Adaptation of anemonefish to their host anemones: From Genetics to Physiology

Nguyen Thi Hai Thanh

Thesis for the degree of Philosophiae Doctor (PhD)
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Abstract

The mutualism between anemonefish and anemones can be studied in terms of permanent or temporary adaptation of fish toward their host anemones. This thesis explores this theme, and aims to test (1) Whether a mutual relationship leads to adaptation of anemonefish symbionts to their host anemones through coevolution (2) Whether *Amphiprion ocellaris* juveniles are able to acclimate to live with multi host anemone species (unnatural hosts), and what the consequences may be in terms of growth and activity, and (3) Whether the acclimation of fish is mediated by biochemical changes in skin mucus.

These questions were explored using a cophylogenetic appoarch, and an experimental system based on *Amphipron occelaris*, a specialist anemone fish, and its response to manipulations of access to its natural host anemone.

The phylogenetic analysis carried out in this thesis did not detect any evidence for historical coevolution between anemonefish and anemones. Although there was some dependence of the symbiont anemonefish on the host anemones, the genetic composition of one species (anemonefish) does not necessarily evolve alongside or in response to the other (anemones), despite the close association between the two species.

The experimental approach provides new insight into the adaptation of *Amphiprion ocellaris* juveniles to recognize their host at the settlement stage, and their capacity and strategy to adapt to an unnatural host in the absence of their natural hosts, in captive conditions. Moving from natural to unnatural hosts affected the fitness of the juveniles, as measured by decreases in growth and changes in swimming activities. Changes in the protein profiles of the skin mucus were also informative; and provide a list of novel compounds in both naive fish and experienced fish living with different anemone hosts. The function of proteins based on gene ontology analysis can broadly be classified as belonging to immunologic defence, molecular transport, stress response, and signal transduction groups.

These findings contribute to understanding the mechanism of anemone – anemonefish symbiosis, and also allow some speculation about the future of coral reefs under a changing environment, especially climate change. If climate change affects anemone species, some anemonefish species such as *Amphiprion ocellaris* might be able to seek alternative hosts but might experience reduced fitness.

Keywords: Mutualism, anemonefish, anemone, coevolution, fitness, proteomics.

List of Publications

Paper I:

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1. INTRODUCTION

1.1 Anemone-Anemonefish associations and the benefits of living together

Partnership between fish and invertebrates was discovered quite early in the scientific exploration of the aquatic environment (Gohar 1934). Among these, the relationship between anemones and anemonefish has been considered an iconic example of mutualism in coral reef ecosystems. This association has been the subject of research to understand its evolution, as well as the chemical and behavioral mechanisms underpinning the relationships, which are still debated (Elliott 1995; Mebs 2009; Titus et al. 2019).

Thanks to the Pixar movie ‘Finding Nemo’, the little anemonefish Nemo is now an iconic image of coral reefs. Millions of children and adults all over the world are fascinated by the star Nemo, who represents one of the 28 anemonefish species that live in a mutualistic relationship with anemones as their hosts.

When first discovered in 1868 (Collingwood 1868), this symbiotic association was described as commensal, without any benefit for either partner. Anemonefish were thought to receive only protection from predators by hiding among the toxic tentacles of their anemone hosts. However, this relationship was redefined as mutualism in 1970, since both partners benefit from living together (Mariscal 1970). There is increasing evidence both from the field and laboratory indicating that rather than commensalism, the anemonefish- anemone association is probably one of the best characterized examples of mutualism.

Anemonefish or clownfish are the group of 28 species belonging to the genera *Amphiprion* (27 species) and *Premnas* (only one species), subfamily Amphiprionidea (Table 1), the family Pomacentridea, the order Perciformes. They are found mostly in warmer waters of the Indian and Pacific oceans, along with the Great Barrier Reef and the Red Sea; but they are totally absent from the Caribbean region. The majority of the species inhabit restricted areas, while others have a wider distribution. They can be recognized by the bold color strokes on their body (from rich purplish brown to bright orange and red and yellow). Anemonefish are omnivorous with a diet of algae, copepods, and other zooplankton (Fautin & Allen 1997). The fish also feed on other small crustaceans and molluscs (Fautin & Allen 1997).

