation

RNA was extracted from the *E. quadricolor* tentacles (non-hosting 0h, n = 6, non-hosting 72h n= 6, hosting 0h n= 6, hosting 72h n= 6) using a RNeasy mini kit (Qiagen Venlo, Netherlands), as per manufacturer's instructions (Appendix S5.2). Tissue samples (n=24) from each sampling point were disrupted using a mortar and pestle and ground to a fine powder under liquid nitrogen and added to the lysate buffer RLT and homogenised. The samples were then transferred to the mini spin column and the column was washed three times with wash buffer by centrifugation to remove contaminants. The RNA was eluted from the column using RNAse-free water before being stored at -80°C. RNA was quality controlled and quantified via LabChip (Perkin Elmer) and Qubit 2.0 (ThermoFisher Scientific). All 24 RNA samples had RIN values between 7 and 9.2 thus appropriate for library preparation which was conducted by Flinders University Genomics Facility

(Appendix S5.1). The TruSeq stranded mRNA library prep kit (Illumina) was used to create each library starting with between 200 ng and 1 µg RNA as per standard protocol. Pooled equimolar libraries were quality checked and sequenced at the Ramaciotti Centre for Genomics, Sydney, NSW on the NovaSeq S4 flow cell, paired end 2x150 bp, to achieve an average coverage of 20 million reads per sample.

5.2.3.2 RNA-seq read quality control

Transcriptome analysis was conducted by Zachary Stewart at Queensland University of Technology (QUT), Australia. Paired-end reads were trimmed to ensure data quality using Trimmomatic v.0.36 (Bolger et al. 2014). Illumina's TruSeq adapter sequences were removed, and parameters otherwise mimicked those used by the Trinity de novo assembler (Haas et al., 2013) i.e., "ILLUMINACLIP:${ADAPTERS}8:2:30:10 SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25". Resulting quality-trimmed reads were used for all downstream analyses.

5.2.3.3 Transcriptome assembly

All 24 transcriptome samples, regardless of treatment were assembled to create a global transcriptome library for *E. quadricolor*. Our process for creating a high-quality transcriptome assembly made use of several transcriptome assemblers, specifically, SOAPdenovo-Trans v.1.03 (Xie et al., 2014) and Oases v.0.2.09 (Schulz et al. 2012) assemblers were used to build transcriptomes with several k-mer lengths (23, 25, 31, 39, 47, 55, and 63 for both and 71 additionally for SOAPdenovo-Trans only). Trinity v.2.14.0 (Haas et al. 2013) was also used with parameters including min_kmer_cov = 2 and SS_lib_type = RF. All resulting transcriptome files were concatenated and sequences shorter than 250 bp were removed to eliminate potentially poor quality and/or fragmented transcripts (Appendix S5.3).

The concatenated file was subjected to the EvidentialGene v.2022.01.20 tr2aacds pipeline (Gilbert 2016). This process is designed to receive a massively redundant transcriptome from multi-k-mer assembly and produce a non-redundant output containing the best-assembled transcripts from each assembler; it additionally predicts coding regions within these transcripts. The resultant transcriptome was assessed for quality using BUSCO v.5.2.1 (Simão et al. 2015).

5.2.3.4 Contaminant removal

PsyTrans (jueshengong 2017) was used to remove transcripts arising from endogenous symbionts. PsyTrans is a script which utilises protein sequences from sea anemone species related to *E. quadricolor* as well as protein sequences from symbionts to identify and remove contaminants from the transcriptome.

We created a custom database of symbiont sequences using published genomic and transcriptomic resources for *Symbiodinium* and related organisms (Levin et al. 2016, Arriola et al. 2018, González-Pech et al. 2021, Camp et al. 2022) (Appendix S5.4). When a data source only provided nucleotide transcripts, we used TransDecoder v.5.6.0 (TransDecoder 2022) to obtain translated coding DNA sequence predictions. We also opted to use solely genomic resources for related sea anemone species, avoiding transcriptomic data which may itself contain symbiont contaminants; the data was sourced from unpublished genome annotations. Data was sourced from other members of the family Actiniidae, namely *Actinia equina* (Wilding et al. 2020), *Actinia tenebrosa* (unpublished data GenBank (GCA_029948245.1)), and *Aulactinia veratra* (unpublished data) (appendix S5.5).

Sequences from related sea anemone species and from symbionts had their redundancy reduced through use of CD-HIT v.4.6 (Li and Godzik 2006) with parameters -c 0.95 -n 5 -aS 0.9. The resulting files were provided to PsyTrans with default parameters; the output FASTA file corresponding to predicted sea anemone host transcripts represented our final transcriptome.

5.2.3.5 Clustering and read counting

To obtain read counts associated to the gene level rather than individual transcripts, we first used salmon v.1.9.0 (Patro et al. 2017) with parameters --libType A --dumpEq --hardFilter --skipQuant to produce equivalence classes for the reads from each sample against the transcriptome file. Following this, Corset v.1.09 (Davidson and Oshlack 2014) used the salmon equivalence classes to cluster transcripts based on shared read alignments and expression patterns and provided read counts associated to each cluster (which putatively represents a gene). Gene clusters were used in downstream analyses rather than individual transcripts and read counts were used in chapter 6 for differential expression.

5.2.3.6 Gene annotation and functional enrichment analysis

Predicted coding DNA sequences (translated proteins) from our transcriptome were queried against the UniRef90 database (Suzek et al. 2014) using MMseqs2 v.fcf5260 (Steinegger and Söding 2017). Gene names for queried sequences were attributed based on their best match, and functional annotation of gene ontology (GO) (Ashburner et al. 2000, Gene Ontology Consortium 2020) was performed by identifying the best match which had GO annotations in UniProt's idmapping_selected.tab file. Annotated GO terms were expanded to include ancestor terms using the Python library goatools (Klopfenstein et al. 2018).

5.2.3.7 Toxin annotation pipeline

Toxin annotation was accomplished using custom scripts available from https://github.com/zkstewart/Various_scripts/tree/master/Toxins_annot. As an overview, this process leverages custom-made hidden Markov models (HMMs) and the HMMer software (Eddy 2011) to predict protein domains located in venom protein sequences. Python scripts assess the results of HMMer searches to determine whether a sequence is likely to be part of a sea anemone-associated toxin family.

The custom-made HMMs were generated from an initial dataset of six host and non-hosting sea anemone venom proteomes i.e., *Actinia tenebrosa*, *Aiptasia pulchella*, *Heteractis malu*, *Macrodactyla doreensis*, *Telmatactis* sp., and *Stichodactyla haddoni* (Stewart Z, Undheim EA and Prentis PJ unpublished data). Multiple sequence alignments (MSAs) of venom families were formed using a mixture of manual inspection of sequences (with an emphasis on visually locating conserved regions likely to be important to protein structure e.g., cysteine residue organisation), assisted by BLAST (Altschul et al. 1990, Camacho et al. 2009) and HMMer searches to find sequence homology. Importantly, this process focused on gene families present in two or more of the sea anemone venom proteomes; genes found in only one species were excluded from further consideration even if they were known to be venom toxins from previous studies. MSAs were manually trimmed to adjust the domain regions from within family alignments and were converted into HMMs. Scripts were created by manually tuning a rule-based process which considers the HMMer results obtained for each sequence including the domains which hit against a sequence and the significance of their E-value, in addition to considering sequence features including the relative positions of the domain hits in a sequence. Through this process, many previously discovered toxin families were modelled and made easily

predictable using this system. Additionally, several toxin families were identified which have not been reported on previously (U# and Z# models) and hence have unknown functionality (Stewart Z, Undheim EA and Prentis PJ unpublished data).

Toxins from the pipeline that were assigned as uncharacterised toxins were manually inspected for toxin domains that could be assigned to a venom category. In addition, toxin families identified by Delgado et al. (2022) as present in the *E. quadricolor* transcriptome but not found through the pipeline were manually added if the appropriate domain was present.

5.2.3.8 Signal P

SignalP v.5.0b (Almagro Armenteros et al. 2019) was used to predict signal peptides in protein sequences using default settings i.e., using Eukarya prediction.

5.2.3.9 Nematocyte orthologs

To identify nematocyte-specific genes, we followed the methods of Kashimoto et al. (2022) by obtaining sequences corresponding to the 410 proteins identified by Balasubramanian et al. (2012) in *Hydra magnipapillata* nematocytes. Orthologs of these genes in our *E. quadricolor* transcriptome were found using OrthoFinder v.2.5.4 (Emms and Kelly 2019). Hydra nematocyte matches were assigned to categories according to Balasubramanian et al. (2012), with hits from the *Hydra* venom category not identified in the QUT toxin pipeline added to our putative toxins list.

5.2.4 Proteomics

To identify all proteins in the venom, DDA analysis with gas fractionation using a Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, Waltham,

Massachusetts, USA) was conducted on a pooled venom sample to create a spectral library of all proteins.

5.2.4.1 Venom protein extraction for mass spectrometry.

Lyophilized venom from four *E. quadricolor* individuals in the non-hosting period and four *E. quadricolor* individuals in the hosting period underwent proteomics analysis at the Flinders University Omics Facility. A 50 µg protein pool of all eight samples (25 µg non-hosting:25 µg hosting), was reduced and alkylated following a standard procedure. Briefly, a mixture of hydrophobic and hydrophilic Sera-Mag Carboxylate SpeedBeads (Cytiva) was used for protein clean up and trypsin digestion following the manufacturer's instructions. Following the trypsin digestion, peptides in each sample were cleaned up with a 200 µl C18 StageTip and eluted in 80% acetonitrile/0.1% formic acid. The sample was then dried down in a Christ RVC 2-25 CD plus vacuum concentrator (Christ, Osterode am Harz, Germany) and resuspended in 5% acetonitrile. Approximately 5.8 µg peptides were recovered from the pooled venom sample as measured by the NanoDrop.

5.2.4.2 Spectral library creation via DDA and GPF

Data dependent acquisition (DDA) was used to create a spectral library, using a Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with a Nanospray Flex™ Ion Source (ES071, Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 UPLC chromatography system (Thermo Fisher Scientific). The pooled venom tryptic peptides (4.8 µg) were injected into a PepMap™ 100 trap column (0.3 x 5 mm, 5 µm C18, Thermo Fischer) and then eluted onto an inhouse pulled column created from 75 µm inner diameter fused silica capillary packed with 3 µm ReproSil-Pur C18 beads (Dr. Maisch, Ammerbuch, Germany) to a length of 15cm. The column was heated to 60°C using a Nanospray Flex™ Column Oven (Sonation

lab solutions, Biberach, Germany) and the flow rate for the gradient pump was 300 nL per minute. The column and trap were equilibrated in Solvent A (0.1% formic acid in water) and eluted with solvent B (79.9% Acetonitrile, 20% Water, 0.1% Formic Acid) using a 2-30% linear gradient over 55 minutes (Table 5.1). Total run time was 85 minutes and internal mass calibration using RunStart EASY-IC™ was enabled.

Table 5.1 HPLC chromatography gradient.

Time	Solvent B
0 min	2%
5 min	2%
10 min	8%
60 min	31.2%
66 min	50%
69 min	100%
72 min	100%
75 min	2%

Gas phase fractionation was employed in conjunction with DDA for this analysis (methods 2-7, table 5.2). Gas phase fractionation (GPF) separates peptides in the gas phase i.e., once peptides have entered the instrument in the gas phase peptides in each phase are run through six identical methods following the DDA protocol. It is implemented by analysing multiple injections of the same sample with 50-400 m/z mass windows analysed in each injection. With only the MS scan range changing for each method, the m/z range overlapped by 10 da between methods to ensure no peptides were missed (Table 5.2).

Table 5.2: m/z scan ranges used in each method performed on each GPF fraction.

Method	m/z
1	350-1200
2	350-500
3	490-550
4	540-610
5	600-710
6	700-810
7	800-1200

All DDA files collected were used to generate a spectral library using Spectronaut software V16.022 with default settings (Biognosys AG, Schlieren, Switzerland). The spectral library was searched against the predicted protein sequences from the assembled *E. quadricolour* transcriptome to identify proteins present in the venom proteome. Gene clusters were used across both datasets to match and compare the tentacle transcriptome to the venom proteome.

5.2.4.3 Data analysis

The venom and nematocyte profiles were visualised using PieDonut from the 'webr' R package (Moon 2020). Bar graphs were created in R using the package "ggplot2" (Wickham 2016).

5.3 Results

5.3.1 Tentacle transcriptome

The assembled tentacle transcriptome of *E. quadricolor*, consisted of 650,353 ORFs after PsyTrans contaminant removal. BUSCO scoring indicated that a high-quality assembly was achieved, with 98.3% of near-universal metazoan single-copy genes predicted (BUSCO short summary = Completeness: 98.3% [Single copy: 13.1%, Duplicates: 85.2%], Fragmented: 0.6%, Missing: 1.1%, n:954). Clustering with Corset resulted in a predicted 279,274 gene clusters, which substantially reduced the number of redundant transcripts according to BUSCO (BUSCO short summary after clustering = Completeness: 94.0% [Single copy: 73.7%, Duplicates: 20.3%], Fragments: 1.5%, Missing: 4.5%, n: 95) (Table 5.3). The majority of gene clusters in the *E. quadricolor* tentacle transcriptome encoded proteins between 5-9kda (49.3%) with only 1.25% gene clusters encoding molecular weight proteins >50 kDa (Fig 5.2A). Of 279,275 gene clusters identified, only 72,218 could be annotated, matching to 18,469 unique protein hits using the UniRef90 database. Thus

over 74% of the *E. quadricolor* tentacle transcriptome represented novel transcripts, that had no sequence similarity or homology to proteins in UniRef90.

Table 5.3: Summary of tentacle transcriptome and venom proteome generated from *Entacmaea quadricolor*. ORFs = Open Reading Frames

	RNA gene clusters	Protein gene clusters
ORF sequences	279,274	2,736
Annotated ORF sequences	72,218	2,686
ORFs with GO terms	46,288	1,988
UniRef90 Hits	18,469	1,928
ORFs with signalP	11,807	709
Putative toxins ORFs	1,251	135
Putative toxin ORFs with signalP	515	97
Putative toxins UniRef90 Hits	296	78
ORF with hydra nematocyte matches	388	106
Hydra nematocyte matches UniRef90 Hits	190	79
Hydra nematocyte matches with signalP	181	57

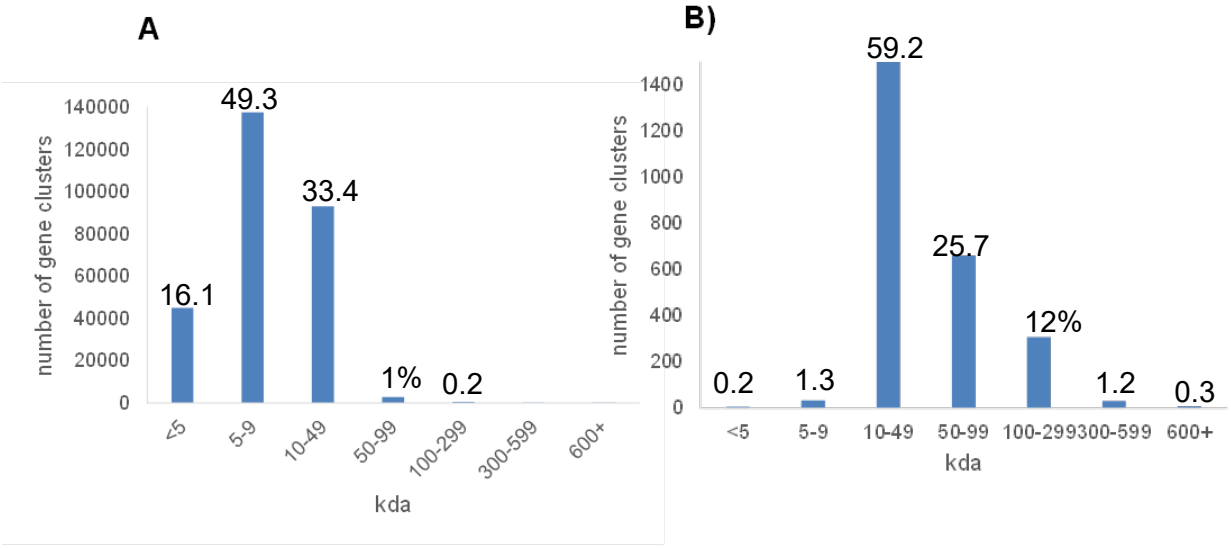


Figure 5.2: Size range of proteins identified in *Entacmaea quadricolor* A) based on the amino acid translation of all tentacle gene clusters B) based on proteins identified in the venom.

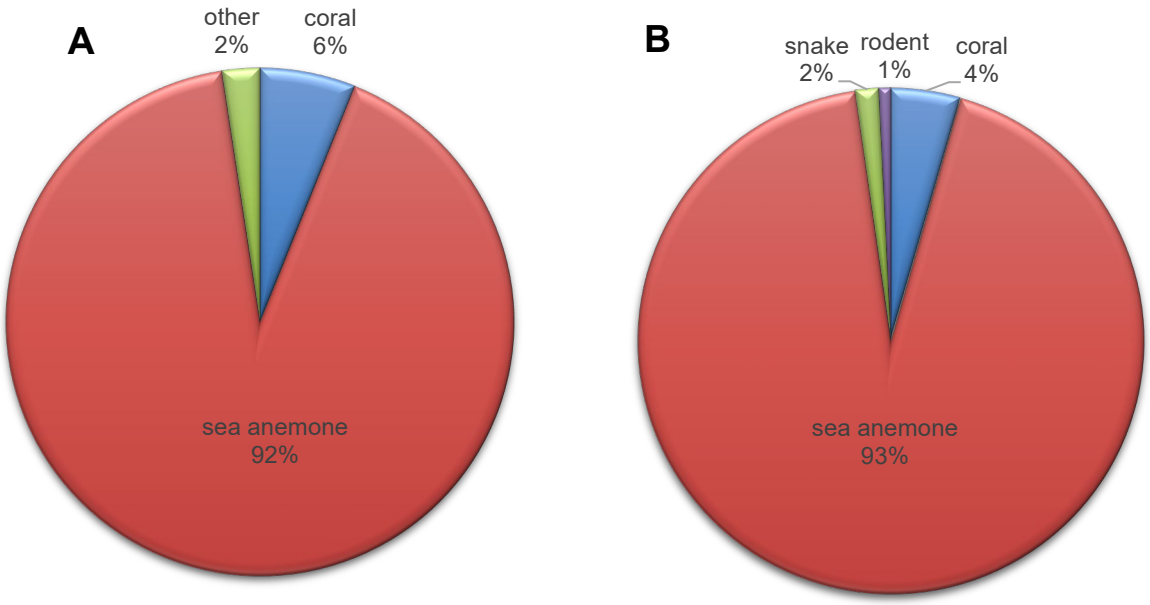


Figure 5.3: Pie chart of taxon from UniRef90 hit matches in A) Venom proteome, B) toxins in venom proteome.

5.3.2 Venom proteome

The spectral library created identified proteins matching to 2,736 gene clusters (0.9% of 279,274 gene clusters in tentacle transcriptome) in *E. quadricolor* venom. Unlike the tentacle transcriptome, 98% of the proteins in the venom were annotated, matching to 1,928 UniRef90 hits, with only 50 proteins having no known protein match (Table 5.3). 59.3% of proteins identified in venom were between 10-49kda in size, and only 1.5% of proteins identified were <10kda (compared to the 65.4% of gene clusters <10kda in the tentacle transcriptome) (Fig 5.2B).

92% of proteins identified in *E. quadricolor* venom proteome matched to proteins present in other sea anemones (Fig 5.3A). Likewise, 93% of toxins identified in the proteome matched to toxins in other sea anemones (Fig 5.3B). Specifically, in the venom proteome 76.5% of proteins (2,094 proteins) matched genes identified in the non-host sea anemone, *Actinia tenebrosa,* genome (Surm et al. 2019). There were 102 *A. tenebrosa* protein orthologs identified as toxins in our pipeline.

5.3.3 Gene Ontology

To better understand the functions of the gene clusters that were present in the tentacle transcriptome of *E. quadricolor*, Gene Ontology (GO) terms were assigned. While there were 46,288 gene clusters (17%) with GO terms assigned, a greater number of gene clusters had no GO terms assigned (232,987, 83%). There were 260 biological process GO terms assigned, with cellular and metabolic processes being the most frequently occurring (Table 5.4A). For the venom proteome there were 1,988 gene clusters with GO terms assigned (72.7%), with 1,368 biological process GO terms assigned (Table 5.4B).

Table 5.4: Top 10 Biological Process GO terms present in the *Entacmaea quadricolor* A) tentacle transcriptome. B) venom proteome

A)

GO terms	RNA gene clusters
cellular process	11,452
metabolic process	9,464
nitrogen compound metabolic process	7,762
macromolecule metabolic process	6,875
cellular nitrogen compound metabolic process	4,517
organic cyclic compound metabolic process	4,247
heterocycle metabolic process	4,181
cellular aromatic compound metabolic process	4,180
nucleobase-containing compound metabolic process	4,070
protein metabolic process	3,824

B)

GO terms	Protein gene clusters
unknown biological process	997
cellular process	706
metabolic process	630
organic substance metabolic process	570
primary metabolic process	521
nitrogen compound metabolic process	474
organonitrogen compound metabolic process	431
cellular metabolic process	380
macromolecule metabolic process	352
protein metabolic process	296

5.3.4 Putative toxins

Less than 0.05% of the *E. quadricolor* tentacle gene clusters were annotated as encoding putative toxins. By combining the automated toxin annotation pipeline with a manual search looking for protein families identified in Delgado et al. (2022), a set of 1,251 putative toxin gene clusters was created. This putative toxin set matched to 296 unique UniRef90 protein hits, with 22 gene clusters with no UniRef90 match. The putative toxins were assigned into eight venom function categories and 41 toxin protein families, but 105 toxin gene clusters still remain uncharacterised in terms of protein family (Figure 5.4A, Appendix S5.6). 42.8% of toxin gene clusters were classified as unknown toxins. The second most abundant venom function category was haemostatic and haemorrhagic toxins accounting for 32.7% of toxin gene clusters. Neurotoxins and toxins of unknown function are the two most diverse venom protein function categories as they included representatives from 9 and 14 toxin families respectively.

Only 5% of proteins identified in the *E. quadricolor* venom proteome were assigned as putative toxins (proteins encoded by 135 gene clusters) which matched to 78 UniRef90 hits. While proteins from all eight venom function categories were identified these were only from 25 toxin families, and 12 proteins were uncharacterised toxins (Figure 5.4B, Appendix S5.10). Unknown toxins accounted for 52.6% of toxin proteins identified, followed by haemostatic and haemorrhagic toxins accounting for 19.3% of toxin proteins. Toxins of unknown function are the most diverse venom function categories containing nine different types of toxin protein families.

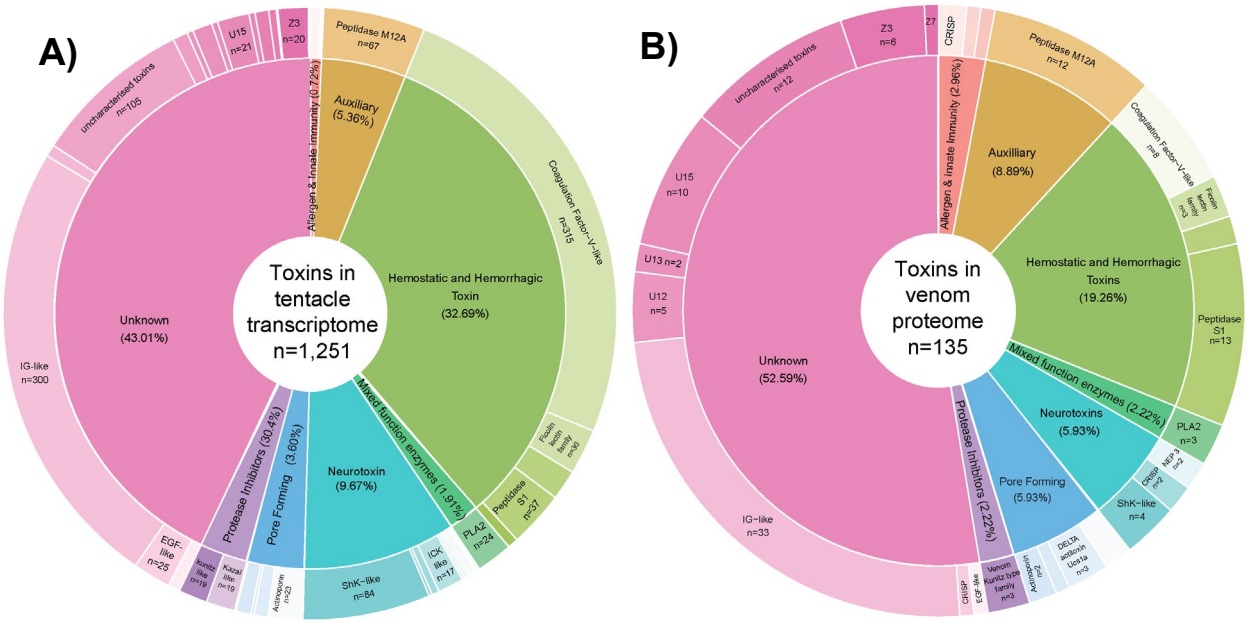


Figure 5.4 Pie donuts visualising venom category (% = percentage of venom composition) (inner circle) and toxin family (n= number of gene clusters) (outer circle) for A) tentacle transcripts, B) venom proteins.

At the toxin family level, results between the tentacle transcriptome and venom proteome were vastly different. Coagulation factor V-like toxin was the most abundant toxin in the tentacle transcriptome, whereas Immunoglobulin-like proteins (IG-like) were the most abundant in the venom proteome and the second most abundant in the tentacle transcriptome. In fact, many of the top ten families present in the venom proteome were not present in the top ten from the tentacle transcriptome (U15, U12, Z3, DELTA-actitoxin-Ucs1a) (Table 5.5), with Ficolin lectin family, EGF-like, PLA2 and Actinoporins all dropping out of the top ten.

Table 5.5: Comparison of the top 10 toxin families based on gene cluster number in A) Tentacle transcriptome B) Venom proteome

A)

Toxin family	RNA gene clusters	Protein gene clusters
Coagulation factor V-like	315	8
IG-like	300	33
Uncharacterised toxins	104	11
ShK-like	85	5
Peptidase M12A	67	12
Peptidase S1	37	13
Ficolin lectin family	30	3
EGF-like	25	1
PLA2	24	3
Actinoporins	23	1

B)

Toxin family	Protein gene clusters	RNA gene clusters
IG-like	33	300
Peptidase S1	13	37
Peptidase M12A	12	67
Uncharacterised toxins	11	104
U15	10	21
Coagulation factor V-like	8	315
Z3	6	20
U12	5	13
ShK-like	5	85
DELTA-actitoxin-Ucs1a	4	9

5.3.4.1 Uncharacterised toxins

In the tentacle transcriptome, there were 300 gene clusters assigned to IG-like proteins and 33 gene clusters in the venom proteome, making IG-like proteins the largest toxin component of the venom. The 33 IG-like proteins found in the venom proteome consisted of 18 different architectures for proteins with Immunoglobulin-like (IG-like) domains (Appendix S5.7). Fifteen of these architectures contained repeats of the IG-like domain ranging from 1-7, with three architectures containing additional functional domains: protein kinase and fibronectin type III. It is currently unclear what function that the IG-like

domain is performing in the venom proteome, but this gene family is conserved and found in the venom of several sea anemone species (Stewart Z, Undheim EA and Prentis PJ unpublished data). Multiple sequence alignment showed that 17 of the IG-like proteins found in *E. quadricolor* venom share homology with venom inhibitors identified in *Didelphis marsupiali* (DM43 and DM64) and *D. virginiana* (Alpha 1B-glycoprotein) (Fig 5.5). The *E. quadricolor* IG-like proteins are homologous to proteins identified in other sea anemones including *Nematostella vectensis* which have been annotated as hemicentin-like, due to their homology with a very large protein secreted by *Caenorhabditis elegans* that is involved in cell adhesion and extracellular matrix that contains 48 tandem IG repeat motifs (Moran et al. 2013).

