

Evolution, Development and Ecology of Anemonefishes

Model Organisms for Marine Science

Edited by
Vincent Laudet and Timothy Ravasi



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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

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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

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

<i>Amphiprion</i> spp:	<i>Heteractis malu</i>	<i>Macroactylia doreensis</i>	<i>Heteractis crispa</i>	<i>Heteractis aurora</i>	<i>Entacmaea quadricolor</i>	<i>Heteractis magnifica</i>	<i>Stichodactyla mertensii</i>	<i>Stichodactyla haddoni</i>	<i>Stichodactyla gigantea</i>	<i>Cryptodendrum adhaesivum</i>	Number of hosts	Number of stripes
<i>clarkii</i>	X	X	X	X	X	X	X	X	X	X	10	3
<i>akindynos</i>	Ø	Ø	X	X	X	X	X	X	X	Ø	7	2
<i>chrysopterus</i>	Ø	X	X	X	X	X	X	X	Ø	Ø	7	2
<i>bicinctus</i>	Ø		X	X	X	X	X	X	X	Ø	7	2
<i>tricinctus</i>	Ø		X	X	X	Ø	X	X	Ø	Ø	5	3
<i>chrysoaster</i>	Ø		X	X	X	X	X	X	X	Ø	4	3
<i>perideration</i>	Ø	X	X	Ø	X	X	Ø	Ø	X	Ø	4	2
<i>melanopus</i>	Ø	Ø	X	Ø	X	X	Ø	Ø	X	Ø	4	1
<i>allardi</i>	Ø	Ø	Ø	X	X	Ø	X	Ø	Ø	Ø	3	2
<i>ocellaris</i>	Ø	Ø	Ø	Ø	Ø	X	X	Ø	X	Ø	3	3
<i>percula</i>	Ø	Ø	X	Ø	Ø	X	Ø	Ø	X	Ø	3	3
<i>polymnus</i>	Ø	X	X	Ø	Ø	Ø	Ø	X	Ø	Ø	3	3
<i>omanensis</i>	Ø		X	Ø	X	Ø	Ø	X	Ø	Ø	3	2
<i>rubrocinctus</i>	Ø		Ø	Ø	X	Ø	Ø	Ø	X	Ø	2	1
<i>sandaracinos</i>	Ø		X	Ø	Ø	Ø	Ø	Ø	Ø	Ø	2	1
<i>akallopisos</i>	Ø	Ø	Ø	Ø	Ø	X	X	Ø	Ø	Ø	2	1
<i>barberi</i>	Ø		X	Ø	X	X	X	Ø	Ø	Ø	2	1
<i>ephippium</i>	Ø		X	Ø	X	Ø	Ø	Ø	Ø	Ø	2	0
<i>latezonatus</i>	Ø		X	Ø	X	Ø	Ø	Ø	Ø	Ø	2	4
<i>frenatus</i>	Ø		Ø	Ø	X	Ø	Ø	Ø	Ø	Ø	1	1
<i>chagosensis</i>	Ø		Ø	Ø	X	Ø	Ø	Ø	Ø	Ø	1	2
<i>pacificus</i>	Ø	Ø	Ø	Ø	Ø	X	Ø	Ø	Ø	Ø	1	1
<i>fuscocaudatus</i>	Ø	Ø		Ø	Ø	Ø	X	Ø	Ø	Ø	1	3
<i>latifasciatus</i>					Ø	Ø	X	Ø	Ø	Ø	1	2
<i>mccullochi</i>			Ø		X	Ø	Ø	Ø	Ø	Ø	1	1
<i>nigripes</i>			Ø	Ø	Ø	X	Ø	Ø	Ø	Ø	1	1
<i>sebae</i>	Ø		Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	1	2
<i>biaculeatus</i>	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).

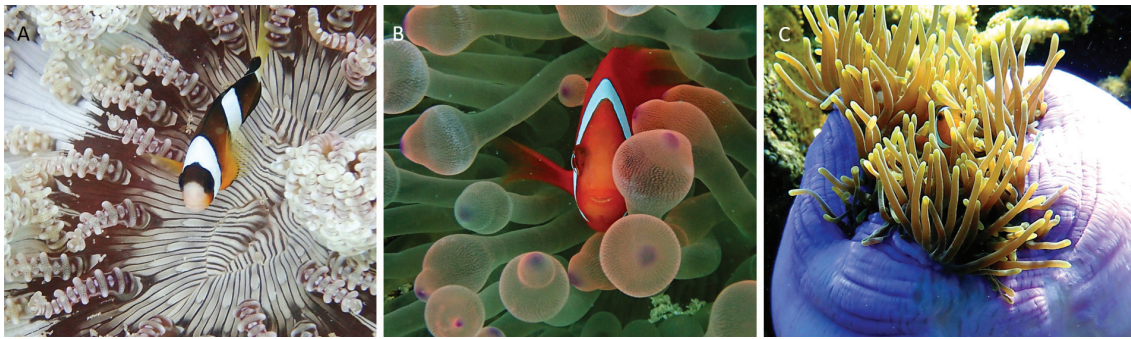


FIGURE 19.1 Various morphology of anemone hosts that aid in camouflaging anemonefish. A) Beaded tentacles of *Heteractis aurora*, B) bulb-like tentacles of *Entacmaea quadricolor*, C) retraction of tentacles by *Heteractis magnifica*. Images: Emily Fobert.