Anemonefish are symbionts on their anemone hosts. The anemonefish swim freely among the tentacles of anemones, unharmed by the nematocyst toxins that can immobilize other prey nearby. In this association, the benefits for the anemonefish include protection from predators (Fautin 1991), removal of external parasites (Allen 1972), gaining additional nutrients from tentacles, and increase in reproductive fitness through egg protection (Allen 1972; Saenz-Agudelo et al. 2011). Anemonefish are long-lived, and are used as an experimental model of exceptional longevity (Holbrook & Schmitt 2005; Sahm et al. 2019). Their lifespan is generally more than 30 years. This is twice as long as other damselfish species, and up to six times longer than other marine fish of a comparable size. .

In nature, anemonefish live in a group in their host anemone, which consists of up to six individuals with a well-defined size hierarchy. The largest fish is a dominant female, the second largest is a breeding male, followed by up to four non-breeding males of gradually smaller size (Buston 2003; Iwata et al. 2008). Removal of any individual fish leads to faster growth of the fish at the next lower rank. For example, if the dominant female dies, the male changes sex to become the female and the largest subordinate becomes the breeding male. This social hierarchy seems to be reinforced by behaviour, in which aggressive sounds and threat postures produced by the dominant fish make the other fish emit submissive sounds and show submissive postures (Iwata et al. 2008; Chen & Hsieh 2017).

Sea anemones are members of the phylum Cnidaria, class Anthozoa, subclass Hexacorallia and order Actiniaria, one of the oldest orders of venomous animals. There are ca.1200 species of sea anemone within Actiniaria, but only 10 species are symbiotic with anemonefish. These belong to three subfamilies: Actiniidae, Stichodactylidea and Thalassianthidae (Table 1). The anemones are not just hosts to anemonefish since they are also found to live with crustaceans and can also live without any external symbiont (Mebs 2009). As a host, the anemones benefit from the symbiosis. Some anemones have been documented to live as long as 100 years (Holbrook & Schmitt 2005) and in general hosting anemones live longer than those without external symbionts (Godwin & Fautin 1992).

The auto territorial defensive behaviour of anemonefish could prevent predators like butterflyfish *Chaetodon fasciatus* from attacking the tentacles of anemones (Fautin 1991; Fautin & Allen 1997; Porat & Chadwick-Furman 2004). The presence of anemonefish has been positively associated with higher growth and reproduction of their anemone host (Philip et al. 2016). By fanning the anemone host at night, anemonefish appear to supplement oxygen for their host, which increases metabolism of both partners and releases a large amount of waste products, such as dissolved ammonia and phosphorus, which are then assimilated by the anemone (Porat & Chadwick-Furman 2005; Porat & Chadwick-Furman 2004, Godinot &Chadwick 2009).

1.2 Host specificity of anemonefish

Most studies that address the issues of host specificity of anemonefish have been based on observations made in the field. Based on these observations, anemonefish have been found to live with up to 10 species of anemones as hosts (Fautin & Allen 1992; Astakhov 2016), but there are differences in host specificity (Table 1). *Amphiprion clarkii* has been observed to live with all 10 host species, and thus is defined as a generalist. Other anemonefish species, such as *A. ocellaris* have been observed to live with only a few host species, and these fish are defined as specialists. There are also extreme specialists, such as *A. frenatus,* which have only been observed living with one host species. There are also differences in symbiont specificity among anemone hosts (Table 1). For example, *Entacmaea quadricolor* serves as host for at least 16 different anemonefish species, whereas other anemones (*Heteractis malu* and *Cryptodendrum adheasivum*) have only a single anemonefish species associated with them and will only be the host if no other anemone is available (Nedosyko et al. 2014).

There are four hypotheses to explain host specificity. (1). The Olfactory hypothesis, by Fautin, suggests that certain host species attract certain species of anemonefish by secreting a ‘chemical’ attractant that enables the fish to find their host (Fautin 1986). (2). The Imprinting hypothesis says that the fish only have an innate or acquired imprinting preference towards some of potential host anemones. Imprinting has been used more generally to explain the ability of juveniles of coral reef fish, including anemonefish, to return to natal reefs, where their parents had settled (Arvedlund & Nielsen 1996). (3). The Resource partitioning hypothesis suggests that specificity has arisen as a mechanism of dividing the microhabitat of the host, reducing competition through specialization (Fautin 1986). (4). The Competitive exclusion hypothesis proposes that the fish will compete for access to potential anemones and settle there, preventing others from settling (Fautin 1986).