There were also 104 gene clusters assigned to uncharacterised toxins in the tentacle transcriptome and 11 gene clusters in the venom proteome. The 11 uncharacterised toxins in the venom proteome consisted of 10 different architectures and 15 different domains (Appendix S5.8). Proteins were allocated to this toxin family when they were unable to be assigned to a functional venom category based on sequence alignment or toxin domain.

5.3.4.2 Peptidase S1

In the tentacle transcriptome, there were 37 gene clusters assigned as Peptidase S1 toxins with 13 gene clusters in the venom proteome making Peptidase S1 toxins the second largest toxic component of the venom. All Peptidase S1 toxins identified in the venom proteome contained a trypsin domain containing the three catalytic site residues, His, Asp and Ser except for Cluster-31550.26173. These Peptidase S1 toxins share homology with known thrombin like snake venom serine proteases such as asperase in *Bothrops asper*

(Terciopelo) and gyroxin in *Crotalus durissus terrificus* (South American rattlesnake) (Fig 5.6A).

5.3.4.3 Pore Forming and Neurotoxins

In the tentacle transcriptome there were 85 gene clusters assigned to ShK-like neurotoxins with five gene clusters in the venom proteome. Only ShK-like toxins with a single ShK-like domain were translated into the venom proteome, despite there being sequences with multiple ShK-like domains present in the tentacle transcriptome. All ShK-like toxins identified in the venom proteome shared homology with *Stichodactyla helianthus* Kappa-stichotoxin-She3a with a conserved cysteine scaffold (Fig 5.6B).

DELTA-actitoxin-Ucs1a didn't make the top ten toxin families in the tentacle transcriptome but was in the top ten venom proteome toxin families with four gene clusters being annotated as this toxin. Further only one actinoporin gene cluster was translated into the venom proteome despite being in the top ten toxin families in the tentacle transcriptome. All five of these gene clusters in the venom proteome had a conserved cytolysin domain as demonstrated by conducting a multiple sequence alignment. All cytolysins identified in the venom proteome shared homology with DELTA-AITX-Ucs1a and actinoporins from *E. quadricolor*, *Urticina crassicornis*, *Heteractis magnifica*, *H. crispa*, *Actinia tenebrosa*, *Anthopleura asiatica*, *Epiactis japonica*, *Cribrinopsis japonica* and *A. sulcata* in UniProt90. The aromatic phosphocholine (POC) site residues which help the actinoporin to interact with phosphocholines present in sphingomyelin of cell membranes and the integrin binding RGD motif that is involved in oligomerisation of actinoporin subunits were mostly conserved in the proteins present in the venom (Fig 5.6C) (Macrander and Daly 2016, Ramírez-Carreto et al. 2020).

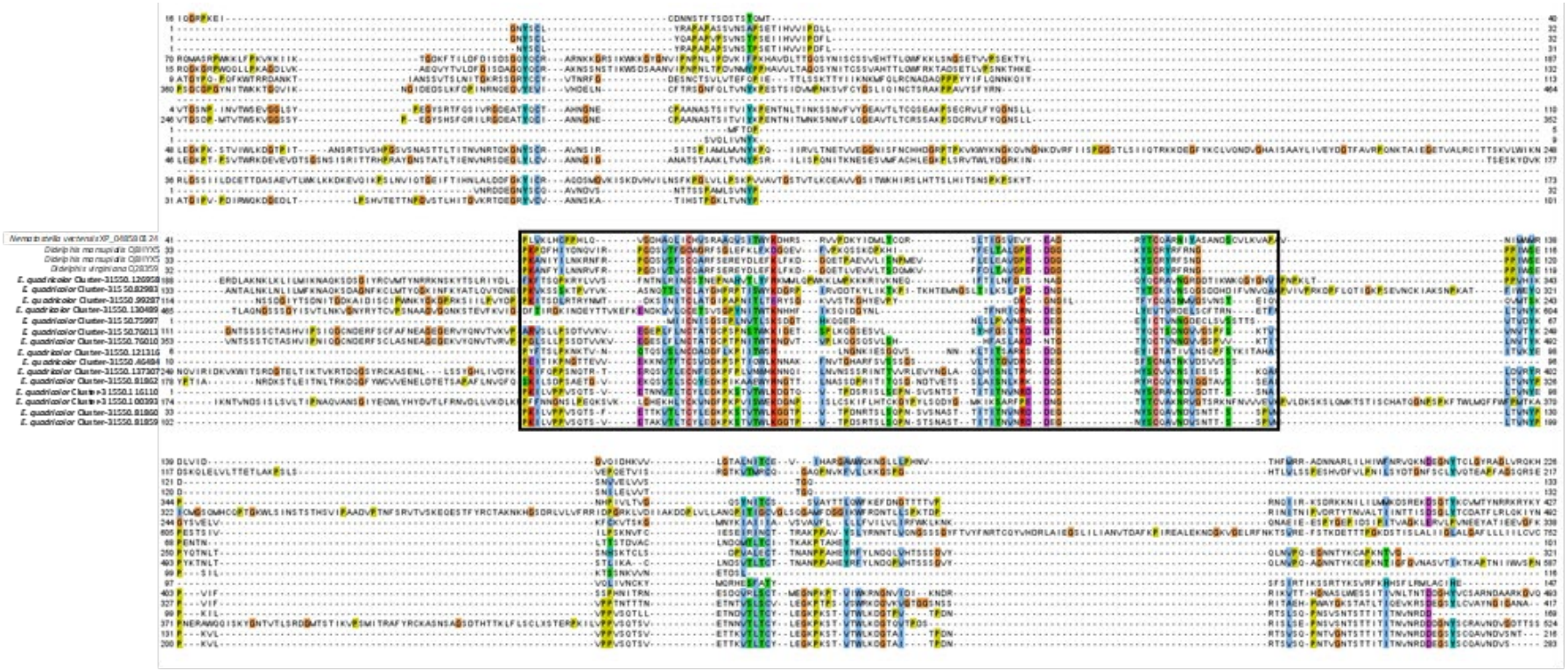


Figure 5.5: Multiple sequence alignment of *E. quadricolor* putative toxins visualised using Jalview with Clustal colour scheme. IG-like family conserved region marked with a black box. Sequences in **BOLD** are from this study, other sequences have their accession number listed

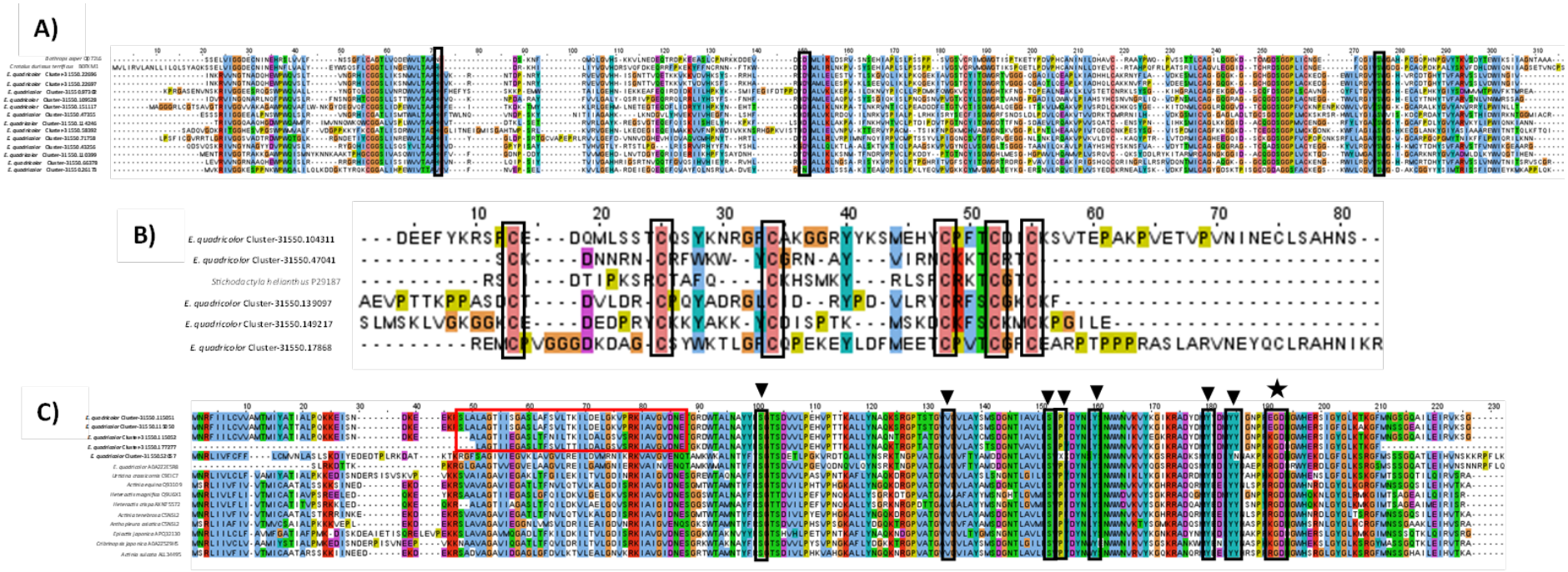


Figure 5.6: Multiple sequence alignment of *E. quadricolor* putative toxins visualised using Jalview with Clustal colour scheme A) Peptidase S1 family members mostly contain the conserved His, Asp and Ser residues of the trypsin domain indicated with a black box. B) ShK neurotoxin family all contain conserved cystines indicated with a black box. C) Cytolysin family mostly contain conserved RGD motif indicated by a star and POC binding site indicated by triangles, red box indicates conserved region matching to *E. quadricolor* sequence from Mebs (1994). Sequences in **BOLD** are from this study, other sequences have their accession number listed.

5.3.5 *Hydra* nematocyte matches

Using the only comprehensive proteome created from *Hydra magnipapillata* nematocytes (410 proteins- Balasubramanian et al. (2012)), we identified 388 gene clusters that when translated matched *Hydra* nematocyte proteins in the tentacle transcriptome of *E. quadricolor.* The nematocyte gene set represented 190 UniRef90 hits (Fig 5.7A, Appendix S5.9). Structural proteins represented 23.2% of nematocyte matches with other ECM motif proteins dominating; followed by peptidases and other enzymes (17.5% and 17% respectively). Ungrouped proteins and other enzymes were the most diverse categories with ten and seven different nematocyte protein families, respectively.

In the venom proteome, proteins from 106 gene clusters matched hydra nematocyte proteins and these represented only 63 UniRef90 proteins. Peptidases represented 26.4% of nematocyte matches with metallopeptidases dominating; followed by other enzymes and structural proteins (18.9% and 17.9% respectively) (Fig 5.7B, Appendix S5.13). Other enzymes were the most diverse category with six nematocyte protein families present in the venom proteome.

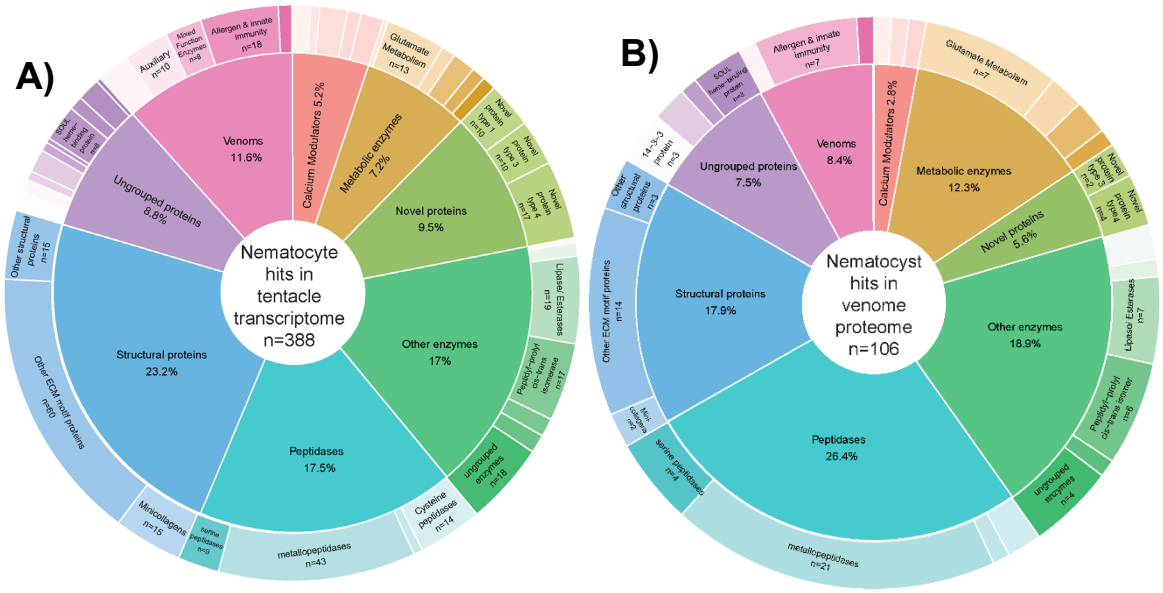


Figure 5.7: Pie donuts visualising nematocyte category (inner circle) and nematocyte functional group (outer circle) for A) tentacle transcripts, B) venom proteins.

5.3.6 Transfer of transcripts into venom proteome

The venom proteome only contained 10.8% of the gene clusters classified as toxins in the tentacle transcriptome. The allergen and innate immunity venom category had the highest transfer of gene transcripts into the venom, with 44.4% of tentacle RNA transcripts from this category, translated into proteins detected in the venom, with gene clusters from all three categories present (Table 5.6). However, we identified four out of five toxin families from the haemostatic and haemorrhagic toxin category found in the tentacle transcriptome to be present in the venom proteome while only 6.4% of gene clusters from this category are translated and transported into the venom arsenal. Neurotoxins had the second lowest rate of gene transcripts appearing as proteins in the venom proteome with only 6.6% and only three out of the nine neurotoxin toxin families identified in the transcriptome, were present in the venom proteome.

Table 5.6: Percentage of toxin gene clusters encoded into proteins by venom category. TF = toxin family.

Venom category	RNA gene clusters	TF	Protein gene clusters	TF	Percentage (%)
Allergen and innate immunity	9	3	4	3	44.4
Auxiliary	67	1	12	1	17.9
Haemostatic and haemorrhagic	409	5	26	4	6.4
Mixed function enzymes	24	1	3	1	12.5
Neurotoxins	122	9	9	3	7.3
Pore forming	45	5	8	4	17.8
Protease inhibitors	38	2	3	1	7.9
Unknown	537	16	70	9	13

The venom proteome contained 27.3% of the gene clusters that matched hydra nematocyte proteins in the tentacle transcriptome. Metabolic enzymes had the highest transfer of genes into the venom proteome with 46.4% (Table 5.7). Novel proteins had the lowest transfer of genes into venom proteins with only 16.2% and ungrouped proteins had the lowest transfer of nematocyte families with only four ungrouped protein families out of ten, present in the sea anemone venom proteome. Nematocyte gene clusters had a more than double the number of proteins appearing in the venom proteome compared to toxin gene clusters.

Table 5.7: Percentage of hydra nematocyte matched gene clusters encoded into proteins by nematocyte category. NF = nematocyte family.

Nematocyte category	RNA gene clusters	NF	Protein gene clusters	NF	Percentage (%)
Calcium Modulators	20	5	3	3	15
Metabolic Enzymes	28	6	13	4	46.4
Novel Proteins	37	3	6	2	16.2
Other Enzymes	66	7	20	6	30.3
Peptidases	68	4	28	4	41.2
Structural Proteins	90	3	19	3	21.1
Venoms	45	6	9	4	20
Ungrouped Proteins	34	10	8	4	23.5

5.4 Discussion

This study has provided the first holistic overview of gene expression and venom from the most popular host sea anemone, *Entacmaea quadricolor*; using proteotranscriptomics. Previously, the majority of studies exploring venom composition of sea anemones and other venomous species have only utilised transcriptomic approaches to profile venom composition. Here, we have provided further evidence to support the assertion that a combined proteomic and transcriptomic approach is essential to accurately profile venom composition (Madio et al. 2017, Zhao et al. 2018, Walker et al. 2020, Levin and Butter 2022), particularly in the context of symbiosis, as anemonefishes interact with host sea anemone venom proteins rather than RNA transcripts.

5.4.1 Venom Proteome

Proteotranscriptomics revealed 2,736 proteins in the venom of the host sea anemone *E. quadricolor*. This accounted for less than one percent of the total transcripts produced in the tentacle transcriptome. Only 5% of these proteins were putative toxins, revealing the majority of proteins in the venom are not of toxic origin. This is a much larger number of proteins identified compared to other sea anemone venom proteome studies, e.g. *S. haddoni* 135 proteins (Madio et al. 2017), *Anthopleura dowii* 156 proteins (Ramirez-Carreto et al. 2019) and *Bunodactis verrucosa* 413 proteins (Dominguez-Perez et al. 2018). Biological process GO terms revealed that the majority of the venom profile consisted of unknown biological processes, which was not in the top ten biological process GO terms in the tentacle transcriptome and as well as various metabolic processes. Sea anemones form a tripartite symbiosis with anemonefishes and internal zooxanthellae where both inorganic and organic carbon and nitrogen compounds are recycled between all three symbionts (Roopin et al. 2008, Cleveland et al. 2010, Roopin et al. 2011, Verde et al. 2015). This may account for the

large number of metabolic processes found in the GO terms of the tentacle transcriptome. The high number of metabolic processes GO terms in the venom proteome is more surprising, the lack of a centralised venom gland may explain the additional proteins we find in the venom, or these proteins may be required for living in an aqueous environment. This environment may pose challenges not encountered by other venomous species such as snakes and perhaps these proteins reduce dilution or maintain viscosity of venom and mucus (Mebs 1994). Overall, we have revealed that the venom profile of sea anemones is more complex than previously thought and that not all proteins in the venom are toxins; in fact, toxins only make up a very small proportion.

5.4.2 Putative toxins

We discovered a high number of toxin genes (1,251 gene clusters from 41 toxin families) via the QUT toxin pipeline used in this study, in comparison to what has been published for other host sea anemones, e.g. *Sticodactyla haddoni*: 508 toxin transcripts from 23 families and 27 toxin proteins (Madio et al. 2017), *Cryptodendrum adhaesivum*: 118 toxin transcripts from 14 families, *Macrodactyla doreensis*: 72 toxin transcripts from 13 families (Ashwood et al. 2021). Delgado et al. (2022) is the only other study to assess the venom profile of the most popular host sea anemone, *E. quadricolor*, finding 328 toxin transcripts from 37 families using six transcriptomes from the NCIB database. We suspect the higher values we found is likely due to the extensive sequencing coverage used in this study (NovaSeq S4 flow cell, paired end 2x150 bp, to achieve an average coverage of 20 million reads per sample), the addition of biological replication (n=6) both with and without anemonefish presence and the inclusion of two tentacle sampling timepoints (0 and 72-hours post venom milking) to assess regeneration (Madio et al. 2017). Furthermore, other studies have previously used higher open reading frames (ORFs) cut-offs e.g. ≥ 50-70 amino acids (Ashwood et al. 2021, Ashwood et al. 2022,

Barua et al. 2022, Delgado et al. 2022, Kashimoto et al. 2022), which may limit the discovery of smaller molecular weight toxins and explain the larger dataset we acquired in this study (ORF cut-off ≥ 30 amino acids).. The subset of these less than 7 kda (7kda is equivalent to an approximately 70 aa amino acid cut off) would not have been recovered by other studies using the higher amino acid ORF cut-offs. We found that 41.9% of gene clusters recovered encoded proteins <7 kda which accounted for 117,222 proteins in the tentacle transcriptome and 14 proteins in the venom proteome.

More importantly our pipeline did not exclude toxins without a signal peptide, as is done in most toxin pipelines. The assumption used in these other studies is that proteins require a signal peptide in order to be secreted and be trafficked into venom. However, we found 40 toxin gene clusters (29%) in the venom proteome that did not have a signal peptide present, with 74% of gene clusters in the venom proteome overall not containing a signal peptide. Many of the toxin gene clusters obtained from our RNA transcriptomics lacked the N-terminal methionine required for the start of protein translation. This is one of the limitations of Illumina sequencing, the 5' end of the mRNA transcript of genes may not be captured in the data and thus the N-terminus of a protein containing the signal peptide may be missing. Moreover, if the ORF prediction is a few nucleotides off from the "true" start, the signal peptide may not be predicted properly. For these reasons the QUT toxin pipeline does not rely on the presence of a signal peptide to assign toxins. In addition, the QUT pipeline included IG-like proteins as a toxin family, and this family has not been included as a toxin in previously published host sea anemone toxin papers, thus this very large family (300 plus gene clusters) increased our reported toxin numbers.

This study was only the second to use a combined transcriptomic and proteomic approach to study the venom of a host sea anemone. A study by Madio et al. (2017), discovered 12 new protein families based on cysteine scaffolds and amino acid sequence similarity in the venom of the host anemone *S. haddoni* by utilising a combined proteomic and transcriptomic approach. We were able to identify five of the 12 novel protein families Madio et al. (2017) found in this *S. haddoni* study (U2, U8, U9, U11, U12) within the *E. quadricolor* tentacle transcriptome, but only U12 was identified in the venom proteome. Interestingly, Madio et al. (2017) detected U2, U3, U12 proteins only in the venom proteome and did not find gene transcripts for these proteins in their *S. haddoni* tentacle transcriptome. Due to our use of the spectral library to ID the proteins in the proteomic pipeline we were unable to identify any toxins or other proteins in the venom proteome that were not found in the tentacle transcriptome, as the amino acid sequence library used to ID the proteome was generated from our transcriptome library.

5.4.3 Dominant Venom Hypothesis

The dominant venom hypothesis proposed by Smith et al. (2023), suggests that species of sea anemones are defined by a venom phenotype with one venom function dominating. In the *E. quadricolor* transcriptome, we found Coagulation factor V-like from the haemolytic and haemorrhagic toxin category to be the dominant venom function present; supporting the dominant venom hypothesis results of Delgado et al. (2022). However, our venom proteome did not reflect haemolytic and haemorrhagic toxins being the dominant venom function, instead IG-like proteins from the unknown toxin category were the dominant functional category. Only 10% of toxin gene clusters were present in both the tentacle transcriptome and encoded into the venom proteome, with a number of toxins families not appearing as proteins in the venom or had a significant reduction in their number. However, all venom

categories were present in both datasets. This reduction in encoded proteins being present in the venom results in the shift of dominant phenotype venom categories between the tentacle transcriptome and venom proteome. According to Madio et al. (2017), the major toxic component of *S. haddoni* venom varied depending on the factor used to predict it. When using the size of the toxin family, enzymatic activity was the dominant toxin component, whereas at both the tentacle transcript and venom proteome level neurotoxins were dominant. However, when Delgado et al. (2022) used Madio et al. (2017)'s *S. haddoni* transcriptomic data in their own toxin pipeline, haemolytic and haemorrhagic toxins were predicted to be the main toxin function at the transcript level. This demonstrates the disparity between different toxin pipelines and the need for a standardised approach across sea anemone venom research. Furthermore, this highlights the importance of proteomics in sea anemone venom research, and that caution needs to be applied when using transcript expression as an accurate predictor of major toxin function in venom (Madio et al. 2017), a methodology which is often used in snake and arthropod venom research. The ability to dissect out the venom gland in snakes probably results in the transcriptomic toxin profile better matching to their venom toxin profile, unlike in sea anemones who lack a centralised venom gland (Madio et al. 2019) and differ in toxin transcript composition depending on tissue type sampled (Macrander et al. 2016, Ashwood et al. 2021, Ashwood et al. 2022). Future studies should also investigate the venom profile of host sea anemone mucus using a proteotranscriptomic approach, as the mucus covering the host sea anemone's tentacles is the main surface that the anemonefish interact with.

5.4.4 Venom proteome toxin functions

This study uncovered that the toxin profile of the venom proteome of the host sea anemone *E. quadricolor* is functionally different to the toxin profile of the transcriptome. Understanding the different venom toxin components and how they function are important to better understand the ability of anemonefish to adapt to this venomous environment.

5.4.4.1 Uncharacterised toxins

Five of the top ten toxin families in the venom proteome were from the toxins of unknown function venom category. The largest component of the venom proteome was Immunoglobulin-like (IG-like) proteins, accounting for 24.4% of toxin proteins in the venom. IG-like proteins are included in the QUT toxin pipeline under the assumption that genes that are highly expressed during venom regeneration (e.g. our 72 hour post milking sample) and are highly conserved across sea anemone species can be used to indicate putative toxins (Madio et al. 2017). While it is unclear without biological assays what the function of IG-like proteins is in the venom, IG-like proteins are the largest group of natural venom inhibitor proteins. IG-like gene superfamily proteins such as oprin, AHF-1 and DMP43 are known to neutralise snake venom metalloendopeptidases (SVMPs) and phospholipases, and are found in plasma, serum or muscle of mammals such as mongoose or opossum that are resistant to some snakebites (Perales et al. 2005, Holding et al. 2016). Natural venom inhibitors allow venomous species such as snakes for example, to be resistant to their own venom (Bastos et al. 2016). The conservation and expansion of IG-like proteins in the venom of sea anemones may enable self-recognition abilities, which prevent the firing of nematocytes when their tentacles touch (Elliot et al. 1994). Molecular mimicry of host sea anemone mucus is proposed as a mechanism of anemonefishes resistance to their hosts toxins, the transfer of IG-like proteins onto anemonefishes' mucus could facilitate this process.

Uncharacterised toxins represented 8.9% of the toxins in the venom proteome. Gene clusters were allocated to this toxin family when they were unable to be assigned to a functional venom category based on sequence alignment or toxin domain. Many of these proteins contained multiple toxin domains making it unclear what function in the venom this protein will perform or if they have the potential to be cleaved by endopeptidases into multiple proteins with different toxic functions. Two representatives from Madio et al. (2017)'s novel U toxins (U15, U12) and a new novel Z toxin from the QUT toxin pipeline (Z3) also featured in the top ten toxin families in the venom proteome. As 48.8% of toxins in the venom proteome belong to the unknown venom category, it is clear that our current understanding about sea anemone venom composition and functionality is still quite limited, and more studies are required to determine the function of these newly identified 'toxins'.

5.4.4.2 Haemostatic and Haemorrhagic toxins and Peptidases

Serine proteases (Peptidase S1) were the second largest toxin component of the venom proteome accounting for 9.6%. As discussed above these toxins can cleave peptide bonds and are responsible for coordinating blood coagulation. The peptidase S1 proteins in the proteome all have trypsin domains, which is the largest family of proteases. In the non-host sea anemone *N. vectensis* trypsin domains have been found to have many putative functions including digestion, wound healing, and blood coagulation; with 17 lineages of trypsin in the common cnidarian ancestor (Madio et al. 2019). However, in comparison to snake venoms there is a distinct lack of different serine proteases in the venom of sea anemones generally as these venomous linages evolved separately.