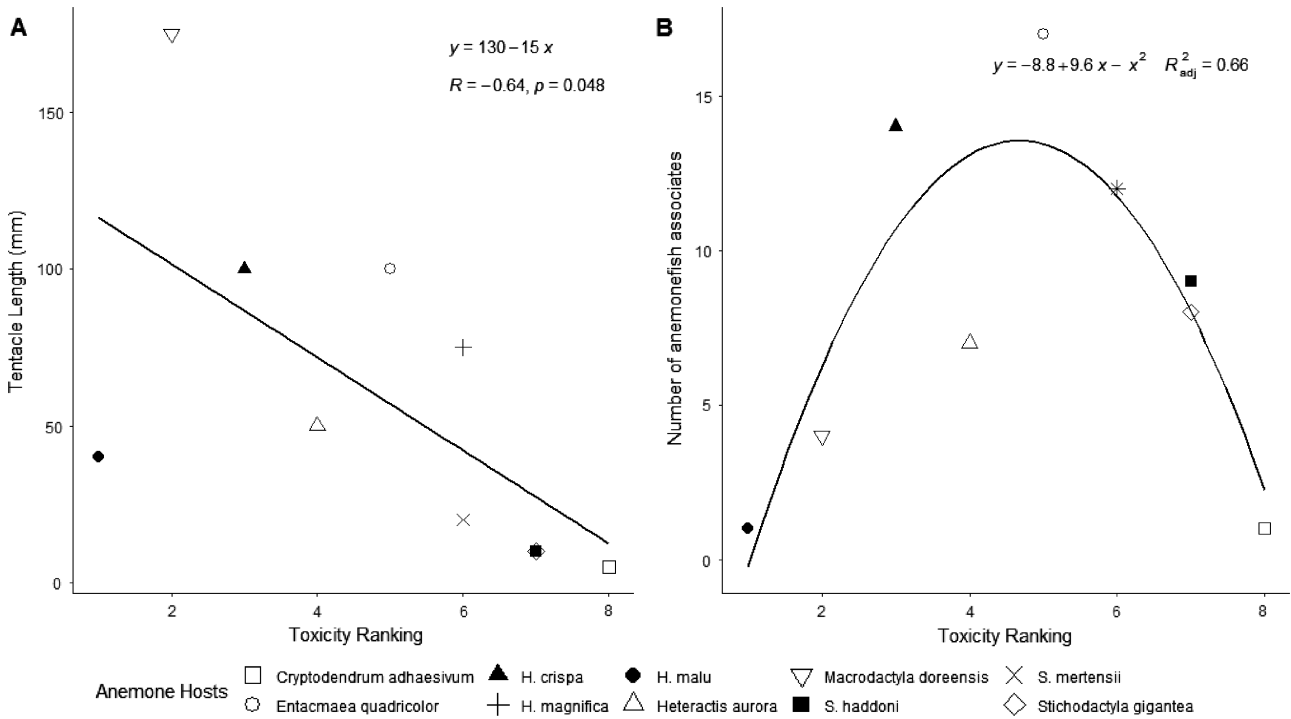


FIGURE 19.2 A) Negative relationship between anemone tentacle length (mm) and overall host anemone toxicity ranking (Merilaita and Kelley 2018). B) Relationship between number of anemonefish associates and overall host anemone toxicity ranking. Updated from Merilaita and Kelley (2018), Nedosyko et al. (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 (*Macroactylia 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 Elliot and Mariscal 1996	Forced contact Forced contact
b	Anemonefish gain protection through an acclimation period	Supported	Davenport and Norris 1958 Mariscal 1970a, b Brooks and Mariscal 1984 Mebs 1994 Balamurugan et al. 2015	Observation of behaviors Observation of behaviors Acclimation time to surrogate anemones Ichthyotoxic activity Observation of behaviors
2	Anemonefish have a thicker mucus layer than other fish	Insufficient evidence	Lubbock 1980	Nomarski optics (<i>A. clarkii</i>)
3	Anemonefish mucus molecularly mimics the composition of anemone mucus	Insufficient evidence	Schlichter 1976 Elliot et al. 1994	Electrophoresis/radiolabelled mucus Antibody assays
4	Anemonefish mucus does not trigger firing of the anemone's nematocysts	Insufficient evidence	Lubbock 1980 Abdullah and Saad 2015	Nematocysts per cm ² 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/mm²) (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 cytolytins (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 α 1B-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) hypothesis 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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