However, none of these hypotheses can fully explain the movement of some anemonefish from one anemone host species as juveniles to another host species as adults. Some anemones, for example *H. crispa* and *H. malu* appear to serve as nursery hosts since they are only found with immature fish (Dunn 1981; Fautin 1991; Karplus 2014). The fish switch to another host species when they enter the reproductive stage. This suggests that different anemone species must provide different fitness levels for the fish (Nedosyko et al. 2014; Nguyen et al. 2019). Anemonefish will choose the anemone host that provides them with the highest quality of refuge and lowest cost in terms of physiological expenditure (Burke & Nedosyko 2016). The quality of the host anemone may be categorized based on morphology (Huebner et al. 2012) and toxicity (Nedosyko et al. 2014). Anemones with longer tentacles and larger body size have been considered the better hosts since anemonefish can find better protection from predators. Anemones with moderate toxicity may be optimal for anemonefish survival and reproduction. Highly toxic anemones are unlikely to attract many fish species, since it would be an existence at the upper toxicity threshold for anemonefish to handle without being harmed. In contrast, low toxicity anemones do not appear to confer protective benefits to the fish. For example, fish associated with bleached host anemones have shown lower fitness in terms of decreased fecundity and spawning frequency (Saenz-Agudelo et al. 2011; Beldade et al. 2017). Fish also have increased oxygen consumption and metabolic rates when residing in bleached host anemones (Norin et al. 2018). There is a link between a high standard metabolic rates and reduced reproductive success and survival in a range of animals, including fish (Norin et al. 2018).

The association patterns of anemonefish and their hosts are mixed; in some cases, specialist fish use generalist anemone partners, and generalist fish use specialist anemone partners (Ollerton et al. 2007). The cross partner usage may help to save specialist species from the likelihood of natural extinction, as in the solely specialist – specialist interaction, the loss of one species certainly leads to the loss of the other (Ollerton et al. 2007). There is a negative correlation between environmental specificity and host specificity anemonefish (Litsios et al. 2014b). Specialist anemonefish have a wide range of habitat usage, whereas generalist anemonefish have very specific habitat preferences. This specialist – generalist trade-off may act as a redundancy mechanism that allows a long-term coexistence of species in presence of conspecifics (Litsios et al. 2014b).

Table 1. Taxonomy and host specificity of anemonefish (Updated from Fautin & Allen 1997; Allen 2010)

	Anemone species										Total number of anemone associates
	Actiniidae		**Stichodactylidea**							**Thalassian -thidae**	
	Entac- maea	*Macro- dactyla*	*Stichodactyla*			*Heteractis*				*Crypto- dendrum*	
Anemonefish species	*E. quadriolor*	*M. doreesis*	*S. mertensii*	*S. haddoni*	*S. gigantea*	*H. magnifica*	*H. crispa*	*H. aurora*	*H. malu*	*C. adheasivum*	
Amphiprioninea											
Genus *Premnas*											
P. biaculeatus	X										1
Genus *Amphiprion*											
sub-genus Actinicola											
A. ocellaris			X		X	X					3
A. percula					X	X	X				3
sub-genus Paramphiprion											
A. polymnus		X		X			X				3
A.latezonatus	X						X				2
A. sebae				X							1
sub-genus Phalerebus											
A.akallopisos			X			X					2
A.leucokranos											
A. nigripes						X					1
A. pacificus											?
A. perideraion		X			X	X	X				4

A.sandaracinos			X				X				2
sub-genus Amphiprion											
Subcomplex: ephippium											
A. barberi	X						X				2
A. ephippium	X						X				2
A. frenatus	X										1
A. mccullochi	X										1
A. melanopus	X					X	X				3
A. rubrocinctus	X				X						2
Subcomplex: clarkii											
A. akindynos	X		X	X	X	X	X	X			7
A. allardi	X		X					X			3
A. bicinctus	X		X		X	X	X	X			6
A. chagosensis	X							X			1
A. chrysogaster		X	X	X		X		X			5
A. chrysopterus	X		X	X		X	X	X			6
A. clarkii	X	X	X	X	X	X	X		X	X	10
A.fuscocaudatus			X								1
A. latifasciatus			X								1
A. omanensis	X			X			X				2
A. tricinctus	X		X	X			X	X			5
Total number of fish associates	16	4	12	8	7	11	14	7	1	1	

1.3 Evolution of anemonefish and their host anemones (at micro and macro scales).

The symbiont-host relationship between anemonefish and anemones is likely to have evolved 10 million years ago, with origins diversifying in the Central Indo-Pacific area (Litsios et al. 2014a). The relationship probably began with anemonefish competing with related damselfish to occupy anemones as a predation refuge. Over time, the relationship is likely to have evolved from a close behavioural association to full contact and immersion into the tentacles, which eventually gives anemonefish protection against the stinging nematocyst of the host anemones (Burke & Nedosyko 2016; Holbrook & Schmitt 2010).