Peptidase M12A proteins were also a dominant toxin component of the venom proteome accounting for 8.89%. Peptidase M12A has an astacin domain and the ability to cleave

peptides. Again, it is unclear what role M12A plays in the venom of sea anemones, in spider venom peptidase M12A is thought to aid in digestion and the permeability of tissue structures to help spread other toxins into the bloodstream (Trevisan-Silva et al. 2010). In snakes zinc-dependent SVMPs hydrolyse extracellular matrix components, leading to capillary rupture and systemic bleeding (Rao et al. 2022).

Despite being the dominant toxin component of the tentacle transcriptome, coagulation factor V-like toxins were only the sixth highest toxin component of the venom proteome. This low translation into the venom profile, highlights the unreliability of the tentacle transcriptome in predicting venom protein composition, with only 2.5% of coagulation factor V-like toxins transcribed into the proteome. Coagulation Factor V-like toxins have a F5/8 type-C domain, which is highly conserved and promotes binding to anionic phospholipids on the surface of platelets and endothelial cells, causing blood coagulation (clotting) (Bos et al. 2009), so clearly these proteins could form part of the anemones' toxic repertoire to kill prey.

5.4.4.3 Pore Forming and Neurotoxins

Neurotoxins were the second most diverse venom category and the third largest toxin component in the tentacle transcriptome with nine toxin families identified. However, only three of these families (ShK-like, CRISP & NEP 3 family) were found in the venom proteome. ShK-like neurotoxins were in the top ten of expressed toxin families and are type one Voltage-gated potassium (K_v) channel blockers. ShK is a 35 amino acid peptide first isolated from *Stichodactyla helianthus* whole body extracts as a potent K_v 1 channel blocker (Castañeda et al. 1995) and this study demonstrate SHK like proteins are present in *E. quadricolor* venom. The ShK domain is known to be found in combination with other toxins domains such as serine peptidases and phospholipase A_2 (Castañeda et al. 1995). An analogue of this peptide

is under clinical trial for the psoriasis treatment (Shafee et al. 2019), which highlights the potential for drug development from these newly discovered novel Shk-like proteins in *E quadricolor.*

The pore forming toxin DELTA-actitoxin-Ucs1a rounded out the top ten toxic components of the venom proteome, despite not making the top ten in the tentacle transcriptome. This cytolysin was purified from the non-host sea anemone *Urticina crassicornis* whole body extracts and permeabilises small lipid vesicles causing hemolysis (Razpotnik et al. 2009). Thus all of the *E. quadricolor* orthologues of this sequence would contribute to the haemolysis activity elicited by *E. quadricolor* whole body extracts (Mebs 1994) and that is regularly observed in venom in our lab (Hoepner et al. 2019) (Appendix S7.2). Moreover, the cytolysin sequence observed by Mebs (1994) is present in four of our sequences.

Twenty six percent of neurotoxins and six percent of pore forming toxins in the transcriptome encoded proteins <10kda but there were not any neurotoxins or pore forming toxins in this range identified in the venom proteome. Overall, there were 182,612 gene clusters with amino acid translations <10kda, but only 43 proteins were identified in the venom proteomic data in this size range. It remains unclear if the lack of neurotoxins and pore forming toxin families in the venom proteome is a result of our proteomic approach being optimal for detecting proteins, and therefore it would require a proteomics workflow optimised to identify peptides/proteins < 10 kDa; or that these toxins are not secreted into the venom proteome which aids the establishing of a symbiotic relationship with sea anemones. Delgado et al. (2022) suggested that host sea anemones produce less neurotoxin transcripts compared to non-hosts and observed that *E. quadricolor* has less neurotoxins that other host sea anemones. Thus, an alternative hypothesis to explain symbiosis could be that host sea

anemones when in association with anemonefishes reduce the number of neurotoxin families secreted into their venom, thereby enabling the association with anemonefishes to exist. This hypothesis is further explored in chapter 6.

5.4.5 *Hydra* Nematocyte matches

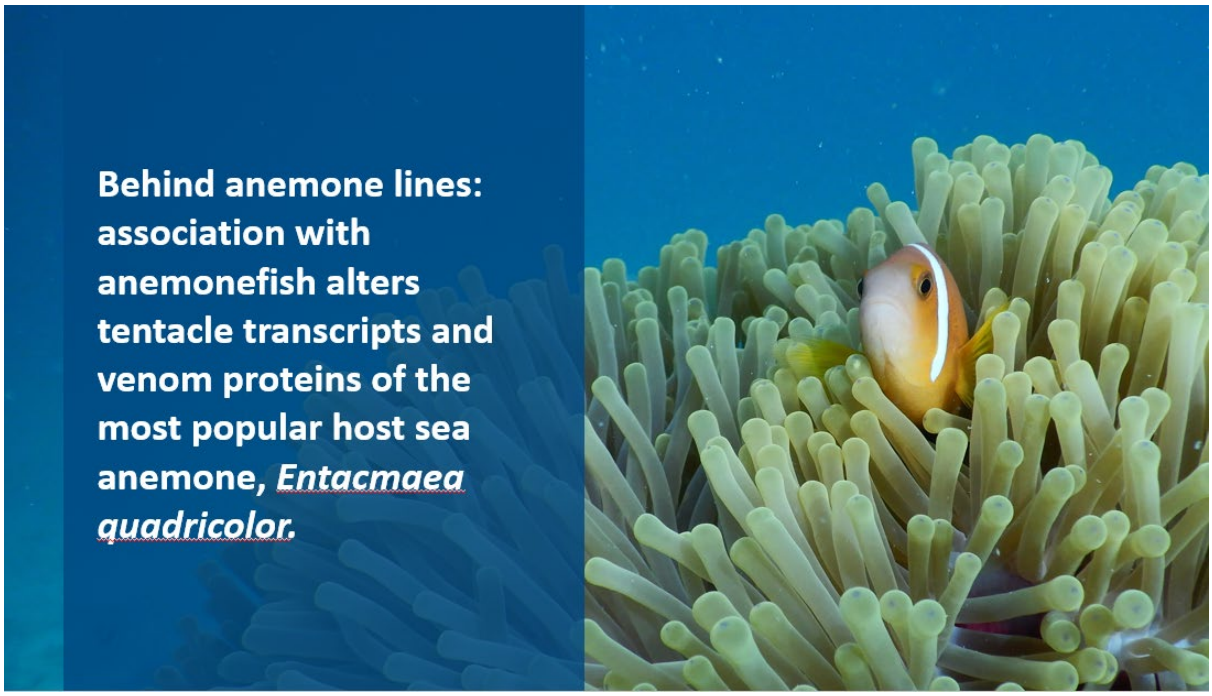
Only 27% of *Hydra* nematocyte matched transcripts were translated into proteins that appear in the venom. Most of the nematocyte families present in the tentacle transcriptome were also present in the venom proteome, albeit at lower numbers. Unlike the loss of multiple toxin families observed during translation into the venom. We did not specifically extract and test the proteome of *E. quadricolor* nematocytes, and this could account for some of the lack of hydra nematocytes matches in the venom proteome. Kashimoto et al. (2022) found several putative nematocyte genes that were relatively uniform across all host sea anemones identifying 20 potentially important nematocyte genes involved in hosting anemonefishes. However, in our findings many of the transcripts for the main structural components of the nematocyte wall identified by Kashimoto et al. (2022) in *E. quadricolor* were not present in our transcriptomic data e.g. CPP-1 and Minicollagen 3,10, 15. Kashimoto et al. (2022) identified only five gene transcripts of minicollagen-21 in the tentacle transcriptome of *E. quadricolor* compared to 8-10 transcripts in *Stichodactyla sp.* and *H. magnifica*. Our study identified 13 gene clusters of minicollagen-21 (structural proteins) in the tentacle transcriptome, this is the only minicollagen observed in our results with no minicollagen proteins identified in the venom proteome. Kashimoto et al. (2022) found one transcript of Lipase-3 (other enzymes) in *E. quadricolor,* whereas our study identified 12 clusters from this gene family in the transcriptome but only one Lipase-3 protein was present in the venom. However our results do support Kashimoto et al. (2022) in the finding that *E. quadricolor* lacks D-galactoside/L-rhamnose-binding SUEL lections (structural proteins/other ECM motif

proteins) with only one gene cluster found in the tentacle transcriptome and this protein not being identified in the venom proteome. Chapter 3 found that although host sea anemones fire fewer nematocytes at acclimated and familiar anemonefishes, they maintain a higher standard level of firing at damselfish or even unacclimated anemonefishes; demonstrating that host sea anemones need to maintain production of the proteins to build nematocytes even when anemonefishes are present.

5.5 Conclusion

This study has provided the first proteotranscriptome profile of the most popular host sea anemone, *E. quadricolor*. Focusing on the toxin and nematocyte gene profile, we found that only a subset of transcripts produced are encoded into toxin or nematocyte proteins that appear in the venom. This work also raises the perils of defining a dominant venom type based on transcriptomics data alone, as we found that the dominant venom type differed between the transcriptome and proteome. Moreover, anemonefishes interact with sea anemone proteins rather than sea anemone RNA transcripts, so it is important when determining the dominant toxin type to examine the actual toxins that are present in host sea anemone venom and mucus which anemonefishes are known to interact. Furthermore, toxins make up such a small proportion of proteins in the venom proteome revealing that there is very little knowledge about other proteins present in venom and the role that they play. This comprehensive venom profile of *E. quadricolor* can be used to inform future investigation into anemonefishes toxin resistance, particularly in relation to IG-like proteins and their potential role as venom inhibitors for anemonefishes.

Chapter 6:



Initial concept **CMH** CAA KBDS, experimental design **CMH** CAA, lab work **CMH** CAA, data analysis **CMH,** ZS, CAA, first draft **CMH**, editing **CMH** EKF CAA KBDS

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6.1 Introduction

Cnidarian venom is well recognised as a potential source for drug discovery and therapeutics; however, little research exists into the ecological factors that may influence toxin production and use (Hoepner et al. 2019, O'Hara et al. 2021, Delgado et al. 2022). Sea anemone venom is the most well-characterised of cnidarian venoms (Prentis et al. 2018), yet only a few in-depth studies on a small number of species exist (*Nematostella vectensis* Putnam et al. 2007, *Aiptasia* Baumgarten et al. 2015, *Exaiptasia pallida* Shum et al. 2022). A review by O'Hara et al. (2021), explored the ecological factors that may impact cnidarian venom composition and found that their venom can be highly plastic and does respond to environmental changes. Many cnidarian species form mutualisms with organisms ranging from microscopic algae to vertebrates, and these associations have been shown to be of evolutionary importance with effects on distribution, ecology and behaviour (Bingham et al. 2014, Titus et al. 2019), however there has been little consideration into the impact of symbiosis on venom production. Venom production has positioned sea anemones to be an ideal symbiotic partner for exosymbionts who are protected from predators as they shelter amongst their venomous tentacles (Mebs 2009, Burke da Silva and Nedosyko 2016). The sea anemone's ability to produce venom has also benefited from another symbiotic relationship, with 85% of their daily nutrient budget provided by endosymbionts (Lonnstedt and Frisch 2014, Cantrell et al. 2015). Thus, sea anemones may not have to exert as much energy sourcing prey and can divert that energy into optimising their venom composition when in association with symbionts.

One of the most iconic symbiotic relationships, is the tripartite symbiosis between host sea anemones, a photosynthetic alga (zooxanthellae) and anemonefishes. Zooxanthellae

(*Symbiodiniaceae* sp.) are intercellular algae that live within the tissue of sea anemones and via photosynthesis provide their host with energy. Sea anemones in return provide inorganic nutrients from their metabolism to the *Symbiodiniaceae* sp. Host sea anemones, have also evolved an association whereby anemonefishes live and reproduce within their venomous tentacles where they are protected from predation (Holbrook and Schmitt 2004). The anemonefishes in return defends their host sea anemone from predators, and when in association increases growth and reproduction of the sea anemone by providing nutrients from faeces and increased oxygenation from swimming amongst the tentacles (Szczebak et al. 2013, Frisch et al. 2016, Schligler et al. 2022). This tripartite symbiosis creates a closed loop of nutrient and energy transfer, where both inorganic and organic carbon and nitrogen compounds are recycled between all three symbionts (Roopin et al. 2008, Cleveland et al. 2010, Roopin et al. 2011, Verde et al. 2015) (Fig 6.1).

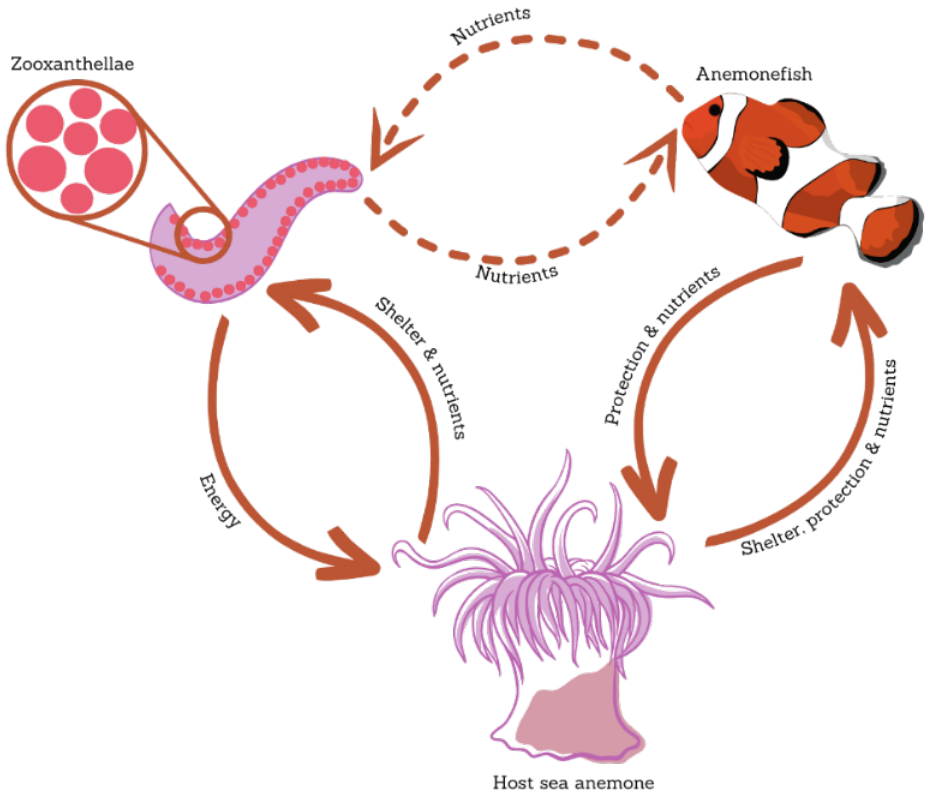

Figure 6.1: Tripartite symbiosis between host sea anemones, zooxanthellae and anemonefishes. Solid arrow indicates direct transfer between symbionts. Broken arrow indicates indirect transfer via host sea anemone. Updated from (Roopin et al. 2011).

Of the approximately 1170 sea anemone species (Rodríguez et al. 2022), only ten species from three unrelated families (*Thalassianthiade, Actinidae, Stichodactylidae*) (Titus et al. 2019), form associations (as hosts) with one or more of the 28 species of anemonefishes (*Amphiprion*) (Fautin 1991, Burke da Silva and Nedosyko 2016, Tang et al. 2021). Despite decades of study into the mechanism behind host sea anemone and anemonefishes symbiosis, it remains unclear how anemonefishes can live unharmed within the venomous tentacles of their host sea anemone (Fautin 1991, Mebs 2009, Burke da Silva and Nedosyko 2016, Hoepner et al. 2022). To-date studies exploring anemonefishes and sea anemone symbiosis have solely focused on the anemonefishes with little to no inquiry regarding the role of the host sea anemone and the evolution of sea anemone venom when in a symbiotic relationship. In order to understand the mechanisms that enable this symbiosis to occur, a more thorough picture of venom composition is required. As both symbionts benefit from their relationship, it is important to consider mechanisms that host sea anemones utilise to enhance their appeal as a host for anemonefishes.

Nedosyko et al. (2014) found variation in toxicity across the ten-host sea anemone species; with host sea anemones with a middle range toxicity forming more associations with anemonefishes species than species with a high or low toxicity. However, it is not known how toxicity of host sea anemones compares to non-host sea anemone species. The dominant venom hypothesis (Smith et al. 2023) suggests that venom of sea anemones is largely influenced by a single toxin family, under high positive selection which causes rapid diversification. Delgado et al. (2022) found that haemolytic and haemorrhagic toxins are the most prevalent and diverse toxin category found at the transcriptome level in species of host sea anemones, however, in chapter five we showed that in the venom proteome unknown

toxins were the dominant venom category and many toxins expressed in the tentacle did not get transferred into the venom as proteins, within the most preferred host sea anemone species, *E. quadricolor*. Alternatively, species of non-host anemones are generally neurotoxin dominant at the RNA transcript level (Macrander et al. 2015, Delgado et al. 2022, Smith et al. 2023). This finding indicates that the development of anemonefishes resistance to host sea anemone venom may be focused on a single venom category, rather than the development of resistance to the venom as a whole. This has led us to the hypothesis that the host sea anemone may adapt their venom to provide an optimal environment for their anemonefish symbionts to exist in. A high neurotoxic environment would be potential lethal for a vertebrate symbiont and thus a reduction in neurotoxin venom content could aid in the establishment and maintenance of a symbiotic relationship. Further, if you have a symbiont, you may not need to invest as much energy into producing protective and hunting toxins, as anemonefishes are known to protect their host sea anemone from predators while also providing added nutrients (Fig 6.1). Non-host sea anemone species likely require more energy and toxin development for capturing prey and for protection compared to host sea anemones that are aided by their symbionts.

In this study, we experimentally investigate the influence of symbiosis with *A. percula* anemonefish on the tenacle transcripts and venom proteins of *E. quadricolor*, the most popular host sea anemone. This study focused on the role the sea anemone itself may play in the establishment of their iconic symbiosis, through the potential alteration of their venom in the presence of anemonefishes using a proteotranscriptomic approach. As host sea anemones benefit from their association with anemonefishes, making changes to their venom composition to provide a more optimal venomous environment for their symbiont

anemonefish to acclimate to, would promote the establishment and evolution of this symbiotic relationship.

6.2 Materials & Methods

Chapter 6 follows on from the animal experiment, transcriptomics and proteomics methods that were described in detail in chapter 5.

6.2.1 Experimental set-up and tentacle collection

See chapter 5 for the experimental design used to collect tentacle and venom samples from *E. quadricolor* during hosting and non-hosting periods with anemonefish (*A. percula*). Briefly, three tentacle samples were taken from each sea anemone (n=6) during the non-hosting period (Fig 1). Each sea anemone was then milked for venom as previously described by Sencic and Macek (1990) & Hoepner et al. (2019). Another three tentacles were collected 72 hours post milking (Madio et al. 2017) after which a pair of *A. percula* were added to each tank for a three week acclimation period. Tentacle and venom sampling was then repeated for the hosting period.

6.2.2 Transcriptomics

6.2.2.1 Transcriptome assembly and differential gene expression analysis

See chapter 5 for details of the RNA sequencing and the assembly of a transcriptome *E. quadricolor*. Differential gene expression (DGE) analysis took place using the DESeq2 v.1.30.1 (Love et al., 2014) package in R 4.0.3 (R Core Team, 2020). RNA seq data from each sampling point was compared back to the assembled transcript to get read counts for transcripts in each individual data set (n=24). Genes with low abundance were filtered to retain only those which obtained a normalised read count of at least 10 within two or more samples (Appendix S6.1). A P-value threshold of 1e-3 was used to determine statistical significance with default

DESeq2 testing i.e., the Wald test. After low abundance gene filtering (normalised read count of at least 10 within two or more samples) and clean up there were 86,336 gene clusters (representing 312,841 transcripts), of which 55.3% (47,738 gene clusters) were unannotated. This set of gene clusters was eligible for differential gene expression analysis of the tentacle transcriptome.

6.2.2.2 Gene annotation and functional enrichment analysis

Enrichment of GO terms within groups of genes identified as being differentially expressed was assessed using GOseq v.1.42.0 (Young et al., 2010) in R. A P-value threshold of 0.05 was used here to determine statistical significance.

6.2.3 Proteomic analysis

The spectral library created in Chapter 5 was used as a database to perform Data Independent Analysis (DIA) MS analysis of individual venom samples to determine if the protein composition of anemone venom changed when hosting anemonefish.

6.2.3.1 Venom protein extraction and trypsin digestion for DIA analysis

Lyophilized venom from four *E. quadricolor* individuals in the non-hosting period and four *E. quadricolor* individuals in the hosting period underwent proteomics analysis at the Flinders University Omics Facility. Following the same methods as chapter 5, 10 ug from each individual sample where reduced and alkylated, approximately 0.9-1.5 ug of peptides was recovered from each of the eight samples as measured by a NanoDrop. See Appendix S6.1 for details.

6.2.3.2 DIA differential protein expression

For differential protein expression analysis, approximately 1ug of tryptic peptides (6.4 uL) from each individual sample was injected into a PepMap™ 100 trap column (0.3 x 5 mm, 5 µm C18, Thermo Fischer) using the methods described in Chapter 5 (5.4.2). In these individual

runs data was acquired in independent mode (DIA) (30 sec peak width, default charge = 3) using RunStart EASY-IC™ as the internal mass calibration. DIA data was collected between 350-1200 Da m/z range in windows ranging between 16-91 Da m/z (Table 6.1)

Table 6.1: M/Z windows used for DIA aquisation.

M/Z range for DIA aquisation	M/Z window	M/Z range for DIA aquisation	M/Z window
350-404	53	784-808	23
402-424	21	807-833	25
422-441 440-459	18	832-859	26
456-474 473-491 490-508	17	831-860 859-888	28
504-521 520-537	16	887-919	31
534-552	17	918-954	35
550-567	16	953-994	40
564-582 581-599 598-616 615-633 632-650	17	993-1044	50
649-668 667-686 685-704	18	1043-1109	65
703-724 723-744 743-764	20	1108-1200	91
763-786	22		

Spectronaut software V16.022 (Biognosys AG, Schlieren, Switzerland) was used for peak detection and deconvolution of the spectra utilising the spectral library created in Chapter 5 and to determine levels of the proteins detected. During the normalisation process one of the four non-hosting data sets was removed from the DIA differential expression analysis due to

too low peak intensities (Appendix S6.2) so the comparison became three non-hosting samples and four hosting samples.

6.2.4 Data Analysis

Statistical analyses were undertaken using the statistical software R (R Core Development Team 2013) using base R functions unless otherwise stated. Generalized principal components analysis for dimension reduction of non-normally distributed data (GLM-PCA) was used to compare the tentacle transcriptome between hosting and non-hosting using the R package "glmpca". PERMANOVA was run using adonis2 from the R package 'vegan' (Dixon 2003). Heatmaps of differential gene expression were created using the R package "pheatmap" with rows scaled using z-scores (x - mean(x)) / sd(x). Differential expression of putative toxins and nematocyte hits were visualised using PieDonut from the 'webr' R package. Venn diagrams were generated using https://bioinformatics.psb.ugent.be/webtools/Venn/.

6.3 Results

6.3.1 Influence of symbiosis on *Entacmaea quadricolor* transcripts and proteins

E. quadricolor tentacle gene expression was significantly different between the hosting and non-hosting periods ($p<0.0001$) (Fig 6.2A, Table 6.2). There was no significant differences in gene expression levels between the tentacle samples taken at 0 and 72 hours after milking venom in both the hosting and non-hosting periods (Fig 6.2A, Table 6.2, Appendix S6.3). Therefore the 0 and 72 hour timepoint were combined for this analysis.

There were 5,633 gene clusters that were differentially expressed ($p<0.001$) between hosting and non-hosting *E. quadricolor* tentacle transcripts (2% of 279,274 gene clusters in tentacle transcriptome – see chapter 5), with 3,649 gene clusters matching to 2,078 UniRef90 hits.

Differential gene expression analysis showed a clear split between gene clusters that increased their expression levels during hosting (54.6%, 3,096 gene clusters) and those that decreased expression levels during hosting (45.4%, 2,537 gene clusters) (Fig 6.2B). There were 827 unannotated gene clusters (26.7% of 3,096 gene clusters) that increased expression with hosting and 1,157 unannotated gene clusters (45.6% of 2,537 gene clusters) that decreased expression with hosting.

Four hundred and eighty-eight venom protein gene clusters were differentially expressed ($q<0.05$) (17.8% of 2,736 gene clusters in the venom proteome - see chapter 5), matching 287 UniRef90 hits. The DIA differential protein expression analysis showed the opposite trend to the tentacle transcriptome with the majority of differentially expressed proteins decreasing with hosting (61.9%, 302 gene clusters) (Fig 6.2C). Differentially expressed venom proteins had only eight unannotated gene clusters, all that decreased with hosting.

6.3.2 Differentially expressed gene ontology

To better understand the functions of differentially expressed tentacle transcripts, Gene Ontology (GO) terms were assigned. There were 270 Biological Process GO terms assigned to gene clusters whose expression decreased with hosting and 98 Biological Process GO terms assigned to gene clusters whose expression increased with hosting. Figure 6.3 shows the top 20 most significant biological process GO terms based on number of gene clusters assigned that decrease with hosting (Fig 6.3A) and increase with hosting (Fig 6.3B). Of the top 20 biological process GO terms that increased with hosting, 11 were present in the top 20 biological process GO terms from the whole *E. quadricolor* transcriptome (orange stars Fig 6.3B) (see chapter 5 table 5.2), however, there were no biological process GO terms that decrease with hosting present in the top 20 of the *E. quadricolor* transcriptome. Nine of the

top 20 biological process GO terms upregulated and one from the top 20 biological process GO terms downregulated were identified by Barua et al. (2022) as co-expressed in the evolution of hosting anemones compared (purple stars Fig 6.3) (Table 6.3).

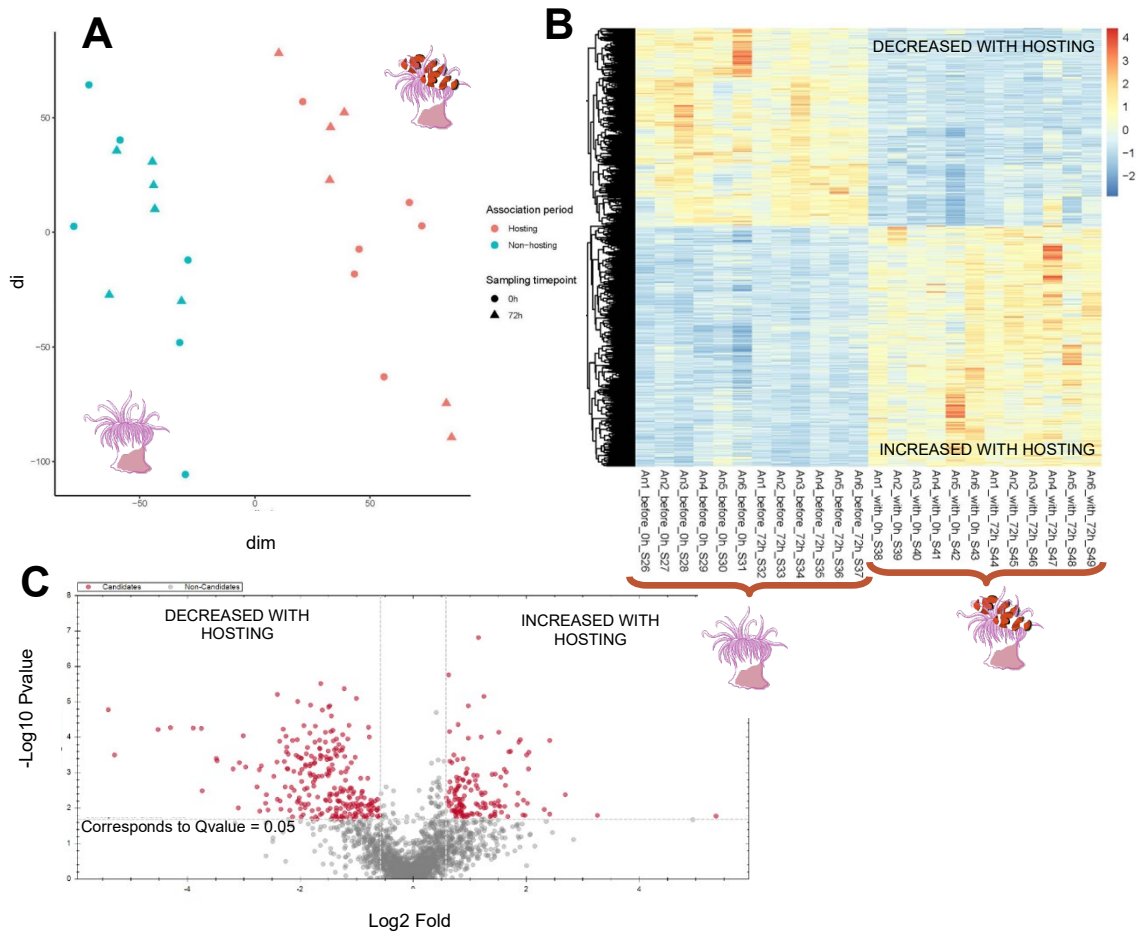

Figure 6.2: Influence of symbiosis on *Entacmaea quadricolor* tentacle transcripts and venom proteins A) Generalized principal components analysis for dimension reduction of non-normally distributed data (GLM-PCA), showing separation between the *E. quadricolor* tentacle transcript levels during the hosting and non-hosting periods. B) Gene clusters that were differentially expressed by the *E. quadricolor* tentacles during the hosting and non-hosting periods. Rows scaled using z-scores (x - mean(x)) / sd(x). P value <0.001. n=5,633 differentially expressed gene clusters (total n=24). C) Volcano plot of differentially expressed proteins from *E. quadricolor* venom proteome. Red dots are the venom proteins that are up or down regulated during hosting (total n=7).

Table 6.2: PERMANOVA of transcriptome expression between hosting and non-hosting periods (**Bold** indicates significance)

	df	Sum of Sqs	R^2	F. Model	Pr(>F)
Symbiosis (Hostng/Non-Hosting)	1	0.023373	0.17101	4.8762	**0.0001**
Hours (0hours/72hours)	1	0.008061	0.05898	1.6818	0.0943
anemoneID	1	0.009403	0.06880	1.9618	0.0584
symbiosis:hours	1	0.004602	0.03367	0.9601	0.4492
symbiosis: anemoneID	1	0.003757	0.02749	0.7838	0.6078
hours: anemoneID	1	0.006775	0.04957	1.4134	0.1744
symbiosis:hours:anemoneID	1	0.004013	0.02936	0.8373	0.5542
Residual	16	0.076691	0.56112		
Total	23	0.136675	1.00000		

Table 6.2: Biological process GO terms identified by Barua et al. (2022) as co-expressed in the evolution of hosting anemones, that were also found in our study as differentially expressed at the RNA level in tentacles.

co-expressed in the evolution of hosting anemones	**GO Term**
downregulated	Small molecule metabolic process
upregulated	RNA metabolic process
	Organic cyclic compound metabolic process
	Nucleobase-containing compound metabolic process
	Nucleic acid metabolic process
	Nitrogen compound metabolic process
	Macromolecule metabolic process
	Heterocycle metabolic process
	Cellular nitrogen compound metabolic process
	Cellular aromatic compound metabolic process

6.3.3 Differentially expressed putative toxins

There were 77 toxin gene clusters that were differentially expressed between hosting and non-hosting *E. quadricolor* tentacle transcripts (6.2% of 1,251 toxin gene clusters – see chapter 5, figure 5.4A), from 22 toxin families across seven venom function categories matching to 41 UniRef90 hits. Comparatively, only 38 toxin gene clusters were found to be differentially expressed between the hosting and non-hosting venom samples (28.1% of 135 putative toxins – see chapter 5, figure 5.4B), from 20 toxin families across seven venom categories matching to 25 UniRef90 hits. Nine toxin families from six venom categories that were found to be differentially expressed at the RNA level were also differentially expressed in the venom samples (Appendix S6.4). There were an additional ten toxin families

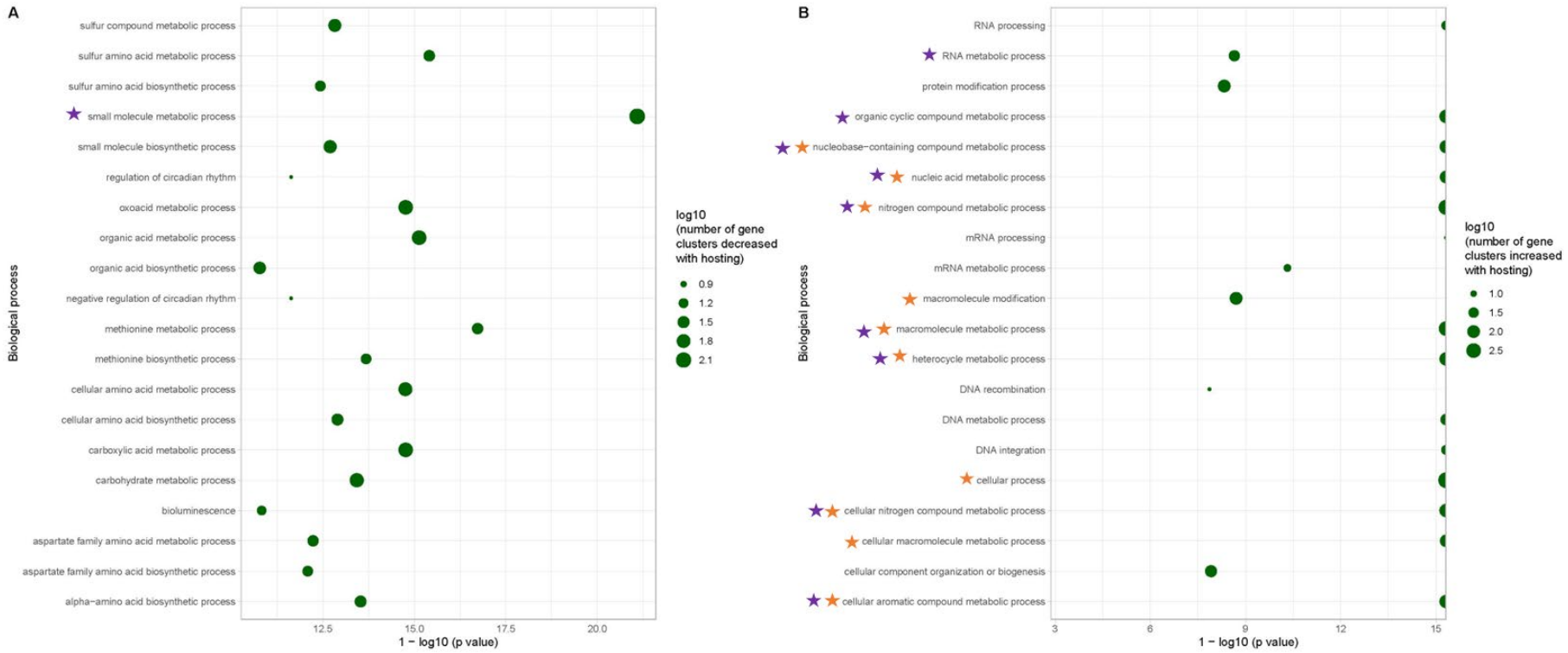

Figure 6.3: A) Top 20 most significant biological processes GO terms for differentially expressed gene clusters that decrease with hosting B) Top 20 most significant biological processes GO terms of differentially expressed gene clusters that increase with hosting. n=5,633 differentially expressed gene clusters. Size of circles represents the number of differentially expressed gene clusters associated with the specific GO terms. Horizontal axis represents increasing significance of the association. GO terms cut off at the x axis are significant beyond the limit of reporting. Orange stars indicate GO terms in the Top 20 GO terms in the *Entacmaea quadricolor* transcriptome (chapter 5 Table 5.4A). Purple Stars indicate Top 20 GO terms identified by Barua et al. (2022) as A) downregulated B) upregulated, and co-expressed in the evolution of hosting anemones compared to non-hosts (see Table 5.1 below).

that were differentially expressed at the protein level in venom where no evidence of differential expression was observed at the RNA level. Moreover 12 toxin families that were differentially expressed at the RNA level in the tentacles, were not observed as proteins in the venom. There were no pore forming tentacle transcripts that were found to be differentially expressed, whereas pore forming venom proteins accounted for 7.9% differentially expressed gene clusters of with two gene clusters decreasing with hosting and one gene cluster increasing with hosting. Allergen and innate immunity toxins were the only venom category not to have any differentially expressed venom families in the venom (tentacle transcripts did show DE in this category).

Overall, there was a clear split in both the tentacle transcripts and venom proteins between gene clusters that increased with hosting or decreased with hosting (Fig 6.4). A lower number of tentacle transcripts gene clusters increased with hosting (28.6%, 22 toxin gene clusters), compared to those gene cluster that decreased with hosting (71.4%, 55 toxin gene clusters) (Fig 6.4A). Haemostatic and haemorrhagic toxins represented the majority of toxin gene clusters that increased with hosting (40.9%, 9 gene clusters) followed by unknown toxins (27.3%, 6 gene clusters) (Fig 6.4C). Only a single neurotoxin type increased with hosting (ICK-like, 3 gene clusters), whereas neurotoxins represented the majority of toxin gene clusters that decreased with hosting (36.4%, 20 gene clusters) after unknown toxins (49.1%, 27 gene clusters) (Fig 6.4B). In the venom the majority of putative toxins differentially expressed decreased with hosting (71.1%, 27 gene clusters) compared to those that increased with hosting (28.9%, 11 gene clusters) (Fig 6.4D). Unknown toxins represented the majority of proteins that decreased with hosting (33.3%, 9 gene clusters) and increased with hosting (72.7%, 8 gene clusters) (Fig 6.4 E/F).

Figure 6.4: Differentially expressed (DE) toxin gene clusters from Entacmaea quadricolor. Inner circle is venom function and outer circle toxin family. A) DE toxin tentacle transcripts B) DE toxin tentacle transcripts that decrease with hosting, C) DE toxin tentacle transcripts that increase with hosting, D) DE toxin venom proteins, E) DE toxin venom proteins that decrease with hosting, F) DE toxin venom proteins that increase with hosting. % = percentage of DE, n= number of gene clusters.

Table 6.3: Comparison of the top toxin families differentially expressed based on gene cluster number in A) Tentacle transcripts B) Venom proteins

A)

Toxin family	RNA gene clusters	Protein gene clusters
Increase with hosting		
Coagulation factor V-like	8	1
IG-like	5	2
ICK-like	3	0
Peptidase M12A	3	1
Decrease with hosting		
Uncharacterised toxins	21	2
ShK-like	10	0
NEP 3	4	2
U12	3	2
Kazal-like	3	0

B)

Toxin family	Protein gene clusters	RNA gene clusters
Increase with hosting		
Z3	4	0
IG-like	2	5
Uncharacterised toxins	2	1
Decrease with hosting		
Peptidase M12A	4	2
U15	4	0
PLA2	3	0
Venom kunitz type family	3	0

At the toxin family level, differential expression between tentacle transcripts and venom proteins were vastly different. Coagulation factor V-like toxins had the most tentacle transcripts that increased with hosting (Table 6.3A), whereas in the venom, toxins of unknown function including Z3 and uncharacterised toxins increased the most with hosting (Table 6.3B). IG-like proteins had both tentacle transcripts and venom proteins that increased with hosting. None of the top tentacle transcript toxin families that decreased with hosting were in the top venom protein toxin families that decreased with hosting. In fact, in the tentacle transcripts Peptidase M12A was one of the top toxin

families that increased with hosting, whilst Peptidase M12A was one of the top toxin families that decreased with hosting at the protein level.

6.3.4 Differentially expressed Hydra nematocyte matches

There were 87 tentacle transcript gene clusters that matched to Hydra nematocysts that were differentially expressed between the hosting and non-hosting *E. quadricolor* host sea anemone tentacle transcripts (22.4% of 388 nematocyte gene clusters – see chapter 5, Figure 5.6A), from all eight nematocyte categories. Whereas there were 39 venom protein gene clusters that are found in Hydra nematocysts that were differentially expressed (36.8% of 106 nematocyte proteins – see chapter 5), from six nematocyte categories. Thirteen tentacle transcript nematocyte families from six nematocyte categories found to be differentially expressed were also differentially expressed in venom (Appendix S6.5). There was only one nematocyte family that was differentially expressed at the protein level in venom where no evidence of differential expression was observed at the RNA level. While five nematocyte categories that were differentially expressed at the RNA level in the tentacles, were not observed to be differentially expressed in the venom.

Again, there was a clear split between tentacle transcripts and venom proteins that matched to *Hydra* nematocytes that increased with hosting or decreased with hosting (Fig 6.5). Only 15% of differentially expressed tentacle transcripts gene clusters that matched to Hydra nematocytes increased expression with hosting (13 gene clusters), with 85% nematocyte gene clusters decreasing expression with hosting (74 gene clusters) (Fig 6.5A). Other enzymes represented the majority of nematocyte gene clusters that increased with hosting (76.9%, 10 gene clusters), and this category was dominated by lipase class 3

domain protein (Fig 6.5B). Novel proteins (as defined by Balasubramanian et al. (2012)) represented 23% (20 gene clusters) of gene clusters that decrease with hosting followed by structural proteins (19.5%, 17 gene clusters) (Fig 6.5C). Myosin motor domain protein, a structural protein, was the only venom protein hydra nematocyte match to increase with hosting, with 38 gene clusters from six venom protein nematocyte categories decreasing with hosting (Fig 6.4D). Metabolic enzymes and peptidases represented 23.7% and 26.3% respectively of hydra nematocyte matched proteins that decreased with hosting, with metallopeptidases dominating (9 gene clusters) (Fig 6.4E).

6.3.5 Matched differential expression of transcripts and proteins

Despite major differences in the number and type of genes differentially expressed in the tentacle and at the protein level in the proteome there were a number of cluster IDs that were shown to have both tentacle transcripts and the venom proteins differentially expressed (Fig 6.5). The matched differentially expressed nematocyte hits and putative toxins are listed in table 6.4. Putative toxins that are differentially expressed across both the tentacle transcriptome and venom proteome, all decreased with hosting and represented neurotoxins auxiliary toxins and a single uncharacterised toxin (also present in the Hydra nematocyte hits). Hydra nematocyte hits that are differentially expressed across both tentacle transcripts and venom proteins were from four nematocyte categories (other enzymes, metabolic enzymes, peptidases, structural proteins). All *Hydra* nematocyte matches (except one) in the venom proteins along with the majority of tentacle transcripts decreased with hosting. Interestingly, lipase class 3 domain protein and ADP-ribosyl cyclase tentacle transcripts gene clusters increased with hosting in the whereas venom protein gene clusters decreased with hosting.

Figure 6.5: Differentially expressed (DE) proteins from *Entacmaea quadricolor* that match to set of nematocyte proteins from *Hydra*. Inner circle is Nematocyte category and outer circle nematocyte family. A) DE nematocyte matched tentacle transcripts, B) DE nematocyte matched tentacle transcripts that decrease with hosting, C) DE nematocyte matched tentacle transcripts that increase with hosting, D) DE nematocyte matched venom proteins, E) DE nematocyte matched venom proteins that decrease with hosting and F) DE nematocyte matched venom proteins that increase with hosting. % = percentage of DE, n= number of gene clusters.

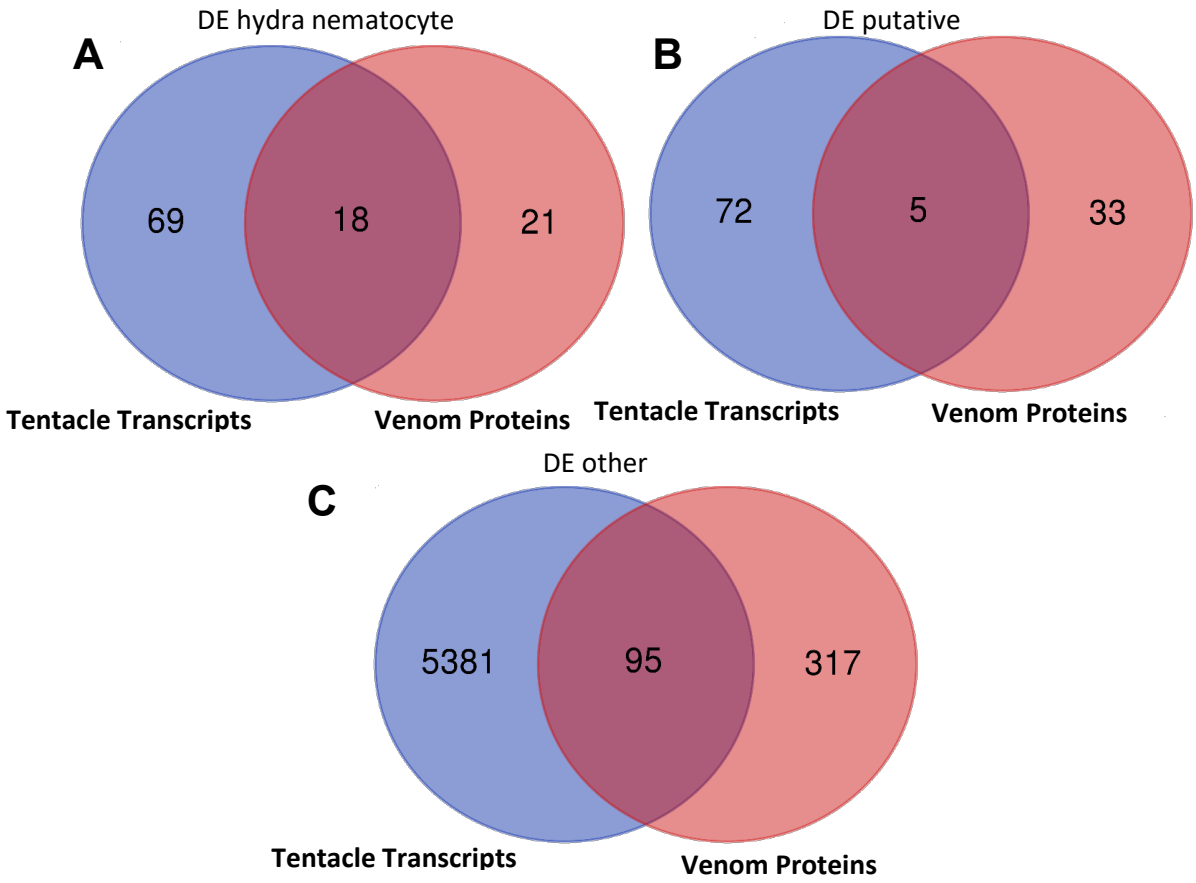

Figure 6.6: Venn diagram of unique cluster IDs differentially expressed (DE) between tentacle transcripts (blue), venom proteins (red) or both (middle). A) DE nematocytes B) DE putative toxins, C) DE other

Table 6.4: Genes clusters that were identified as differentially expressed between tentacle transcripts (TT) and venom proteins (VP). *DE in both nematocytes and venom profiles

	Cluster ID	Protein Hit	Category	TT	VP
Hydra nematocyte matches	Cluster-31550.83352/ Cluster-31550.83348	Lipase, class 3 domain protein	Other Enzymes	+2	-
	Cluster-31550.54624/ Cluster-31550.54621	NOWA-like protein	Structural proteins	-2	-
	Cluster-31550.83191	vWFA domain protein	Structural proteins	-	-
	Cluster-31550.135312	T4NP3	Novel proteins	-	-
	*Cluster-31550.87127	sT4NP21	Novel proteins	-	-
	Cluster-31550.52463	Peptidase M13 protein	Peptidases	-	-
	Cluster-31550.151378	Peptidase M1 protein	Peptidases	-	-
	Cluster-31550.137281	Peptidase family M2 Angiotensin converting enzyme	Peptidases	-	-
	Cluster-31550.104700	Peptidase S8 protein	Peptidases	-	-
	Cluster-31550.108555	ADP-ribosyl cyclase protein	Other Enzymes	+	-
	Cluster-31550.101665	Arylsulfatase B related	Other Enzymes	-	-
	Cluster-31550.119061	Cap A2 protein	Metabolic enzymes	-	-
	Cluster-31550.141798	Peptidase M14 protein	Peptidases	-	-
	Cluster-31550.112117	Gamma-glutamyltranspeptidase	Metabolic enzymes	-	-
	Cluster-31550.61837	Peptidase M13 protein	Peptidases	-	-
	Cluster-31550.149429	Bile acid glucosidase domain protein	Metabolic enzymes	-	-
Putative toxins	Cluster-31550.107594	protein piccolo-like	Neurotoxin	-	-
	Cluster-31550.107670	uncharacterized protein LOC116291117	Neurotoxin	-	-
	*Cluster-31550.87127	uncharacterized protein LOC116301037	Uncharacterised toxin	-	-
	Cluster-31550.137819	blastula protease 10	Auxiliary	-	-
	Cluster-31550.136264	Metalloendopeptidase	Auxiliary	-	-

6.4 Discussion

This study is the first to provide experimental evidence showing significant changes that occur within host sea anemone transcripts and proteins when in the presence of a symbiotic partner, the anemonefish. Work previously done to understand the mechanisms that enable this relationship, have focused on understanding adaptations that occur within the anemonefish; with very little consideration for the role the sea anemone host may play in the establishment and maintenance of this symbiosis. We have categorically demonstrated here that sea anemone hosts play a highly active role in this symbiotic relationship by making extensive changes to their transcriptome and proteome when anemonefish are present, thereby adjusting their hostile venomous environment to a more habitable site for hosting anemonefish.

We found that after three weeks of living symbiotically with an anemonefish partner, both tentacle transcripts and venom proteins of a host sea anemone are significantly different from the transcripts and proteins present before association with anemonefish. Approximately 2% of tentacle transcript gene clusters were identified as differentially expressed, with a much greater proportion of venom proteins (18%) differentially expressed. Marcionetti et al. (2019) concluded that the establishment and maintenance of sea anemone and anemonefish symbiosis may only have required a few gene changes and small variations in expression level, thus indicating that the number of differentially expressed genes we found could be sufficient to explain the association between anemonefish and host sea anemones.

Barua et al. (2022) found that symbiotic associations with anemonefish and *Symbiodiniaceae* influenced gene expression in the tentacles of host sea anemones,

particularly in relation to metabolism and biosynthesis of organic compounds. This tripartite symbiosis creates a closed nutrient loop, with each symbiont providing the other partners with a combination of nutrients, shelter and protection (Roopin et al. 2008, Cleveland et al. 2010, Roopin et al. 2011, Verde et al. 2015). Sea anemones are provided with up to 85% of their daily nutrient budget by endosymbionts (Lonnstedt and Frisch 2014, Cantrell et al. 2015), which enables them to be less reliant on catching their own food. Venom composition can subsequently be less targeted for hunting (neurotoxins) and more targeted towards defence or in the case of host sea anemones, in providing the optimal venomous environment for their exosymbioant, the anemonefish. Dutertre et al. (2014) found that the venom composition of cone snails differed significantly between defensive and predatory stimuli, with very little overlap in venom profile. Proteotranscriptomic analysis showed that both predation and defence-evoked venom were produced in the venom duct of the cone snail, with stimulus-dependent spatiotemporal release of toxins from different segments of the venom duct (Dutertre et al. 2014). This allows the cone snail to rapidly produce and release two distinctive venom types depending on the need that arises. Similarly, sea anemones have been shown to have distinctive venom profiles in different tissue types (Macrander et al. 2016, Ashwood et al. 2021, Ashwood et al. 2022), as they do not have a centralised venom gland (Madio et al. 2019). Thus, hosting sea anemones may have developed mechanisms that allow them to optimize their venom profile when anemonefish are present.

Altering their venom profile may help sea anemones to maintain associations with their symbionts, especially during suboptimal environmental conditions. For example, anemonefish increase host sea anemone resilience to bleaching events that result in the

loss of their zooxanthellae endosymbionts (Pryor et al. 2020) and thus ensuring that this relationship is maintained is essential to host sea anemone survival. Hoepner et al. (2019), found that *E. quadricolor* was able to maintain venom toxicity even when bleached. The mechanism that they use is size reduction (up to 74% reduction in size due to bleaching (Hobbs et al. 2012)) Indicating that the maintenance of their venom composition is importance not only for the sea anemone but for their continuing association with anemonefish who in return supply nutrients to support the recruitment of new endosymbionts and recovery of size (Pryor et al. 2020). Demonstrating that venom is essential to the establishment and maintenance of the tripartite symbiosis between host sea anemones, *Symbiodiniaceae* and anemonefish particularly when exposed to bleaching events that are predicted to increase in frequency into the future (Pryor et al. 2020).

Eleven of the top 20 tentacle transcript biology process GO terms that increased with hosting, were also present in the top 20 most frequently occurring biology process GO terms in the *E. quadricolor* transcriptome (as discussed in chapter 5), indicating that many of the gene clusters that increase with hosting are important to the function of the sea anemone tentacle. Barua et al. (2022) assessed the transcriptome of eight species of host sea anemones to identify genes co-expressed in the evolution of hosting anemonefish compared to sea anemones that are non-hosts. Nine of the top 20 biology process GO terms that we identified as increased whilst hosting, were also present in the top 20 co-expressed GO terms that were upregulated in the evolution of sea anemones that host anemonefish (Barua et al. 2022). There was only one downregulated GO term from our study that was found to be downregulated and co-expressed in the evolution of hosting anemones (Barua et al. 2022). Biological processes that were upregulated in the

transcriptome of host sea anemones during their evolution with anemonefish (Barua et al. 2022), were also found to further increase when anemonefish were present in our study. However, this was not seen in the biological processes that decreased during the evolution of the symbiotic relationship. We found that different biological processes decreased when anemonefish were present, such as bioluminescence, compared to the downregulated biological processes found by Barua et al. (2022). A reduction in bioluminescence, may aid in the camouflage of anemonefish symbionts at night by reducing visibility to predators thus being an adaption of host sea anemones utilise to protect their symbionts. Further, there were 95 gene clusters with tentacle transcripts and venom proteins that were differentially expressed. Exploring the GO terms associated with these proteins could provide further insight into important elements that host sea anemone alter beyond toxins and nematocytes to facilitate their association with anemonefish.

Seventy-seven tentacle transcripts and 38 venom proteins differentially expressed were putative toxins, with the majority of differentially expressed putative toxins gene clusters decreasing with hosting. Specifically, a large proportion of gene clusters that decreased with hosting were neurotoxins in the tentacle transcripts (36.4%), despite accounting for only 11% of gene clusters assigned as toxins in the whole tentacle transcriptome (chapter 5, Figure 5.4A). Only three neurotoxin venom protein gene clusters out of nine in the whole venom proteome (chapter 5, Figure 5.4B) were differentially expressed. Delgado et al. (2022) indicated that neurotoxins were underrepresented in the venom of hosting sea anemones compared to non-hosting anemones based on transcriptomics data from mixed tissues but provided no venom protein evidence for this theory. This study

demonstrates that hosting sea anemone species like *E. quadricolor* could have less neurotoxins in their venom as a whole, and when they form a symbiotic relationship with anemonefish their neurotoxin stocks could be lowered further. Again, as discussed in chapter 5 it is unclear if the proteomics methods used were inefficient for detecting low molecular weight proteins/peptides like neurotoxins or if less neurotoxins are translated into the venom to facilitate their ability to form symbiosis with anemonefish when they encounter them. Many toxin gene clusters that were down regulated in the venom proteins are involved in membrane disruption including: Peptidase M12A, Phospholipase type A_2 (PLA_2) and pore forming toxins; in paralysing prey like the Venom kunitz type family (Madio et al. 2019) or are toxins of unknown function. Downregulated toxins may be those that would have a negative impact on their anemonefish symbionts or be proteins which are difficult for the anemonefish to develop resistance to.