Anemonefish likely evolved from specialist forms that lived with only one or few host species, such as the species complex *P. biaculeatus*/*A. ocellaris*+*A. percula*, to generalist forms that live with a wide range of hosts, represented by the *A. clarkii* complex (Elliott et al. 1999; Litsios et al. 2012; Santini & Polacco 2006). Characteristics of those that have evolved can be seen by their body shape, their caudal fin shape, the numbers of bars in the adult fish, the number of hosts, the dependence on host for shelter and distribution range (Elliott et al. 1999, Santini and Polacco 2006).

Satini and Polacco (2006) suggested general trends of anemonefish evolution in three main steps: 1). The ancestor diversified into a number of species with specialized characters related to body and tail shape. 2). The newly evolved species increased the distribution of their natural habitat. 3). These species further differentiated into extant species. These suggested steps could explain the wide range of groups of fish species with a wide geographical distribution. However, Litsios suggested an alternative; that anemonefish have diversified through an adaptive radiation process, which was driven by the symbiotic association with anemones (Litsios et al. 2012, 2014a). As species spread geographically, they likely encountered more potential anemone hosts and new anemone-anemonefish combinations developed. Over the time, the morphological characteristics and behaviour of the fish adapted to the new anemone hosts, and this process might have shaped anemonefish evolution. Anemonefish have had a faster species diversification rate than their close relatives (other damselfish) without anemone association (Litsios et al. 2012).

Anemonefish behavior is also specialized and adapted to a mutualistic lifestyle (Mariscal 1970). Physical and behavioural adaptations can be seen in the way anemonefish change in response to their hosts at night. Specifically, *Amphiprion* (*A. akallopisos*, *A. nigripes*, *A. percula* and *A. xanthurus*) settle into the tentacles of their host and become immobile at night. Their body color lightens so that their contrasting white-color bars blends in with the background color of the anemone's tentacles (Mariscal 1970). When the fish are exposed to light, they immediately revert to their normal diurnal coloration.

Although anemonefish have adapted to live with anemone hosts, both in morphology and behavior, the hypothesis of co-diversification in this mutualistic pairing has never been tested explicitly. A thorough phylogenetic analysis is needed to examine any evidence for co-evolution between anemonefish and their anemone hosts.

1.4 Mechanism of anemone-anemonefish associations

All Cnidarians, including sea anemones, are venomous animals. There are currently 250 compounds identified in cnidarian venom, most of them are peptides, proteins, enzymes and proteinase inhibitors (Frazão et al. 2012). These compounds are basically divided in two groups: (1) the Neurotoxins are peptides which rarely exceed 10kDa in size. They are only released from nematocysts and injected into a potential prey or predator after a mechanical or chemical stimulation. By binding with neurotoxin receptor sites and then inactivating Na^+V and K^+V channels, these toxins cause paralysis, loss of coordination and tissue damage; (2). the Cytolysins are larger molecules of about 10kDa to 80kDa (Frazão et al. 2012), which are found in the mucous coat covering the anemone's body. These compounds are very effective haemolysins and ichthyotoxins, which can kill fish within one hour at concentrations <0.5ug/ml. Potential prey suffers gill damage due to pore formation in the epithelial membranes of the lamellae, and ultimately die due to breakdown of the organ physiology functions, such as osmoregulation and gas exchange.

Although there have been numerous studies devoted to understanding the mechanisms underlying the protective elements, a single unifying explanation has yet to be determined (Jem & Brooks 1984; Miyagawa 1989; Elliott et al. 1994; Elliott & Mariscal 1997; Karplus 2014; Burke & Nedosyko 2016). There are two main factors proposed to contribute to the fish protection from an anemone's sting:

1. Protection by acclimation

The discovery of acclimation behavior provided the first insight into the protection mechanisms of anemonefish. Whereas non-acclimated anemonefish are often stung by sea anemones, acclimation allows fish to live freely among stinging tentacles.