Delgado et al. (2022), also proposed that haemostatic and haemorrhagic toxins were the dominant toxin category in the transcriptome of host sea anemones. Whereas we found that haemostatic and haemorrhagic toxins followed unknown toxins as the dominant component of the tentacle transcriptome and venom proteome in chapter 5. Not only do toxins of unknown function dominant the tentacle transcriptome and venom proteome of the *E. quadricolor*; they also dominant venom protein gene clusters that increase with hosting. This indicates that these proteins might not function as toxins exclusively and may also facilitate association with anemonefish. Specifically, the major protein with unknown toxin function are the IG-like proteins, which had both tentacle transcripts and venom proteins that increased with hosting. As discussed in chapter 5, IG-like proteins are known to work as natural snake venom inhibitors in opossums (Bastos et al. 2016) and

the presence of many similar IG-like proteins in sea anemones suggest they could be involved in the host sea anemones' ability to self-recognise and be resistant to their own venom as has been found in snakes. Under the molecular mimicry hypothesis (chapter 1) (Elliot et al. 1994), anemonefish cover themselves in their host sea anemones' mucus to prevent detection. It could be suggested that anemonefish incorporate elements from their host sea anemone mucus or venom into their own mucus, in order to aid their resistance and acclimation to their host. The increase in IG-like proteins we observed in this study indicates that host sea anemones could increase their supply of natural venom inhibitors in the presence of anemonefish to support their symbionts resistance to venom.

Eighty-seven hydra nematocyte matches were found to be differentially expressed at the RNA level in tentacles and 39 at the protein level in the venom with the majority decreasing when in association with an anemonefish. All eight nematocyte categories had tentacle transcript gene clusters that decreased with hosting and six nematocyte categories had venom proteins that also decreased with hosting. Specifically, there were 17 nematocyst gene clusters of interest that were differentially expressed at both the RNA and protein level, representing five nematocyte categories (Metabolic enzymes, Peptidases, Other Enzymes, Structural proteins, Novel proteins). In the whole tentacle transcriptome hydra nematocyte matches accounted for <1/3rd of toxin gene clusters (488 hydra nematocyte matched gene clusters vs 1,251 toxin gene clusters chapter 5), whereas hydra nematocyte matches accounted for a similar level of differential expression to toxin gene clusters (87 DE hydra nematocyte matched gene clusters vs 77 DE toxin gene clusters). This indicates that host sea anemones may be putting more energy into altering their nematocyte composition in the presence of anemonefish. Thus, not only do host sea

anemones fire significantly fewer nematocytes at familiar and acclimated anemonefish, while maintaining a high level of firing at non-symbiotic damselfish (chapter 3), they may be able to decrease production of key nematocyte proteins and RNA transcripts when in the presence of anemonefish.

6.5 Conclusion

The most popular host sea anemone, *E. quadricolor* was able to adjust their hostile venomous environment to be a more habitable site for hosting *A. percula* anemonefish. This study is the first to provide experimental evidence that sea anemone hosts' play a highly active role in their relationship with anemonefish through extensive changes to their tentacle transcripts and venom proteins. Specifically, we found that neurotoxin tentacle transcripts and venom proteins responsible for membrane damage, pore formation, and paralysis were downregulated during hosting with anemonefish. We also found that both natural venom inhibitor tentacle transcripts and venom proteins were upregulated in the presence of anemonefish. Chapters 5 and 6 have provided us with a comprehensive profile of the *E. quadricolor* venom that symbiotic anemonefish are exposed to. This can be used to narrow down the search for toxin resistance in anemonefish by testing isolated toxin fractions in anemonefish exposure assays. Investigation into the role of the many diverse IG-like proteins found in the venom of host sea anemones, and the potential for transfer into the mucus of anemonefish enabling them to reduce their detection amongst the tentacles of a host sea anemone is a new avenue for symbiosis studies.

Chapter 7

7.1 Introduction

First recorded in 1868 (Collingwood 1868), anemonefishes and host sea anemones exhibit one of the most well-known and iconic symbiotic relationships (Mebs 2009, Hobbs et al. 2012, Nedosyko et al. 2014). Most research examining the mechanism for the establishment and maintenance of anemonefishes and sea anemone symbiosis has focused primarily on anemonefishes. Two main hypothesises exist that focus primarily on the anemonefishes' mucus layer: hypothesis 1: anemonefishes' mucus molecularly mimics the host sea anemone's mucus and hypothesis 2: anemonefishes' mucus prevents nematocysts from firing (Hoepner et al. 2022) (chapter 1). Although our understanding of the mechanisms that enable sea anemone and anemonefishes symbiosis has improved through previous research, the complete answer remains unresolved. In this thesis I have applied a range of omics techniques to further understand the molecular associations between these symbionts, with a particular focus on the role of the host sea anemone. In

addition, I also examined additional benefits for anemonefishes association with host sea anemones including a reduction in both ectoparasite load and nematocyte firing, all which have contributed to furthering our understanding of this iconic relationship.

7.2 Key findings and their significance

7.2.1 Additional benefits of associating with host sea anemones for anemonefishes.

There are many well-known mutual benefits gained from the association between anemonefishes and host sea anemones, including providing a safe site for reproduction and protection from predation for anemonefishes and growth, reproduction, protection and survival benefits for host sea anemones (Mariscal 1970b, Godwin and Fautin 1992, Nedosyko et al. 2014, Frisch et al. 2016) (chapter 1). This thesis has provided evidence for additional benefits for anemonefish that have not been previously demonstrated or measured. For example, *A. ocellaris* are largely lacking ectoparasites, thus there is no need to visit cleaning stations or have cleaner wrasse visit at their host sea anemone site (chapter 2). This novel observation suggests that their association with host sea anemones has the added benefit of reducing anemonefishes ectoparasite load and it is likely that the venomous environment of the sea anemone prevents the initial attachment or allows for the removal of ectoparasites (e.g., rubbing against tentacles). There is also a significant reduction in the number of nematocytes fired at mucus from *A. percula* that were acclimated and familiar to *E. quadricolor*, compared to a non-symbiotic damselfish (chapter 3). This finding suggests that the anemonefishes' mucus layer, once acclimated to a host sea anemone, has the potential to reduce the number of nematocytes triggered. However, it did not eliminate the nematocyte trigger completely.

7.2.2 Delayed adaptation of anemonefishes' mucus to host sea anemone presence.

Anemonefishes undertake a range of acclimation behaviours to enter and live within a host sea anemone that need to be repeated if they are removed or lose contact with their host (Balamurugan et al. 2015). These behaviours are thought to trigger the acclimation of the anemonefishes' mucus to allow for it to withstand the venomous sting of their host sea anemone. However, chapter 4 found that the lipidome of *A. percula* mucus is not altered by the presence of *E. quadricolor* and is instead in a state of flux and that the glycan profile of *A. percula* mucus was only significantly different after three weeks of association with *E. quadricolor*. As found in chapter 3, nematocytes are still fired at acclimated anemonefishes but the numbers are significantly reduced after three weeks of association, which coincides with the significant changes found in the glycan profile of the *A. percula* mucus layer (chapter 4). This finding provides support to the hypothesis that glycans in the mucus of anemonefishes are important to their ability to associate with host sea anemones. Previous research has found that Neu5Ac (a glycan side chain) triggers the firing of nematocytes (Ozacmak et al. 2001), and that *A. ocellaris* mucus has less Neu5Ac in their mucus compare to other coral reef fish (Abdullah and Saad 2015). More recently it has been proposed that during the evolution of symbiosis, anemonefishes have positively selected genes that may have the potential to cleave glycan side chains (Marcionetti et al. 2019), which could reduce frequency of host sea anemone nematocytes being triggered. Upon removal of *E. quadricolor*, chapter 4 showed that the significant changes to *A. percula* glycan composition was lost within 24 hours to 1 week. While this loss of glycan alteration after host removal matches with the requirement of re-acclimation behaviours, three weeks for glycan changes to occur is beyond the 30 minutes to 48 hours experimentally observed for initial anemonefishes acclimation. While

the findings in this chapter are extremely interesting, we would expect to see alterations to glycans in the anemonefishes' mucus layer in the first 30 minutes to 48 hours of association if this is the mechanism that enables anemonefishes to enter the sea anemone host. This delay in adaptation of the glycan profile of anemonefishes mucus when in association with a host sea anemone indicates there is a primary mechanism yet to be identified that enables association in the initial 30 minutes – 48 hours of association.

7.2.3 Discerning between key hypotheses on anemonefishes' mucus function.

In this thesis I was working towards discerning between two key hypotheses in the literature on the mechanism of anemonefishes' mucus; hypothesis 1: anemonefishes' mucus molecularly mimics the host sea anemone's mucus and hypothesis 2: anemonefishes' mucus prevents nematocysts from firing (Hoepner et al. 2022) (chapter 1). In chapter 3, I examined hypothesis 2 and showed that *A. percula* anemonefish mucus not acclimated to a host sea anemone triggers *E. quadricolor* nematocysts and that once acclimated the number of nematocysts triggered by *A. percula* mucus was significantly reduced. These results both support and question this hypothesis. The standard composition of anemonefishes mucus does not prevent the firing on host sea anemone nematocysts but once acclimated after 3 weeks of association with a host sea anemone it does significantly reduce the number of nematocysts fired, indicating that the mucus layer does play a role in this reduction. In chapter 4 I further explored how anemonefish mucus composition might allow for this significant reduction in nematocyst firing (hypothesis 2). After three weeks association with a host sea anemone the glycan profile of *A. percula* mucus was altered. Previous research has indicated that glycans can trigger sea anemone nematocyte firing.

Hypothesis 1 was also planned to be addressed in chapter 4, unfortunately, due to COVID-19 delays preventing access to the lab in Melbourne where the samples were for over two years and the subsequent loss of some of the key samples during this time this was not completed. However, I was able to compare the lipid composition of *A. percula* and *E. quadricolor* mucus to assess if their compositions became more similar when they were in association (appendix 7.1). This data showed that the lipid compositions of *A. percula* mucus and *E. quadricolor* did not merge or become more similar with association. However, future studies focused on assessing both the protein and glycan mucus composition of anemonefishes and host sea anemones when in association may enable this hypothesis to be disproved or to give direction for future studies in this important area.

7.2.4 Alteration of *Entacmaea quadricolor* venom profile with anemonefish presence.

The above hypothesises fail to consider the role of the host sea anemone in the establishment of the symbiotic relationship with anemonefish. As the host sea anemone benefits just as much from this mutualism as the anemonefish it would make sense that the host also played an active role in attracting and maintaining their symbiotic anemonefish. With decades of research into the role of the anemonefish in this relationship failing to pinpoint the mechanisms used beyond the general trope of the anemonefish mucus layer, perhaps the key lies with the host sea anemones. In order to understand how anemonefishes can withstand their venomous host environment it is important to develop an in-depth profile of host sea anemone venom to which the anemonefishes must develop resistance to. Chapter 6 revealed that *E. quadricolor* host sea anemone alters its transcriptome and proteome in the presence of acclimated *A.*

percula anemonefish, including toxin and nematocyte production, and that not all toxin transcripts produced were recruited into the venom proteome (chapter 5). The majority of differentially expressed tentacle transcript gene clusters increased with hosting, whereas the majority of differentially expressed venom proteins genes decreased with hosting. The trend of differential expression for toxins, however, was the same for the transcriptome and proteome, with the majority of toxins decreasing with hosting. These results highlight the importance of a combined transcriptomic and proteomic approach (proteotranscriptomics), particularly for sea anemones as transcript expression is not an accurate indicator of protein presence in the venom (Madio et al. 2017). This study is only the second to apply both transcriptomics and proteomics to a host sea anemone (following Madio et al. (2017) who analysed the venom of the host sea anemone *Stichodactyla haddoni*) and the first to assess the impact of anemonefish presence on venom composition. Overall, this research indicates that host sea anemones take an active role in providing an optimal habitat for anemonefishes and facilitate the formation and maintenance of this symbiotic relationship through the alteration of their venom composition.

7.3 Future directions

7.3.1 Improving toxin pipelines

Omics technologies are improving year on year and are becoming cheaper, more widely applied, and with increased sequencing coverage. In the study of venoms, venomics (the study of venom using proteomics and transcriptomics) is the dominant method used to understand the complexity and diversity of venoms across species such as snakes, spiders and sea anemones. However, the research presented in chapter 5 and 6 follows on from Madio et al. (2017) and is only the second to apply a combined transcriptomic and

proteomic approach to the study of host sea anemone venom. Annotation of toxins found via venomics relies on prior knowledge of that toxin to be present in toxin databases. From the 1170 species of sea anemones there are only 285 toxin sequences from 50 species reported in Tox-Prot (out of 7,579 reported for all venomous species) as of Mar 2023 (Jungo et al. 2012), emphasising the lack of toxin data for sea anemones. When analysing proteomic and transcriptomic data from sea anemones, venom toxin pipelines are frequently utilised in order to quickly and efficiently process and analysis high volumes of sequence data However, as each study uses slightly different methods, pipeline cut-offs and steps often including manual alignment and categorisation; this makes it difficult to compare results across studies particularly in relation to categorisation of venom function and toxin identification.

Many pipelines remove sequences from their analysis that do not have signal peptides (SignalP) present, with the assumption that sequences without a signal peptide cannot be secreted into the venom proteome. However, chapter 5 showed that 74.1% of gene clusters found to be present in the venom proteome were missing a signal peptide, including 29.2% of putative toxins. Traditional pipelines that exclude these proteins from toxin pipelines will significantly reduce the number of toxins that can be identified. Further, many pipelines use a cut-off of ≥ 50-70 amino acids (Ashwood et al. 2021, Ashwood et al. 2022, Barua et al. 2022, Delgado et al. 2022, Kashimoto et al. 2022). While simplifying and refining the number of proteins found has value in making the analysis process easier; many small molecules may be removed with this restrictive cut-off (Delgado et al. 2022).

Smith et al. (2023), has proposed a dominant venom hypothesis, which is common in the study of venom from other species, in particular snakes, and thus could be applied to sea anemones. The dominant venom hypothesis suggests that the venom of sea anemones would have one toxin function that was dominant, and Delgado et al. (2022) suggests that this theory may define species that can act as host sea anemones. Currently the classification of toxins in sea anemone venom is driven by snake sequences, after years of intensive research into the toxins composition of snake venom for anti-venom development. Due to the lack of sea anemone toxins in the Tox-Prot database, toxin pipelines often rely on snake venom sequences or are built for snakes and other better classified species and then applied to sea anemones. While there may be some overlap, this reliance could be problematic, especially when assigning function to a particular toxin sequence that has not been biologically assayed for function within sea anemone venom. Chapter 5 revealed that there were many toxin protein sequences that contained multiple domain types. For example, there were sequences protein sequences with both ShK domains and metalloendopeptidase domains, as often observed in other venomous species such as snakes. Multidomain proteins are actually more common, since extra domains increases the versatility of protein function (Babonis et al. 2019). These sequences could be miss-assigned a function based on snake venoms and may alter our understanding of how sea anemone toxin's function. Further, many proteins with multiple domains could be post translationally cleaved by the many endoproteases that are present in venom into multiple toxins each with a different function (Dutertre et al. 2013). Thus, by categorising protein function on single dominant domain alone may oversimplify the complexity of sea anemone venom and miss key toxin functionality. This is particularly important when you consider the dominant venom hypothesis for sea anemone venoms

proposed by Smith et al. (2023) . If we are incorrectly assigning function to particular toxins, or only one function to a protein that has multiple toxin functions that will also misinform grouping of sea anemone species based on their dominant venom function. This is particularly important if we are using this hypothesis to explain which species of sea anemones have evolved to host anemonefishes.

The toxin pipeline used in chapters five and six was developed by researchers at the Queensland Institute of Technology (QUT) with extensive experience working with sea anemones and was based upon an initial dataset of venom proteomes from six sea anemone species i.e., three non-host species; *Actinia tenebrosa*, *Aiptasia pulchella*, *Telmatactis* sp., and three host species: *Heteractis malu*, *Macrodactyla doreensis*, and *Stichodactyla haddoni*. Gene families needed to be present in two or more of the sea anemone venom proteomes; genes found in only one species were excluded from further consideration even if they were known to be venom toxins from previous studies. Importantly, this pipeline is based on toxins present in venom proteomes. For example, in chapter five it is apparent that not all toxins present in the transcriptome end up in the venom proteome of sea anemones. However, in order to compare our results to the literature more broadly and specifically to those found by Delgado et al. (2022), we manually added in toxins families that were not identified via the QUT pipeline.

Moving forward, I would encourage the use of toxin pipelines such as the QUT toxin pipeline designed especially for sea anemones, be applied to sea anemone toxin research. An important element of future pipeline use is the inclusion of transcripts without a signal peptide and a ORF cut-off ≥ 30 amino acids, as the absence of the signal peptide does not automatically mean it will not be secreted into the venom proteome or that it is not

present. If the ORF prediction is a few nucleotides off from the 'true' start, the signal peptide may not be predicted properly. Therefore, implementing long read next gen sequencing is important to ensure the entirety of the sequence can be captured (Amarasinghe et al. 2020) and the start and end codons can be predicted accurately. I would also recommend gene clusters be assigned to multiple venom categories, based on all toxin domains present, to more accurately reflect the complexity of the venom's functionality. The venom proteome data in chapter five will contribute to improving the QUT pipeline and may contribute to additional toxin gene families being added to the pipeline, with the sea anemone *E. quadricolour* being the second species with particular toxin gene family present that did not make the current pipeline cut-off (gene families are required to be identified in two species to be counted in the QUT toxin pipeline). Furthermore, the expansion of IG-like proteins in both the transcriptome and proteome indicate that these proteins could play an important role for sea anemones. However, it is currently unclear if they act as a toxin despite being in the venom proteome, or if they contribute to the sea anemone's ability to self-recognise and withstand its own venom (Williams and Williams 1982, Elliot et al. 1994, Holding et al. 2016, Hoepner et al. 2022). Thus, biological assays of the IG-like fraction are also important to develop in order to provide an insight into the role of IG-like proteins in the venom, and given their similarity to venom metalloproteinases inhibitors the ability of these IG-like proteins to inhibit M12 family toxin present in host sea anemone venom could be tested in future.

7.3.2 Venom proteotranscriptomics of host sea anemones

In order to understand the co-evolution of this symbiotic relationship, it is important to match phylogeny to phenotype. Distinctions as to why anemonefishes form symbiotic relationships with only ten species of anemones out of the 1170 species of sea anemones

that exist (Rodríguez et al. 2022), and why some anemonefish species are able to form associations with more than one host sea anemone species are unclear. Nedosyko et al. (2014) demonstrated that host sea anemones with high or low toxicity (based on three toxicity assays) had fewer anemonefish species that formed symbiotic relationships than sea anemones with a mid-range toxicity. This pattern did not equate to a relationship between taxonomic groups. In recent years the taxonomy of host sea anemones has been called into question (Titus et al. 2019, Nguyen et al. 2020, Kashimoto et al. 2022), with three papers proposing alternative lineages for host sea anemones. Traditionally, sea anemones that host anemonefishes were thought to have evolved on three occasions: Thallasianthidae (*Cryptodendrum adhesium*), Actiniidae (*Enctamaea quadricolour* and *Macrodactyla doreensis*) and Stichodactylidae (*Stichodactyla –gigantea, haddoni, mertensii* and *Heteractis – aurora, crispa, magnifica, malu*). All three studies found that *H. magnifica* does not sit with the other members of *Heteractis* genus but instead clusters with *Stichodactyla* via the most recent phylogenic assemblies. Titus et al. (2019) used three mitochondrial (partial 12S rDNA, 16S rDNA, and CO3) and two nuclear (18S rDNA, and partial 28S rDNA) gene markers across 5,887 base pairs to build their phylogeny. In contrast Nguyen et al. (2020) used three mitochondrial markers (16S rDNA, Cytochrome b (Cytb) and COI mtDNA) covering 1,709 base pairs and achieved a similar phylogenetic tree. Most recently, Kashimoto et al. (2022) used 1,365 orthologous gene sequences obtained via RNA transcriptomic data to build their tree. Titus et al. (2019), proposed a new phylogeny for host sea anemones: 1) *Heteractina – aurora, crispa, malu*, and *M. doreensis*, 2) *Entacmaea – E. quadricolor*, and 3) *Stichodactylina –gigantea, haddoni, mertensii* and *H. magnifica*. Titus et al. (2019) remains supportive of three separate evolutionary events but proposed different species within each occurrence. Interestingly,

these new groupings now match the groupings according to toxicity ranking and number of anemonefish species known to host in different sea anemone species as proposed by Nedosyko et al. (2014) (Figure 1). Further, it highlights *E. quadricolor* – the most popular host sea anemone – as belonging to a single lineage within host sea anemone evolution. If the *Entacmaea* linage was the first association with anemonefishes this may explain its status as the most popular host sea anemone as it would have over six million years of co-evolution with anemonefishes (Litsios et al. 2012) and refinement of its venom profile to enhance the optimal balance of protection from predators and cost/benefit of venom production and anemonefishes safety. Thus, taxonomic revision of host sea anemones will be important to reframe current and future findings in terms of the relationship between phylogeny and phenotype. Currently there is no full genome available for any of the ten host sea anemones, with the closest genome being that of the non-host *Actinia tenebrosa* (Surm et al. 2019). Genomes of host sea anemones will aid in taxonomic revision and identification of genes selected for under purifying selection at each of the three evolution events of hosting sea anemones, as Marcionetti et al. (2019) showed in anemonefishes, and may deliver further insights into the evolution of symbiosis.

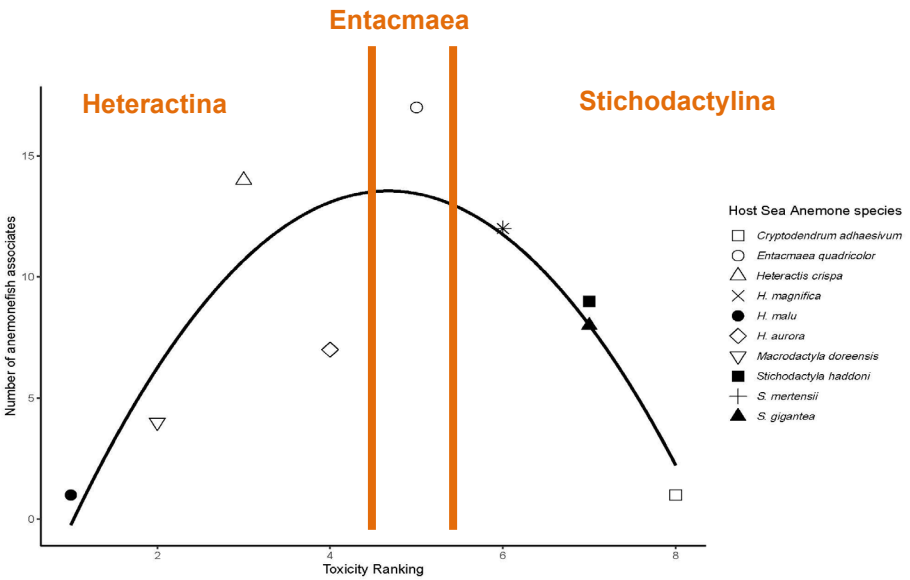

Figure 7.1: Proposed host sea anemone taxonomy from Titus et al. (2019) imposed over relationship between number of anemonefishes that associate with host sea anemones according to their toxicity ranking from Nedosyko et al. (2014) and updated in Hoepner et al. (2022) (see chapter 1).

To further refine why symbiotic associations only occur between particular host sea anemone species despite geographic overlap with sea anemones that do not host anemonefish, both proteomic and transcriptomic analysis with biological replication need to be conducted. A comparison across all ten host sea anemone species as well as some non-hosts such as *Heterodactyla hemprichii, S. helianthus, Actinia equina, A. tenebrosa* (who share geographic overlap with anemonefishes) and *Thalassianthus hemprichii* (who purportedly lost symbiosis with anemonefish (Titus et al. (2019)) could elucidate characteristics of what makes a sea anemone a suitable host for anemonefishes. As demonstrated in chapter 5, host sea anemones play a more active role in the establishment of the symbiotic relationship than previously thought, not only benefiting from the relationship but also facilitating their association with anemonefishes. By comparing both host sea anemones and non-host sea anemones by the same methods in a single study it could be determined if it is the dominant venom hypothesis that defines

host sea anemones or if it is their ability to alter the toxin composition of their venom proteome when in association with anemonefishes that sets host apart from non-hosts.

7.3.3 Mucus proteomics

7.3.3.1 Mucus proteomics of anemonefishes

In chapter 4, I had planned to compare the mucus proteome of *E. quadricolor* and *A. percula*; unfortunately, COVID-19 severely impacted the integrity and analysis of those samples, as travel to Melbourne was repeatedly delayed due to state border closures (for over two years). As both the lipid and glycoprotein profile of the *A. percula* mucus failed to reveal molecular mimicry of the *E. quadricolor* mucus or changes in composition during the initial association between the symbionts (glycoproteins took three weeks of association to reveal significant changes in composition), perhaps the answer remains hidden in my lost protein samples. As toxin proteins and peptides are secreted into the host sea anemone mucus, it could be possible under the molecular mimicry hypothesis that anemonefishes are able to acquire and incorporate some of their host's toxins into their own mucus in order to disguise themselves when in contact with their host sea anemone. Alternatively, the external fish mucus layer may not be the key to the initial entry of the anemonefishes into the venomous environment of their host. As discussed in chapter 4, Balamurugan et al. (2015) found that in the first 30 minutes of association, *A. sebe* anemonefish produce an internal mucus layer that could act as a barrier to nematocyte penetration while their external mucus layer is altered (after three weeks – chapter 4). Balamurugan et al. (2015) has been the only study to apply histology (skin fixed with Bouin's solution, sectioned with microtome to 5 µm and stained with hematoxylin and eosin) to understanding anemonefishes and sea anemone symbiosis. I would recommend repeating this experiment with additional association timepoints e.g. 30

minutes, 24 hours and 3 weeks of association, and comparing it to damselfish skin also exposed to a host sea anemone, to better understand the damage a host sea anemone would normally inflict and how that compares to anemonefishes skin. Following this, scanning electron microscopy (SEM) of the external and internal mucus layer could also provide insight into its protection capabilities and would reveal if nematocysts are trapped in the external mucus layer or penetrate the skin, potentially getting trapped by the internal mucus layer. This could provide a clearer indication of the role that the internal mucus layer may play in the initial stages of anemonefishes entering their host sea anemone.

Of the ten species of host sea anemones and 28 species of anemonefishes that form symbiotic associations, this thesis only tested one combination; therefore, care needs to be taken when applying the results of this research more broadly across symbiotic partners. *Entacmaea quadricolor* was selected as the host sea anemone species in this thesis as it is the most popular host sea anemone (forming associations with 17 anemonefish species) with the broadest relevance to understanding the association. *Amphiprion percula* was utilised as the anemonefish species throughout the thesis due to stock availability at the university and the ease of acquiring additional pairs from Australian-based suppliers. Although it is not common for *A. percula* to associate with the host sea anemone *E. quadricolor* in the wild, it is known to exist (pers. obs) and as it is one of the smallest anemonefish species they are likely competitively excluded from *E. quadricolor* in the wild by larger conspecifics inhabiting the same reef system (Fautin 1985). This pairing commonly occurs in captivity and A. *percula* have been observed to enter *E. quadricolor* within 6-48 hours of introduction (*pers. obs*). In an ideal world, all

host sea anemone and anemonefish combinations would be assessed to determine species level differences and commonalities across the symbiosis, however from a time, aquarium space, and cost perspective, it is out of the reach of this and most other research institutions. It would be beneficial to repeat these experiments with the generalist anemonefish species, *A. clarkii* (who forms associations with all ten host sea anemones) and the sea anemone hosts *C. adhesium and H. malu* (who have the highest and lowest toxicity see Fig 7.1), to determine if the findings of this thesis are consistent across the extreme ends of anemonefishes and host sea anemone symbiosis.

7.3.3.2 Mucus proteomics of host sea anemone mucus

A better understanding of the venom composition of host sea anemone mucus and any composition changes that occur during symbiosis would provide greater depth to the findings in chapter six. It is currently unclear how the toxin composition of host sea anemone nematocysts and mucus differs, if at all. As the host sea anemone's mucus secretions are the main component that the anemonefishes interact with it is important to know how similar or different it may be to the venom packaged into the nematocysts (Madio et al. 2019) – as they have different functions (nematocysts – prey acquisition and protection, mucus - protection). As not all toxins found in the tenacle transcriptome were recovered in the venom proteome (11%), we would assume that at least some of these toxins would be secreted into the mucus. Evidence for differential expression of toxin proteins in the mucus secretions of host sea anemones with anemonefish presence would provide further support for the results found in chapter 6; that host sea anemones can alter the expression of their venom composition to facilitate symbiosis with anemonefishes and would also provide further insight into the toxin composition of the mucus secretions, which are the main component anemonefishes interacts with.

7.3.4 Research avenues from other venomous predator and prey species interactions.

Just as we use anemonefishes as a model species for other research applications (Roux et al. 2020), study into the symbiotic relationship of anemones and anemonefishes may benefit from the application of concepts and knowledge from venom transcriptomic and proteomic studies from other venomous species (Sunagar et al. 2016, Madio et al. 2017) and the study of evolution of toxin resistance in prey species, particularly of prey to snake venoms (Gibbs et al. 2020). Prey species that develop resistance to venom are often able to withstand bites and the direct transfer of venom into the bloodstream. Methods of toxin resistance generally take one of three forms: venom inhibitors, target alteration and repurposed toxins (Holding et al. 2016, Arbuckle et al. 2017, Hoepner et al. 2022) (chapter 1). Typically, investigation into anemonefishes symbiosis with host sea anemones has focused on the mucus layer as a barrier rather than the evolution of resistance to host sea anemone venom. Both Mebs (1994) and Abdullah and Saad (2015) have undertaken ichthyotoxicity assays, where anemonefishes were exposed to host sea anemone extracts in seawater at different dosages and found contrasting survival outcomes Mebs (1994) used semi-purified haemolytic extract 0-1µg/ml; Abdullah and Saad (2015) used whole sea anemone extracts 0-1mg/ml. It is unclear from these studies if anemonefishes were acclimated to the host sea anemone species venom, which may account for the differences in survival found between these studies. I would recommend that this experiment be repeated with both acclimated and unacclimated anemonefishes, with exposure to host sea anemone venom both in seawater as was done in Mebs (1994) and Abdullah and Saad (2015), as well as injecting the venom directly into the bloodstream to observe whether the venom resistance mechanisms are at play rather the anemonefishes' mucus simply acting as a barrier. In addition, SEM of anemonefishes' mucus, scales and

skin when in association with a host sea anemone, will reveal if nematocysts are trapped in the mucus layer or penetrate the skin. These experiments will determine if anemonefishes are in fact resistant to host sea anemone venom or if it is the mucus layers (both internal and external) that provide a barrier from host sea anemone venom and nematocytes.

We can also learn from sea anemone predators and the mechanisms they use to navigate the sea anemones venomous sting as avenues to further explore anemonefishes resistance. Nudibranchs prey upon sea anemones by consuming tentacles and sequestering unfired nematocytes through their gut and into cnidosacs at the tip of their cerata (dorsal appendages), where they can deploy the kleptocnidae (stolen nematocytes) for their own defence (Greenwood 2009). Nudibranchs have chitin cuticles that line their mouth and throat as well as spindles (granular chitin disks) in their skin and stomach cells that act as a physical barrier to discharging nematocytes (Greenwood 2009). Similarly to anemonefish (chapter 3), nudibranchs have a mucus layer that is thought to acclimate to sea anemone venom and is able to reduce nematocyte firing by 60% (Greenwood et al. 2004). Balamurugan et al. (2015) included biting host sea anemone tentacles in the acclimation behaviours of anemonefishes, with Mariscal (1970c) and Verde et al. (2015) finding that anemonefishes will actually eat tentacles from their host sea anemone and that nematocytes were found in both stomach and faecal contents (Mariscal 1970c). None of these studies have considered how anemonefishes are able to consume tentacles that not only contain nematocytes filled with venom but are covered in toxic mucus secretions. SEM of the mouth, throat, skin and stomach of anemonefishes could reveal potential

structures used internally by the anemonefishes to withstand the consumption of toxic mucus and nematocytes.

7.3.4.1 Learnings for other symbiotic relationships

As we can apply concepts from other venomous species interactions with prey species, we can also learn from this venomous symbiotic relationship to inform symbiotic interactions more broadly. As we showed in chapter 6, host sea anemones play a really important and active role in establishment and maintenance of their relationship with anemonefishes. Previously, the research focus has laid solely on the role of anemonefishes and largely ignored any participation from host sea anemones. This thesis has highlighted the importance of equal consideration and research into both symbiotic partners, especially in mutualisms where both species benefit from the relationship and thus likely have evolved and adapted to support the other symbiotic partner and allow the relationship to occur. Further, chapter 6 has added to an emerging body of research into the impact of mutualistic relationships on gene expression. Previous papers have shown that mutualistic relationships can influence differential gene expression in both the host (Mathew and Lopanik 2014, Riesgo et al. 2014, Russell and Castillo 2020) and endosymbiont (internal symbiont) (Maor-Landaw et al. 2020, Smith and Moran 2020). My work in chapter 6 is the first to find significant differential expression of a host (sea anemone) in a mutualism with an exosymbiont (anemonefish). Host-symbiont differential gene expression shows how closely entwined symbiotic partners become when in association. Further research should explore differential gene expression in the anemonefish when in association and separated from a host sea anemone.

7.4 Conclusion

Finding Nemo brought global attention to a quirky fish and its venomous home, yet our understanding of how symbiosis between the pair can occur continues to elude us. Here, each data chapter has provided further insight on the benefits of and mechanisms behind the iconic symbiosis between anemonefishes and host sea anemones, thus meeting the overarching aims of this thesis. Given the impact of climatic changes on host sea anemones, including bleaching and size reduction, it is important to better understand how these species associate in order to ensure that this relationship persists into the future and can be maintained under a range of future climate scenarios. By applying a variety of omics techniques and using a combination of field and laboratory experiments, a stronger understanding of the role of host sea anemones in this symbiosis has been achieved. Overall, the findings of this thesis add to a growing body of literature utilising omics to uncover the mechanisms behind this symbiotic relationship. While the anemonefishes mucus layer is integral to the formation of this relationship, this thesis has demonstrated that host sea anemones play a much bigger role in the establishment and maintenance of their symbiosis with anemonefishes than previously thought. By applying novel techniques to century-old questions, this thesis has revealed that there is still so much more to uncover in this symbiotic relationship and that more work is required to truly understand how these species function together, beyond the trope of the anemonefishes' mucus layer is key.

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Appendix 1: Additional publication

Initial concept EKF, fieldwork **CMH** EKF, first draft EKF, editing **CMH** EKF

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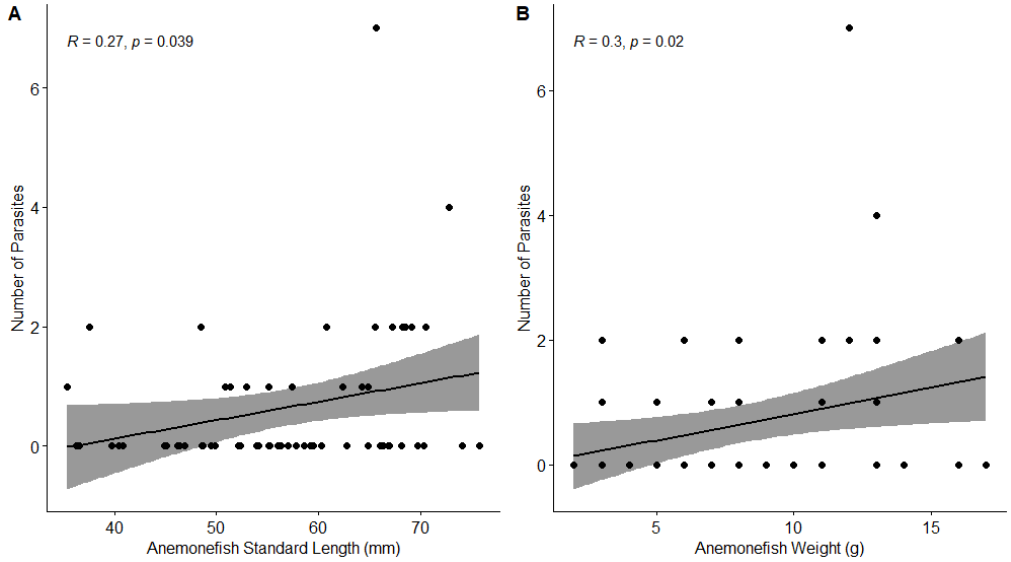
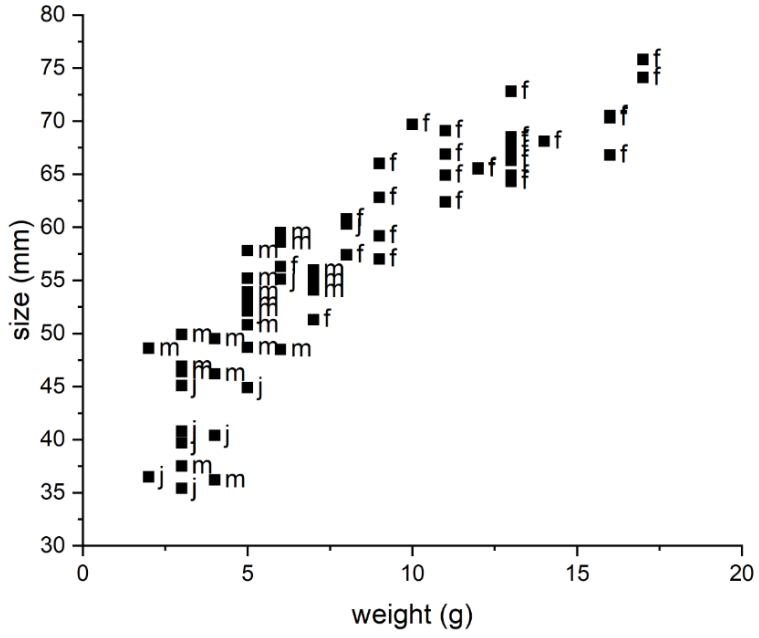
This study was published in Ecology

Hoepner, C. M., and E. K. Fobert. 2022. Just keep swimming: long-distance mobility of tomato clownfish following anemone bleaching. Ecology:e3619.
https://doi.org/10.1002/ecy.3619

APPENDIX 2: SUPPLEMENTARY MATERIAL FOR CHAPTER 2



S2.1: Correlation between ectoparasites and A) length (mm), B) weight (g) of anemonefish

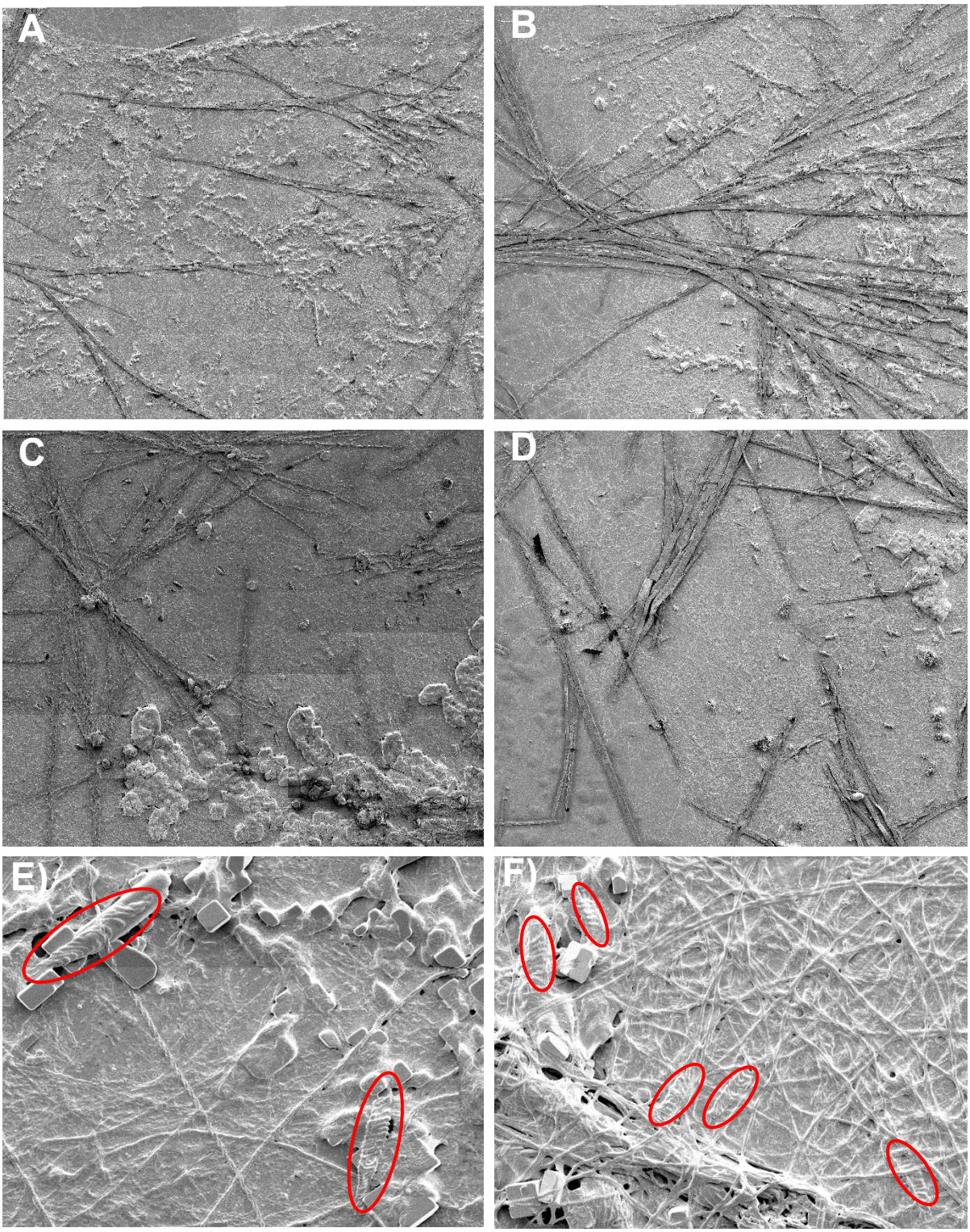


S2.2: Correlation between anemonefish size (mm) and weight (g).

APPENDIX 3: SUPPLEMENTARY MATERIAL FOR CHAPTER 3

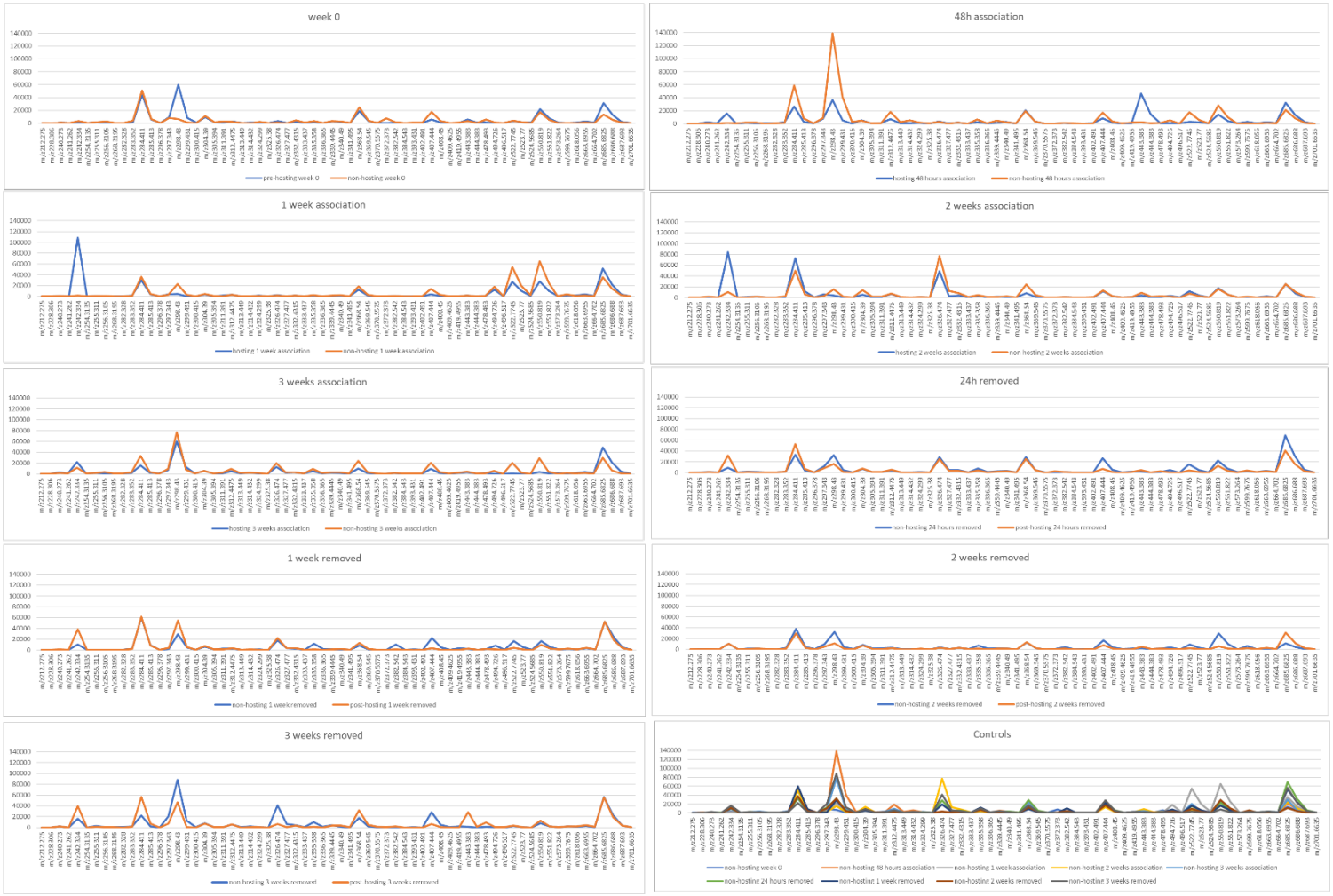
S3.1: Quantification of discharged and undischarged fired nematocytes in 1mm^2 from SEM images

Treatment	Spirocysts	Number of discharged nematocyte capsules	Number of undischarged nematocyte capsules	Total
Damselfish Before	16	242 (70%)	105	347
Prawn Before	7	373 (96%)	16	389
Anemonefish Before	2	354 (91%)	36	390
Anemonefish With	1	104 (73%)	38	142

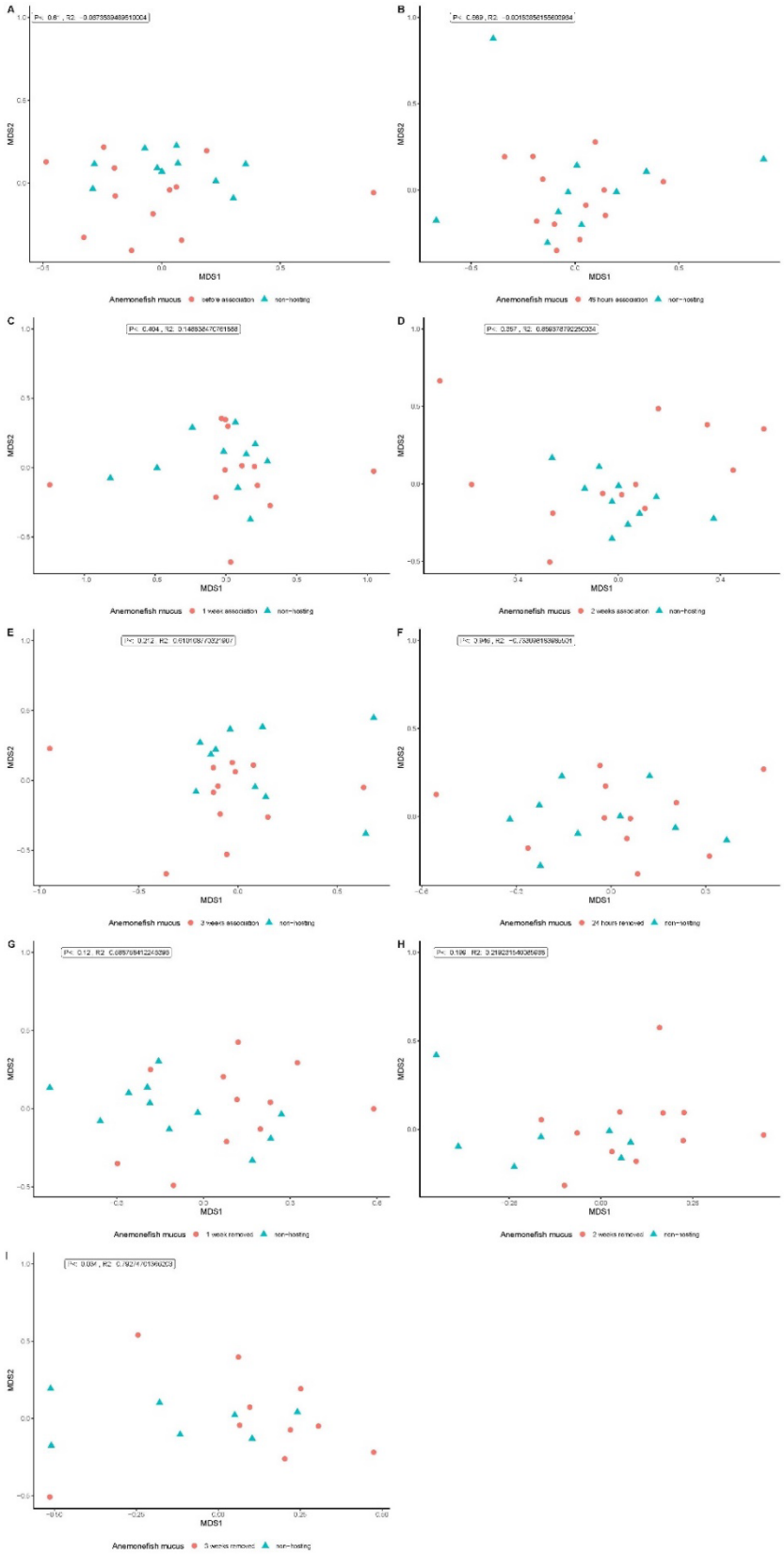


S3.2: 100 SEM images stitched together to represent 1mm^2 from each glass slide A) prawn before, B) damselfish before, C) anemonefish before, D) anemonefish with, E) example of spirocysts on the prawn before slide F) example of spirocysts on the damselfish before slide. Red circles indicate spirocysts.

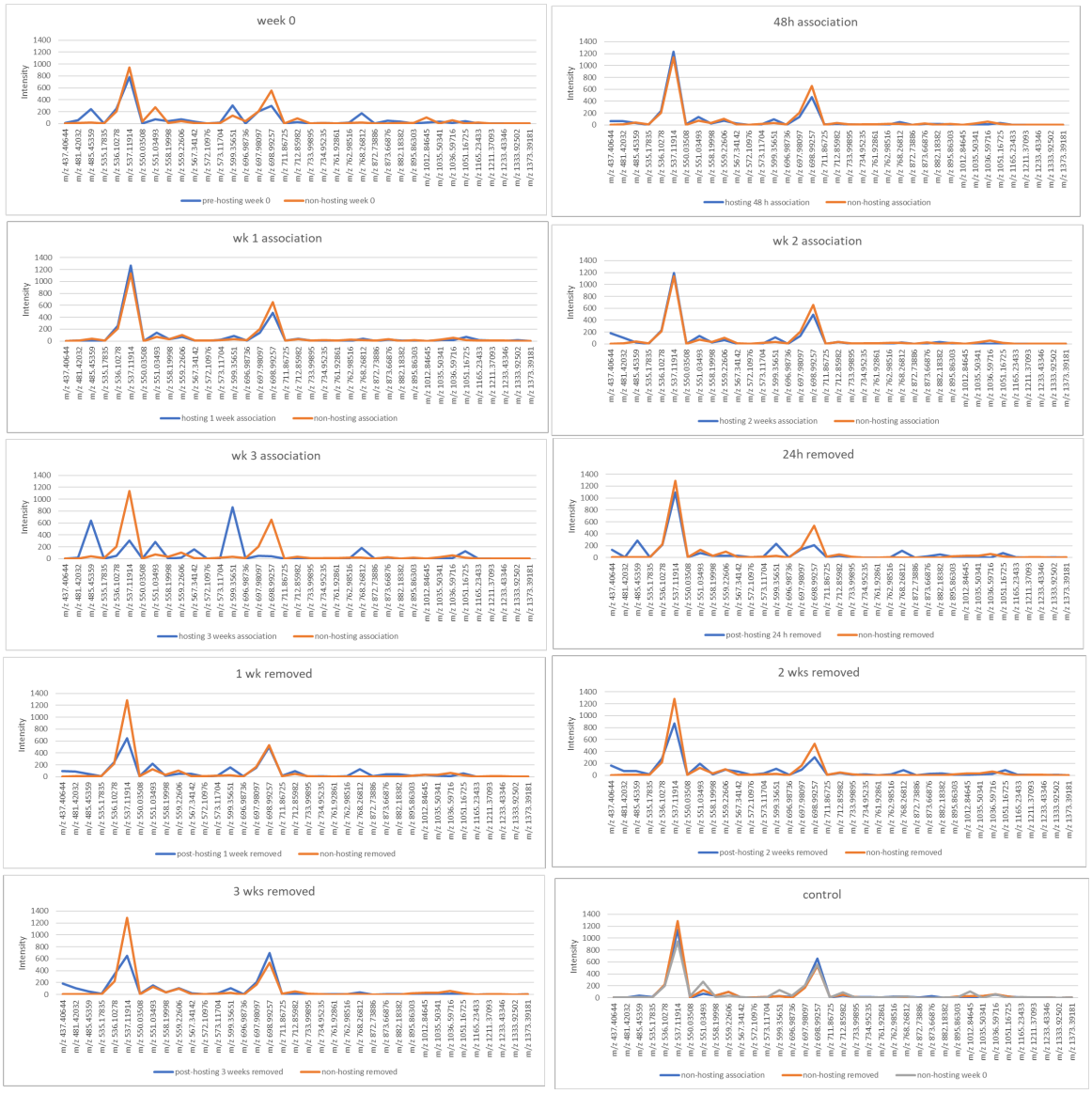
APPENDIX 4: SUPPLEMENTARY MATERIAL FOR CHAPTER 4



S4.1: Lipid intensity over the nine-week experimental period. Blue is the mean treatment group and orange the mean control group.



S4.2: Comparison of lipid Hosting and Non-Hosting profiles via nMDS. A) Pre-Hosting (week 0), B) 48 h association, C) 1 week association, D) 2 weeks association, E) 3 weeks association, F) 24h removed, G) 1 week removed, H) 2 weeks removed, I) 3 weeks removed.



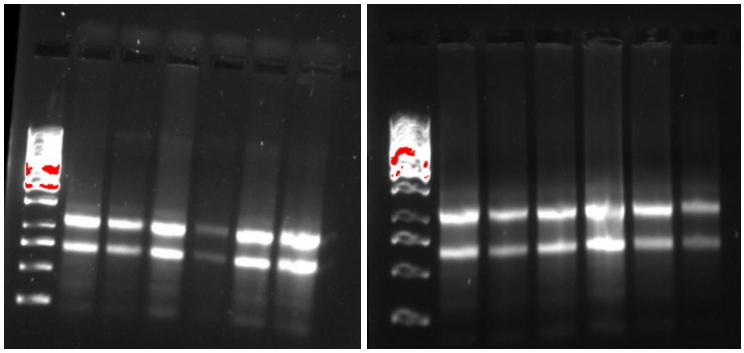
S4.3: Glycan intensity over the nine-week experimental period. Blue is the mean treatment group and orange the mean control group. Mean treatment group is the average of two pooled samples each with 3-4 samples. Mean control group is the average of three pooled samples each with 7-11 samples.

APPENDIX 5: SUPPLEMENTARY MATERIAL FOR CHAPTER 5

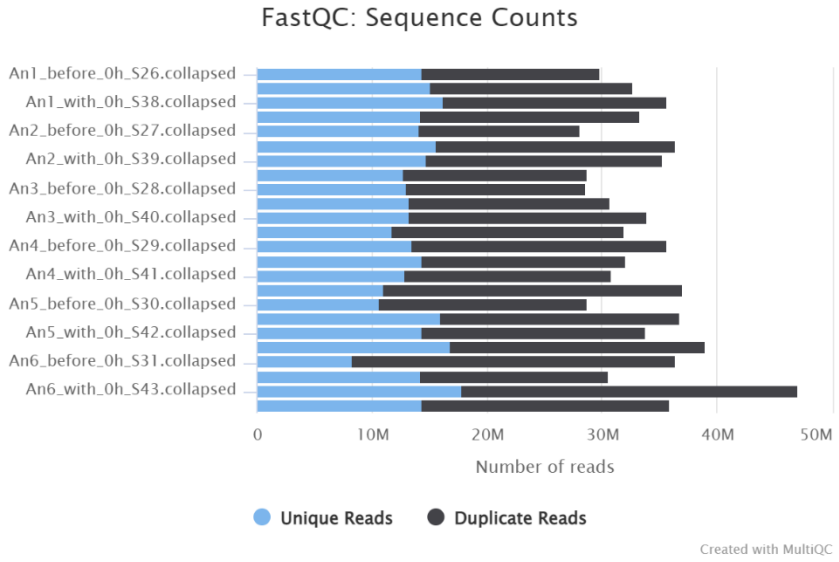
S5.1: Quality control results for RNA samples.

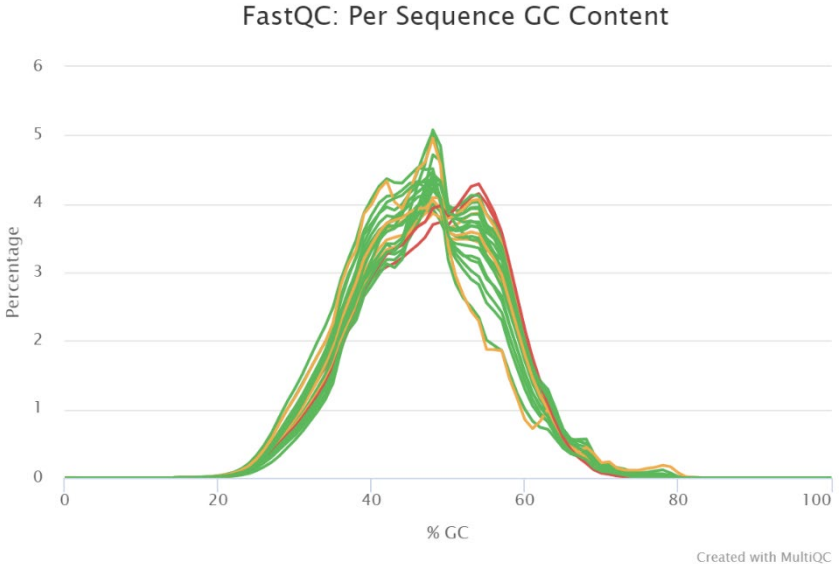
Anemonefish Association & Time period	*RNA concentration (ng/ul)*	*RNA volume*	*RIN*	*% Dups*	*% GC*	*Length (bp)*	*M Seqs*
Before 0h	71	1ug	8.9	52.00	47	169	29.8
	17.2	500ng	9.1	49.80	47	176	28.1
	47.8	1ug	9.1	54.40	46	171	28.6
	87	1ug	8.5	62.00	45	171	35.6
	28.8	1ug	8	62.70	44	172	28.7
	9.17	500ng	7.1	77.20	45	171	36.4
Before 72h	69	1ug	8.7	53.70	46	163	32.7
	41.9	1ug	8.7	57.10	47	163	36.4
	84	1ug	8.8	56.80	47	177	30.7
	95	1ug	8.4	55.00	46	174	32
	64	1ug	8.7	56.50	47	175	36.8
	47.2	1ug	8.8	53.60	47	177	30.6
With 0h	15.3	500ng	8.8	54.50	46	164	35.6
	83	1ug	8.4	58.40	47	170	35.3
	60	1ug	9.1	61.00	48	170	33.9
	55	500ng	8.6	58.40	46	175	30.8
	25.2	500ng	8.3	57.40	47	171	33.8
	47.5	1ug	9	62.10	47	171	47.1
With 72h	42.7	1ug	9.2	57.50	45	168	33.4
	23	500ng	9	55.70	47	176	28.7
	50	1ug	9.2	63.30	47	168	32
	7.85	200ng	7.8	70.20	47	174	37
	48.8	1ug	9	56.80	46	166	39
	56	1ug	9	59.90	46	169	35.9

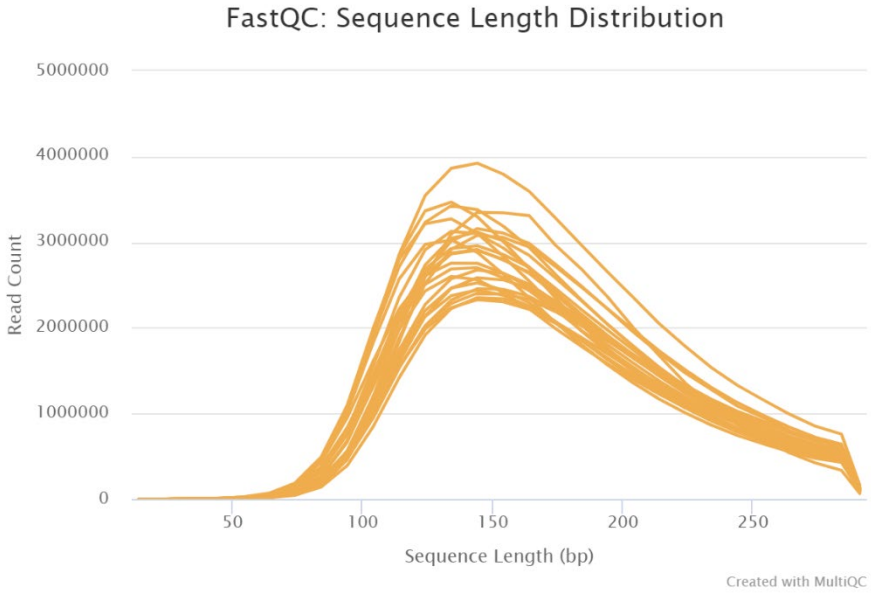
S5.2: Agarose gel showing RNA quality.



S5.3: Quality control graphs for RNA sequencing.







S5.4: Symbiont data sources

Citation	Species
(González-Pech et al., 2021)	• *S. linucheae* (genome) • *S. microadriaticum CassKB8* (genome) • *S. microadriaticum 04-503SCI.03* (genome) • *S. natans* (genome) • *S. necroappetens* (genome) • *S. tridacnidorum* (genome)
(Levin et al., 2016)	• Mixed *Symbiodinium* population isolated from *Acropora tenuis* from South Molle Island (transcriptome) • Mixed *Symbiodinium* population isolated from *Acropora tenuis* from Magnetic Island (transcriptome)
(Camp et al., 2022)	• *Cladocopium goreaui* (transcriptome) • *Durusdinium trenchii* (transcriptome) • *Breviolum* sp. (transcriptome)
(Arriola et al., 2018)	• *Micractinium conductrix* (genome) • *Chlorella sorokiniana* UTEX 1602 (genome)

S5.5 Anemone data sources

Citation	Species
(Wilding et al., 2020)	• *Actinia equina* (genome)
(Unpublished, ePGL)	• *Actinia tenebrosa* (genome) • *Aulactinia veratra* (genome)

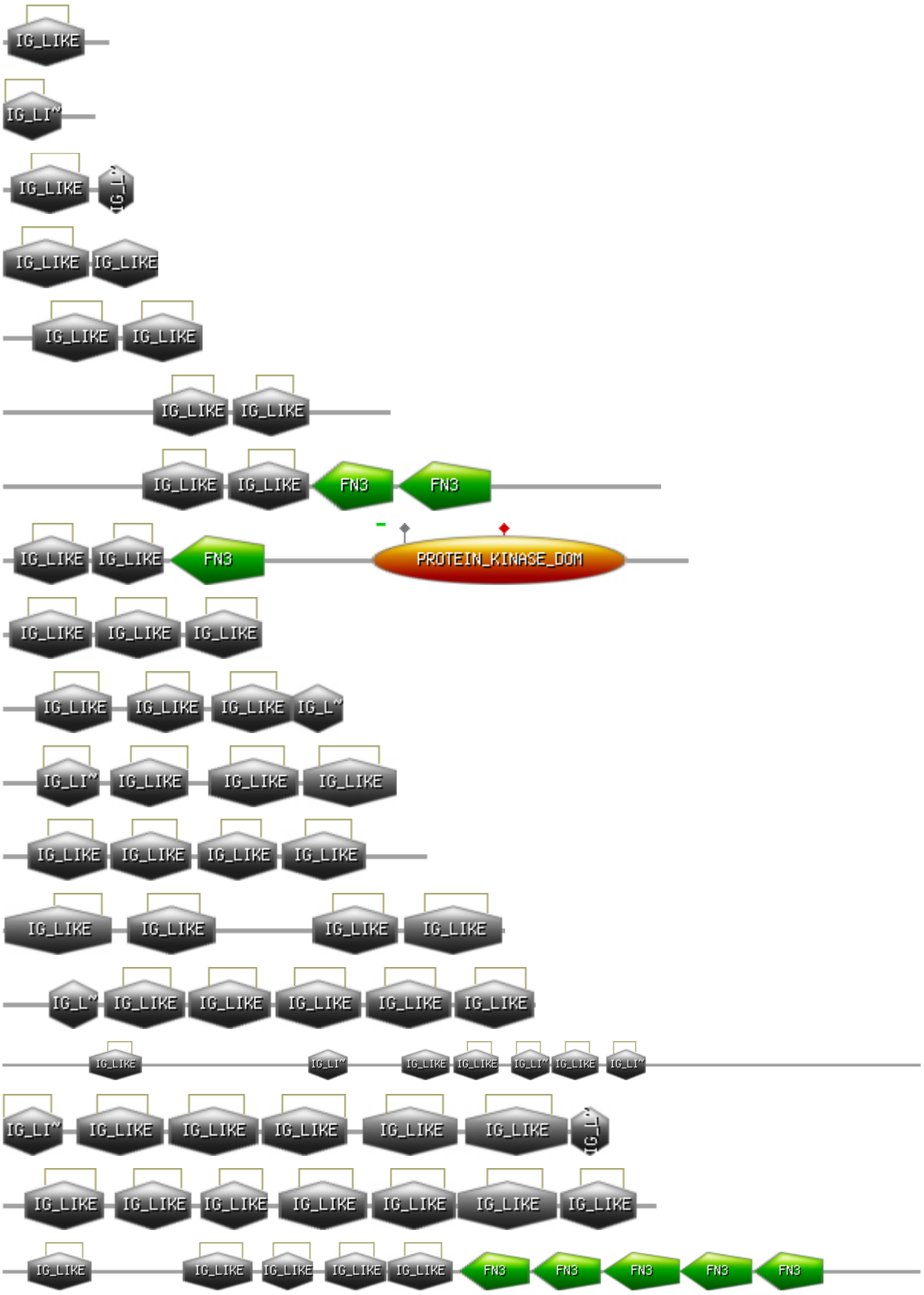
S5.6: Putative toxins present in *Entacmaea quadricolor*. % indicates percentage of gene clusters in the tentacle transcriptome that were found in the venom proteome. * indicates toxin families that were manually identified.

Toxin Family ID	Domain	Tentacle transcriptome	Venom proteome		
		Gene Clusters	Proteins	%	
ALLERGEN AND INNATE IMMUNITY		**9**	**4**	**44.4**	
CRISP	CAP	7	2	28.6	*
DPP IV/FAP	Peptidase S9	1	1	100	*
HYAs	/	1	1	100	*
AUXILIARY		**67**	**12**	**17.9**	
Peptidase M12A	Astacin	67	12	17.9	
HAEMOSTATIC AND HAEMORRHAGIC		**409**	**26**	**6.4**	
True venom lectin family	C-type lectin	7		0	*
Coagulation factor V-like	F5/8 type-C	315	8	2.5	
Ficolin lectin family	Fibrinogen C-terminal	30	3	10	*
Peptidase M12B	TSP type-1	20	2	10	*
Peptidase S1	Trypsin	37	13	35.1	
MIXED FUNCTION ENZYMES		**24**	**3**	**12.5**	
Phospholipase A2	PLA2	24	3	12.5	
NEUROTOXIN		**122**	**9**	**7.3**	
Acrorhagin	/	3		0	
Delta-actitoxin-Eqd1a	ATX- III	2		0	*
BßH-like (Type IV Kv channel)	/	4		0	
ß-Defensin (Type III Kv channel)	Defensin	2		0	
CRISP	CAP/ShKT	2	2	100	*
ICK-like (Type V Kv channel)	/	17		0	
NEP 3 Family	ShKT	5	2	40	*
SCRiP (TRPA1)	/	2		0	
ShK-like (Type I Kv channel)	ShKT	85	5	5.8	
PORE FORMING		**45**	**8**	**17.8**	
Actinoporin (Type II cytolysins)	Cytolysin	23	2	8.7	
DELTA-actitoxin-Ucs1a	Cytolysin	9	3	33.3	*
DELTA-alicitoxin-Pse2a-like	MAC/PF	2	1	50	*
DELTA-alicitoxin-Pse2b-like	MAC/PF	10	2	20	*
DELTA-thalatoxin-Avl2a-like	MAC/PF	1		0	*
PROTEASE INHIBITOR		**38**	**3**	**7.9**	
Venom Kunitz-type family	Kunitz-BPTI	19	3	13.6	
Kazal-like	Kazal	19		0	

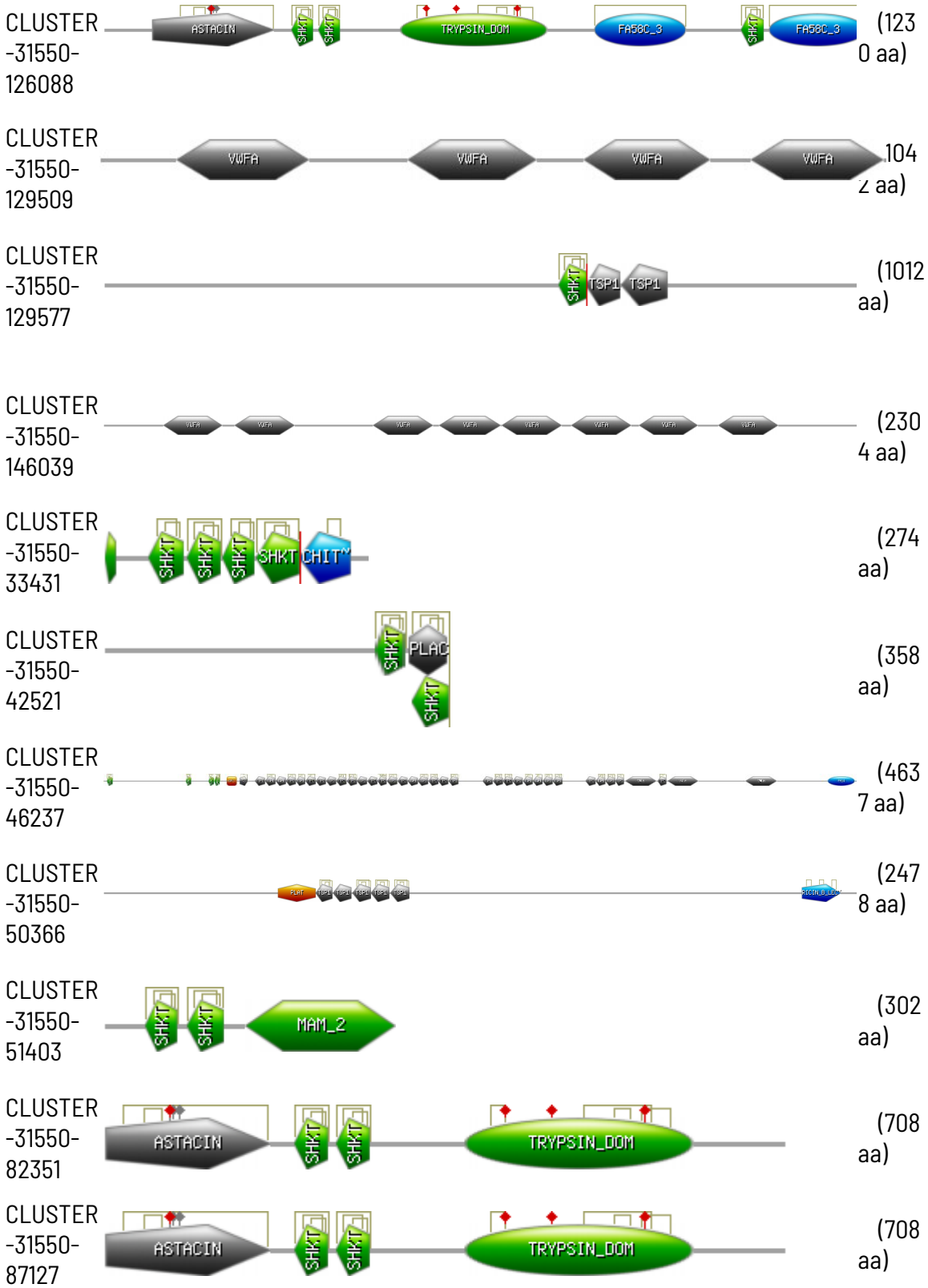
S5.6 cont.

Toxin Family ID	Domain	Tenacle transcriptome	Venom Proteome	
		Gene Clusters	Proteins	%
UNKNOWN		**537**	**70**	**13**
CREC	EF-hand	8	1	12.5
EGF-like	EGF-like	25	1	4.2
Immunoglobulin-like	IGC2-like	300	33	11
Lipase maturation factor	LMF1	8		0
Sea anemone 8 toxin family	/	10		0
U2		9		0
U8		5		0
U9		1		0
U11		4		0
U12	Folate receptor	13	5	38.5
U13	Folate receptor	4	2	50
U15		21	10	47.6
U16		4		0
Z3	Zona pellucida	20	6	30
Z7	Zona pellucida	1	1	100
uncharacterised toxins	/	104	11	10.6
Total		**1,251**	**135**	**10.8**

S5.7 IG-like domain architecture in venom proteome.



S5.8 Uncharacterised toxin domain architecture in venom proteome.



S5.9 Putative nematocytes proteins present in Entacmaea quadricolor. % indicates percentage of gene clusters in the tentacle transcriptome that were found in the venom proteome.

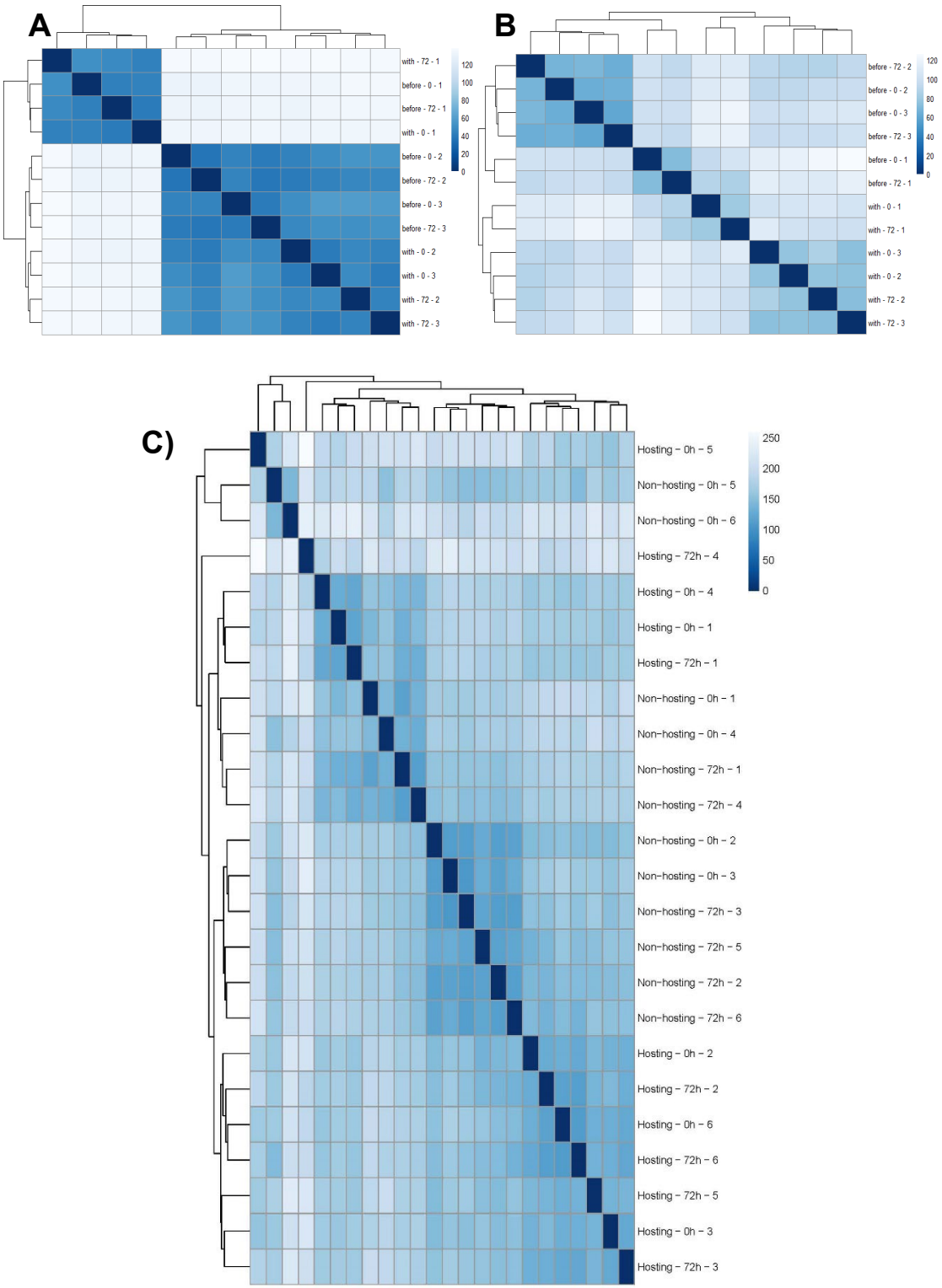
Nematocyte Family ID	Tentacle Transcriptome	Venom Proteome	
	Gene Clusters	Proteins	%
CALCIUM MODULATORS	**20**	**3**	**15**
Calcium-binding EF-hand protein	2	1	50
Annexin	4	1	25
EGF-Ca (2) Formate/nitrite transporter domain protein	5		0
EGF-like type 3 protein	3		0
EGF-Ca domain protein	6	1	16.7
METABOLIC ENZYMES	**28**	**13**	**46.4**
Carbonic Anhydrases	1		0
Glutamate Metabolism	13	7	53.8
Glycoside Hydrolases	3	2	66.7
Other Carbohydrate-modifying Enzymes	5	3	60
Protease-associated Glycosidase	3	1	33.3
Ungrouped proteins	3		0
NOVEL PROTEINS	**37**	**6**	**16.2**
Novel Protein type 1	10		0
Novel Protein type 3	10	2	20
Novel Protein type 4	17	4	23.5
OTHER ENZYMES	**66**	**20**	**30.3**
ADP-ribosyl hydrolase	1	1	100
ATPase	3	1	33.3
Lipase/ Esterases	19	7	36.8
Peptidyl-prolyl cis-trans isomerase	17	6	35.3
Phosphodiesterase / nucleotide pyrophosphatase	4		0
Protein Disulfide Isomerase	4	1	25
Ungrouped enzymes	18	4	22.2
PEPTIDASES	**68**	**28**	**41.2**
Cysteine peptidases	14	2	14.3
Glycoside hydrolases	2	1	50
Metallopeptidases	43	21	48.8
Serine peptidases	9	4	44.4
STRUCTURAL PROTEINS	**90**	**19**	**21.1**
Minicollagens and Proline-rich proteins	15	2	13.3
Other ECM motif proteins	60	14	23.3
Other structural proteins	15	3	20

S5.9 cont

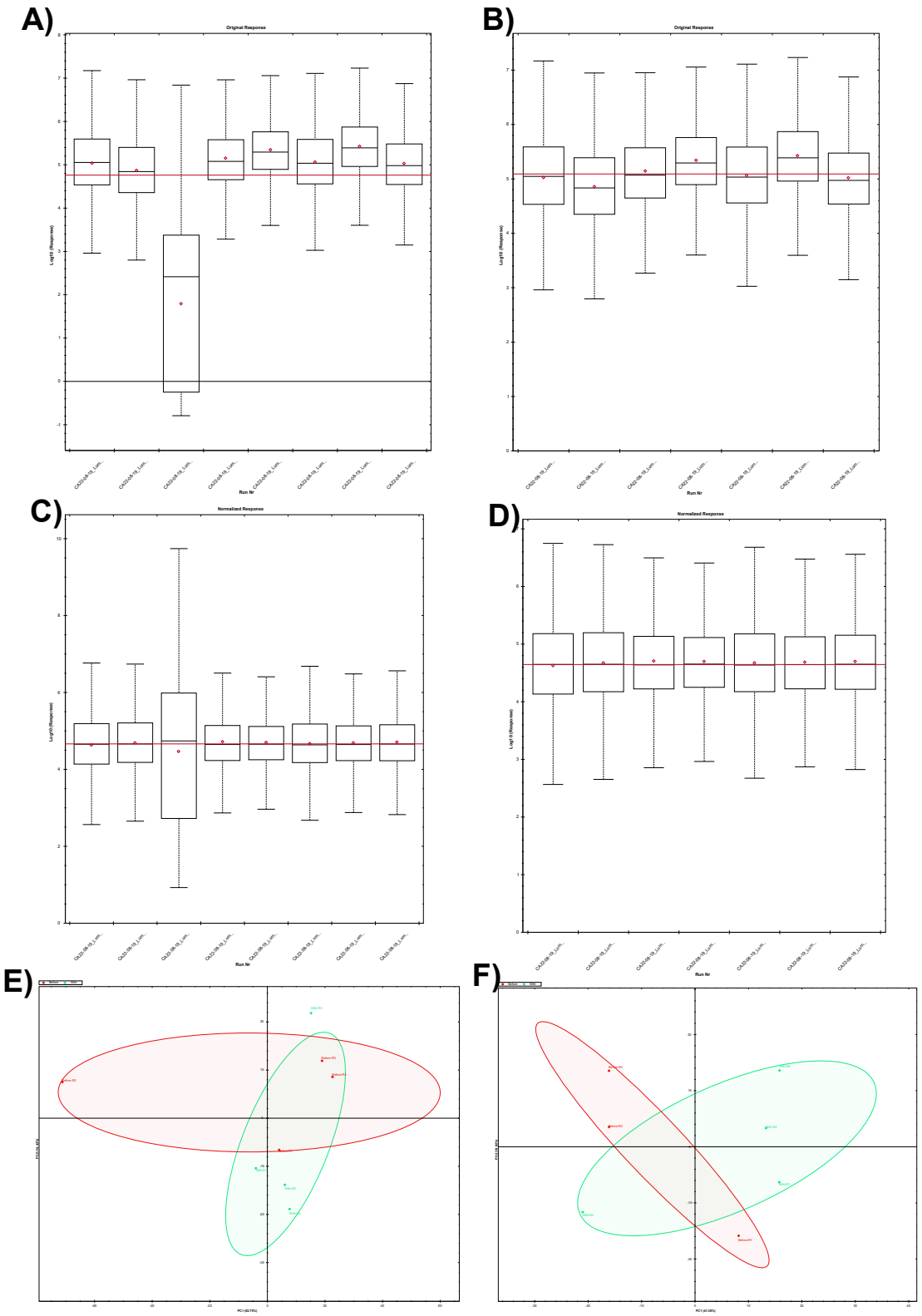
Nematocyte Family ID	Tentacle Transcriptome	Venom Proteome	
	Gene Clusters	**Proteins**	**%**
VENOMS	**45**	**9**	**20**
Allergen and innate immunity	18	7	38.9
Auxiliary	10	1	10
Mixed Function Enzymes	8		0
Protease Inhibitor	6		0
Unknown	3	1	33.3
UNGROUPED PROTEINS	**34**	**8**	**23.5**
14-3-3 protein	4	3	75
ADP/ATP-translocator protein	3		0
GST N Metaxin-like protein	2	1	50
Mef2 myocyte enhancer factor 2-like protein	5		0
Phosphatidylinositol transfer protein	2	1	50
Solute carrier family 25 protein	1		0
SOUL heme-binding protein	8	3	37.5
Translocon-associated protein 1	3		0
TRAP beta domain protein	5		0
Voltage-dependent anion channel 2 related	1		0
Total	**388**	**106**	**27.3**

APPENDIX 6: SUPPLEMENTARY MATERIAL FOR CHAPTER 6

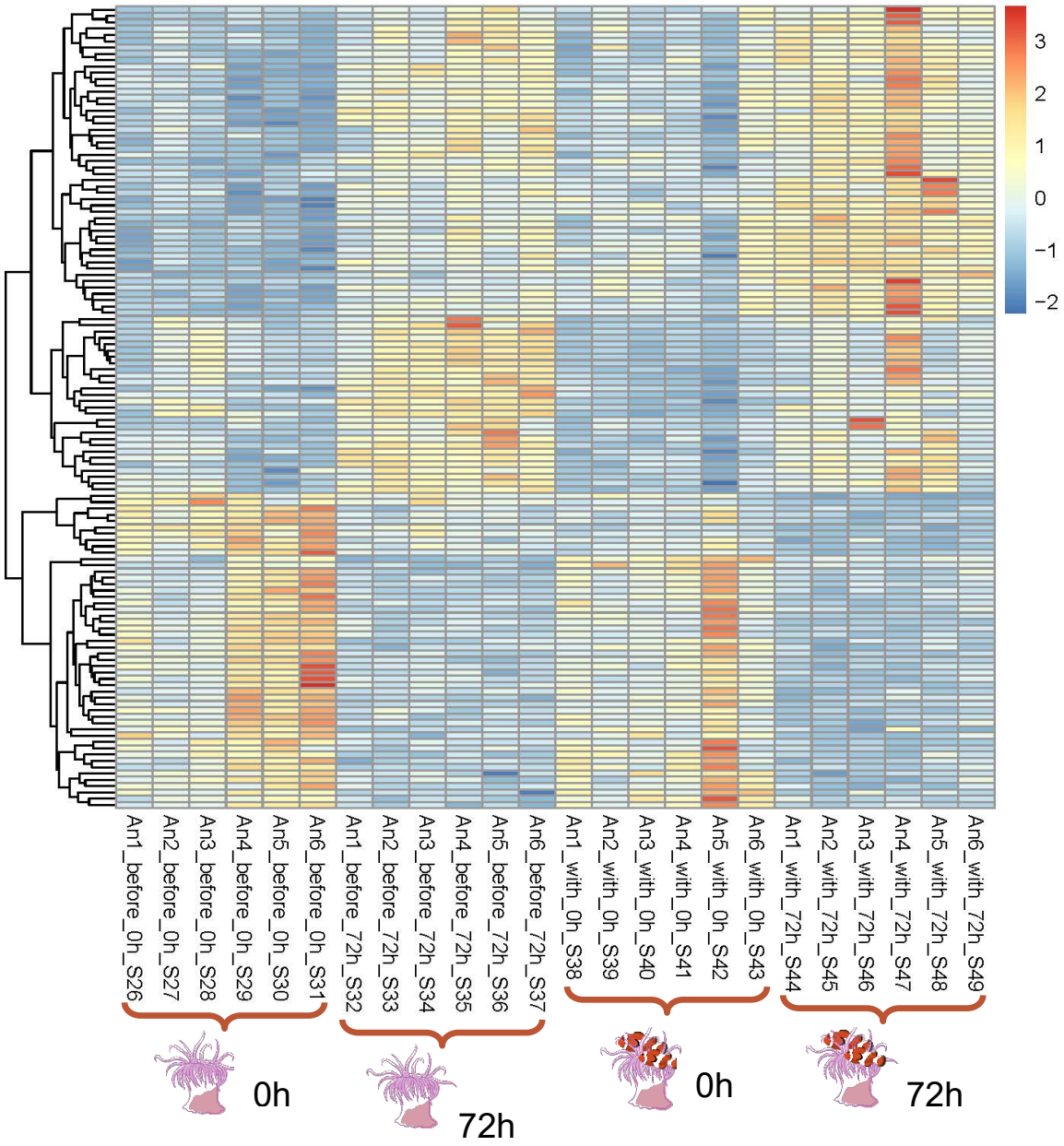
S6.1: Normalisation of transcripts A) before normalisation B) after Combat-Seq normalisation C) after Combat-Seq normalisation all files.



S6.2: Normalisation of venom proteome A) pre-normalisation all files, B) Pre normalisation minus An6 Before, C) Post normalisation all files, D) Post normalisation minus An6 Before, E) PCA all files, F) PCA minus An6 Before



S6.3: Differential gene expression of the *Entacmaea quadricolor* tentacle transcriptome comparing sampling at 0 and 72 hours. Rows scaled using z-scores (x - mean(x)) / sd(x).



S6.4: Differentially expressed putative toxin tentacle transcripts and venom proteins from Entacmaea quadricolor. **Bold/underlined** putative toxins indicate differential expression in transcripts and proteins. DE=number of gene clusters differentially expressed. + indicates an increase with hosting, - indicated a decrease with hosting.

Toxin Family ID	Tentacle Transcriptome			Venom Proteome		
	Gene Clusters	DE	Change	Gene Clusters	DE	Change
ALLERGEN AND INNATE IMMUNITY						
CRISP	**7**	**1**	**-**	**2**	**1**	**-**
DPP IV/FAP	1			1	1	-
HYAs	1			1	1	-
AUXILLIARY						
Peptidase_M12A_L1	28			3		
Peptidase_M12A_L2	5			3	2	-
Peptidase_M12A_L3	1					
Peptidase_M12A_L4	**1**	**1**	**-**	**1**	**1**	**-**
Peptidase_M12A_L5	3			1		
Peptidase_M12A_L6	6	2	+			
Peptidase_M12A_L7	4			1		
Peptidase_M12A_L8	2	1	+			
Peptidase_M12A (UC)	**17**	**1**	**-**	**3**	**2**	**+1/-1**
HEMOSTATIC AND HEMORRHAGIC TOXIN						
Coagulation factor V-like L1	238	2	+	2		
Coagulation factor V-like L2	46	1	+	1		
Coagulation factor V-like L3	**31**	**5**	**+**	**5**	**1**	**+**
Ficolin lectin family	30			3		
Peptidase M12B	20	2	-	2		
Peptidase S1 L1	26	1	+	9		
Peptidase S1 L2	1					
Peptidase S1 (UC)	10			4		
True Venom Lectin Family	7					
MIXED FUNCTION ENZYMES						
PLA2 L1	**16**	**1**	**+**	**3**	**3**	**-**
PLA2 L2	5					
PLA2 (UC)	3					
NEUROTOXIN						
Acrorhagin	3					
BßH-like_L2	3					
BßH-like_L3	1	1	-			
ß-Defensin (Type III Kv channel)	2					
CRISP	**2**	**1**	**-**	**2**	**1**	**-**
Delta-actitoxin-Eqd1a	2	1	-			
ICK-like (Type V Kv channel)	17	6	+3/-3			
NEP 3 Family – NEP 3	**5**	**4**	**-**	**2**	**2**	**-**

S6.4: cont:

Toxin Family ID	Tentacle Transcriptome			Venom Proteome		
	Gene Clusters	DE	Change	Proteins	DE	Change
NEUROTOXIN cont.						
SCRiP	2					
ShK-like L1	9					
ShK-like L2	11					
ShK-like L3	1					
ShK-like (UC)	64	10	-	5		
PORE FORMING						
Actinoporin	23			2	1	-
DELTA-actitoxin-Ucs1a	9			3	1	-
DELTA-alicitoxin-Pse2a-like	2			1	1	+
DELTA-alicitoxin-Pse2b-like	10			2		
DELTA-thalatoxin-Avl2a-like	1					
PROTEASE INHIBITOR						
Kazal-like L1	2					
Kazal-like L2	17	3	-			
Venom Kunitz-type family L1	16					
Venom Kunitz-type family L2	3			3	3	-
UNKNOWN						
CREC	8			1		
EGF-like	25			1		
IG-like L1	12					
IG-like L2	100			6		
IG-like L3	**188**	**7**	**+5/-2**	**27**	**2**	**+**
Lipase maturation factor	8					
Sea Anemone 8 toxin family	10					
U2	9					
U8	5					
U9	1					
U11 L1	4					
U12	**13**	**3**	**-**	**5**	**2**	**-**
U13	4			2	1	-
U15	21			10	4	-
U16	4	1	-			
Z3	20			6	4	+
Z7	1			1		
Uncharacterised toxin	**105**	**22**	**+1/-21**	**12**	**4**	**+2/-2**
Total	**1,251**	**77**		**135**	**38**	

S6.5: Differentially expressed Hydra nematocyte matched tentacle transcripts and the venom proteins from Entacmaea quadricolor. **Bold/underlined** nematocyte match indicate differential expression in transcripts and proteins. DE=number of gene clusters differentially expressed. + indicates an increase with hosting, - indicated a decrease with hosting.

Nematocyte Family ID	Tentacle Transcriptome			Venom Proteome		
	Gene Clusters	**DE**	**Change**	**Proteins**	**DE**	**Change**
CALCIUM MODULATORS						
Calcium-binding EF-hand protein	2			1		
Annexin	4			1		
EGF-Ca (2) Formate/nitrite transporter domain protein	5					
EGF-like type 3 protein	3					
EGF-Ca domain protein	6	3	-	1		
METABOLIC ENZYMES						
Carbonic Anhydrases	1					
Glutamate Metabolism	**13**	**6**	**+1/-5**	**7**	**4**	**-**
Glycoside Hydrolases	3			2	1	-
Other Carbohydrate-modifying Enzymes	**5**	**1**	**-**	**3**	**3**	**-**
Protease-associated Glycosidase	**3**	**1**	**-**	**1**	**1**	**-**
Ungrouped proteins	3					
NOVEL PROTEINS						
Novel Protein type 1	10	6	-			
Novel Protein type 3	**10**	**3**	**-**	**2**	**1**	**-**
Novel Protein type 4	**17**	**11**	**-**	**4**	**3**	**-**
OTHER ENZYMES						
ADP-ribosyl hydrolase	**1**	**1**	**+**	**1**	**1**	**-**
ATPase	3			1		
Lipase/ Esterases	**19**	**11**	**+9/-2**	**7**	**4**	**-**
Peptidyl-prolyl cis-trans isomerase	17			6		
Phosphodiesterase / nucleotide pyrophosphatase	4	1	-			
Protein Disulfide Isomerase	4			1	1	-
Ungrouped enzymes	**18**	**2**	**-**	**4**	**1**	**-**
PEPTIDASES						
Cysteine peptidases	14	1	-	2		
Glycoside hydrolases	2			1		
Metallopeptidases	**43**	**9**	**+1/-8**	**21**	**9**	**-**
Serine peptidases	**9**	**3**	**-**	**4**	**1**	**-**
STRUCTURAL PROTEINS						
Minicollagens and Proline-rich proteins	15			2		
Other ECM motif proteins	**60**	**16**	**-**	**14**	**3**	**-**
Other structural proteins	**15**	**1**	**-**	**3**	**1**	**+**

S6.5: cont:

Nematocyte Family ID	Tentacle Transcriptome			Venom Proteome		
	Gene Clusters	**DE**	**Change**	**Proteins**	**DE**	**Change**
VENOMS						
Allergen and innate immunity	**7**	**6**	**-**	**7**	**5**	**-**
Auxiliary	10			1		
Mixed Function Enzymes	8					
Protease Inhibitor	6					
Unknown	3			1		
UNGROUPED PROTEINS						
14-3-3 protein	4			3		
ADP/ATP-translocator protein	3					
GST N Metaxin-like protein	2	2	-	1		
Mef2 myocyte enhancer factor 2-like protein	5	1	+			
Phosphatidylinositol transfer protein	2	1	-	1		
Solute carrier family 25 protein	1					
SOUL heme-binding protein	8	1	-	3		
Translocon-associated protein 1	3					
TRAP beta domain protein	5					
Voltage-dependent anion channel 2 related	1					
Total	**388**	**87**		**106**	**39**	

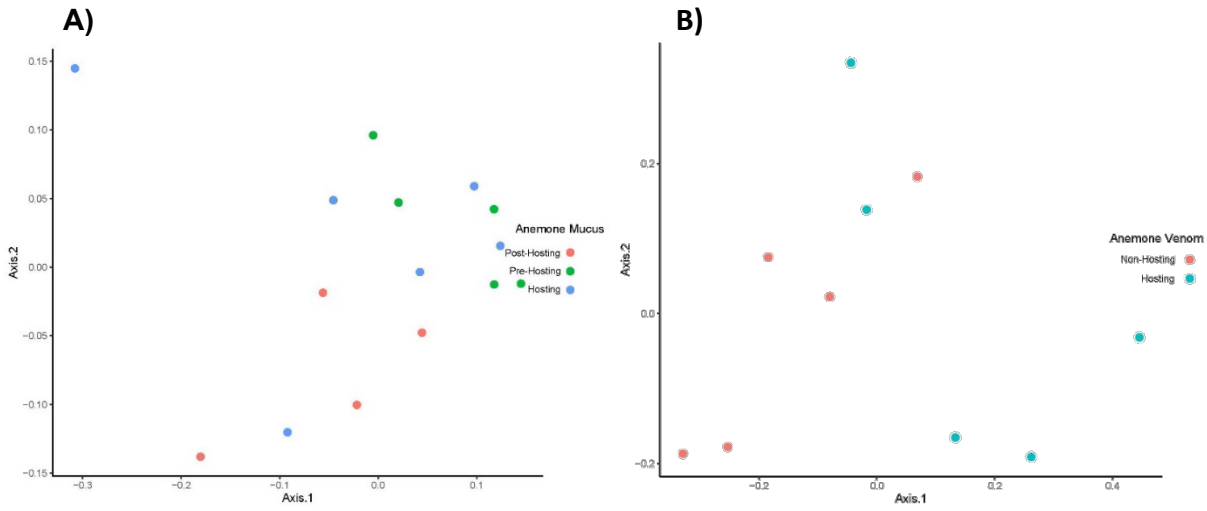
APPENDIX 7: ADDITONAL EXPERIMENTS

7.1 Lipid profiles from anemone mucus and venom

Chapter 4 explored the mucus metabolome of anemonefish, to assess changes during symbiosis with anemone hosts. During this experiment samples of anemone mucus and venom were also collected to perform metabolite analysis, unfortunately during extended COVID-19 lockdowns in Melbourne, where the samples were stored, the anemone glycan samples were misplaced during a freezer clean out leaving only the lipid samples.

The original idea behind chapter 4 was to differentiate between the hypothesises that (1) the mucus layer of the anemonefish lacks the stimulatory compounds to trigger the nematocytes of the anemone, that are present in the mucus layer of non-symbiotic fishes (Lubbock 1980) and (2) the anemonefish mucus layer acts to molecularly mimic the anemone's mucus, preventing the detection of the anemonefish amongst the anemone's tentacles (Elliot et al. 1994). If molecular mimicry was to occur, we would expect the metabolome profiles of the anemone and anemonefish mucus to merge or become more similar during symbiosis. Hence the collection of anemone mucus for comparison to the anemonefish mucus samples, across the different hosting periods.

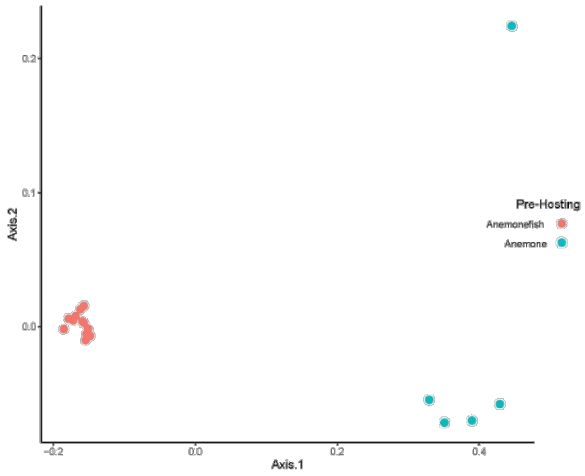
As was seen in the anemonefish lipid profiles, the anemone mucus and venom lipid samples do not change with hosting, as there is no separation between the lipid profiles of hosting and non-hosting anemones (Fig S7.1.1)



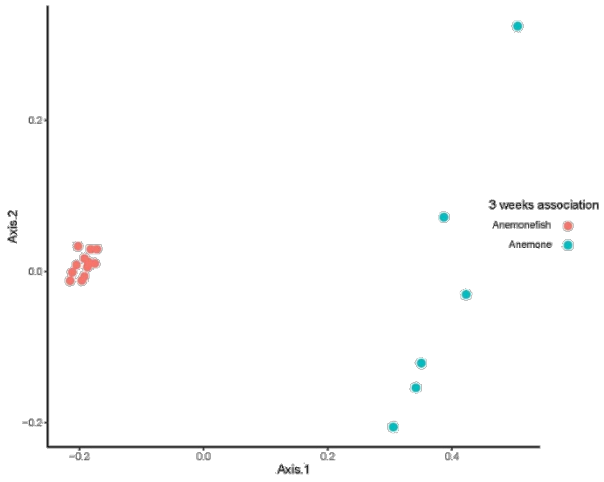
S7.1.1: Principal Coordinate Analysis (PCoA) of anemone mucus and venom lipidome A) anemone mucus, B) anemone venom

Throughout the association periods (hosting and non-hosting) the lipid profile of both anemonefish and anemone mucus remain separated regardless of symbiosis (Fig S7.1.2). In the molecular mimicry hypothesis, we would expect the profiles of host sea anemone and anemonefish mucus to merge during hosting periods, however for the lipidome we do not see this (Fig S7.1.2B). If we also had the glycan samples from the host sea anemone and saw the same result, we could be more confident with rejecting the molecular mimicry hypothesis but at this stage we need more evidence.

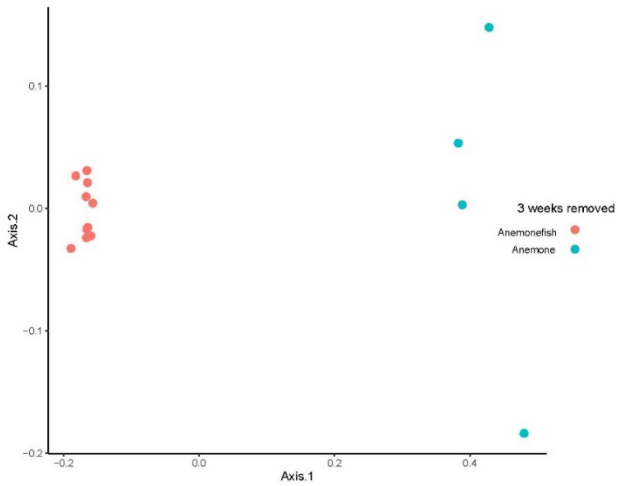
A)



B)



C)



S7.1.2: Principal Coordinate Analysis (PCoA) of anemonefish and anemone mucus lipidome A) pre-hosting, B) 3-weeks association, C) 3-weeks removed

7.2 Haemolysis activity with anemonefish presence

Further to the molecular mimicry hypothesis, we were interested to see if anemonefish could incorporate venom proteins from their host sea anemone into their own mucus for venom inhibition and self-recognition purposes. As an initial step in testing this hypothesis we compared mucus from anemonefish, host sea anemones and a non-symbiotic damselfish (control) alongside host sea anemone venom to assess haemolysis activity.

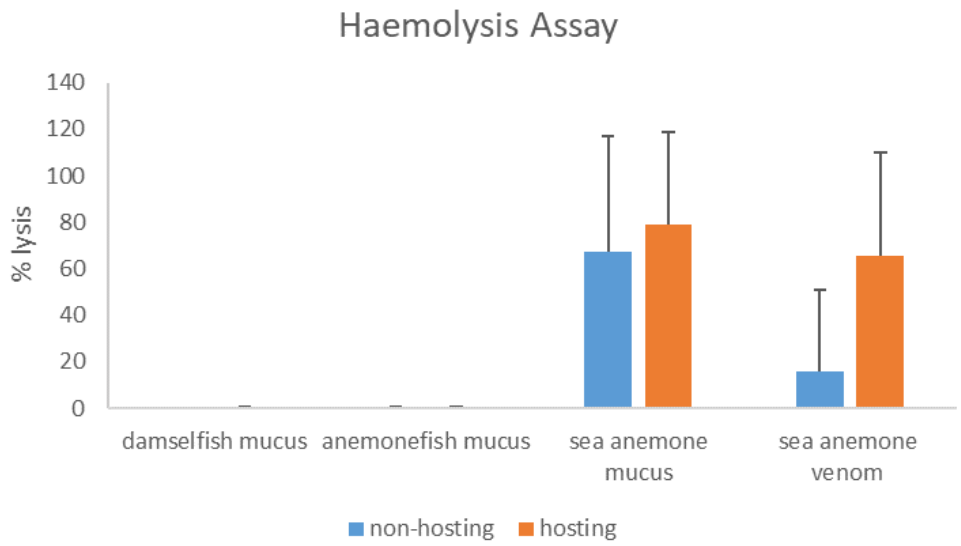


Figure S7.2.1: haemolysis from mucus and venom during hosting and non-hosting periods between a host sea anemone and anemonefish.

We did not find any haemolysis activity from anemonefish mucus when in association with a host sea anemone (Fig S7.2.1). However, we did find that the mucus of host sea anemones produced a slightly higher haemolytic response than their venom did, and that the haemolytic response of host sea anemone venom was altered by anemonefish presence. Haemolysis from host sea anemone venom increased in the presence of anemonefish.

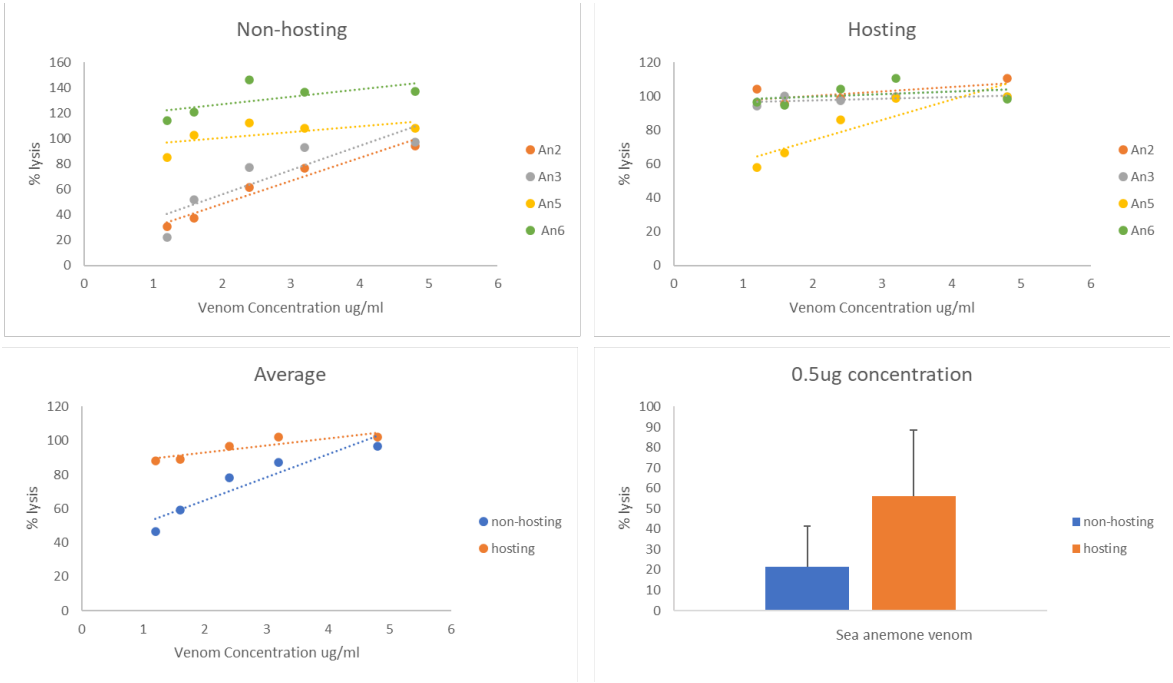


Figure S7.2.2: haemolysis from host sea anemone venom during hosting and non-hosting periods with an anemonefish.

This increase in haemolysis activity when in association with anemonefish, matches results found in chapter 6, that showed DELTA-alicitoxin-Pse2a-like increased in the venom proteome of host sea anemones with anemonefish presence. However, there were also two other pore forming toxin that decreased with anemonefish presence in the venom proteome (Appendix S6.5).

This assay was highly variable between replicates (Fig S7.2.2). Therefore, future studies require further refinement of this method to produce more standardised results.