Acclimation behavior was first described by Gohar (1948), followed by a detailed quantitative description of Davenport and Norris (1958), including a series of stereotype behaviors of fish toward their host anemones. In this process, the first contact of fish toward an anemone is often initiated by nosing or nibbling the tentacles cautiously, then touching tentacles with their fins. Acclimation is completed when the fish penetrate and bathe freely among the tentacles without any response from the anemone. Mariscal (1972) showed that naïve or non-acclimated *A. clarkii*, were stung by three species of anemones (*Stichadactyla gigantea*; *Anthopleura xanthogrammica* and *Anthopleura elegantissima*) regardless of whether or not the anemones had been living with the fish before. The anemones attacked by reaching the fish's skin and adhering their tentacles to it, discharging large numbers of nematocysts, which were seen on the fish's skin when it was examined with a light or electron microscope (Karplus 2014). If the mucous was removed carefully from the fish's skin, deacclimation of partially or fully acclimated fishes could happen, and the fish could be attacked by their former anemone hosts unless they were able to learn to re-acclimate (Win & Olla 1972).

The acclimation behavior of fish has been described to vary with individuals and with different natural and unnatural anemone hosts. The time needed to complete acclimation also depends both on the species of fish and anemone, from a few minutes to several days (Karplus 2014). Acclimated fish can freely move from one anemone to another of the same species but may either succeed or fail when switching to a host of a different species.

2. Protection by mucus.

In the acclimation process, the skin mucus components of the anemonefish may undergo a change and acquire a certain resistance to the host venom. There are three hypotheses to account for any change in the fish skin mucus; 1) Camouflage, 2) Alternation of fish skin mucus, and 3) Incorporation of anemone mucus elements.

1) Camouflage. During acclimation, the fish acquire a form of "camouflage" or "macromolecular mimicry" from the anemone to avoid being recognized as "not self", which can result in an attack by the anemone (Schlichter 1976, Elliott et al. 1994).

2) Alteration of fish skin mucus. Lubbock (1980, 1981) found that *A. clarkii*, the generalist anemonefish, which can be found living with all ten host species, was able to settle into *Stichodactyla haddoni* immediately. He assumed that this was because the chemical components in the mucus of *A. clarkii* are different from those found in other non-symbiotic damselfish (Pomocentridae). Most of those components are glycoproteins, containing neutral polysaccharides, which do not elicit anemone nematocyst discharge (Lubbock 1980). Therefore, it was thought that *A. clarkii* produces skin mucus that does not have the substances which would otherwise trigger nematocyte release. According to Lubbock (1980, 1981), the transfer of anemone mucus to the fish might increase the thickness of fish mucus layer, but it was a minor contribution to a fish's protection. He claimed that the mucus of *A. clarkii* acted as an inert layer that separated stinging cells of the anemones and provided the fish with a "cloak of invisibility".

The hypothesis was also supported by the findings of Miyagawa (1989), in which she suggested that *A. clarkii* had an innate protection produced in their mucus coat. However, observations by Burke & Nedosyko (2016) showed that *A. clarkii* which had been separated from their host anemone for two months were stung by their former host when reintroduced, as they did not have their acclimated behavior yet. Elliott et al (1994) also found anemone antigens in the mucus coat of acclimated *A. clarkii* and those antigens were not present in those that did not inhabit a host anemone.

Developmental and interspecific variations in the anemonefish protection was also investigated (Elliott & Mariscal 1996). Whereas fish eggs were protected, the larvae were captured and killed by the symbiotic anemones. The metamorphosed juveniles were not stung, suggesting that the protection developed during ontogeny (Mebs 2009; Karplus 2014).

3) Incorporation of anemone mucus into the fish mucus. Foster (1975) found a unique protein in the mucus of acclimated fish, which was absent from either non-acclimated fish mucus or anemone mucus or toxins. Mebs (1994, 2009) described a specific mechanism of “immune response” mediated through the skin that helps the fish to survive among stinging tentacles. In this process, there would be a two-way signal transmitted between fish and anemone through the water over a short distance during acclimation (Mebs 2009).

In recent research, analysis of the mucosal microbiome of symbiont anemonefish showed significant variation from that of non-symbiont fish, suggesting a potential microbial role in mediating the fish-anemone interaction (Pratte et al. 2018).

To sum up, anemonefish protection from anemones is complex and differs among anemonefish species. The mechanisms may also depend on the host species (Elliott 1991) and may have evolved independently in relation to anemone toxicity, tentacles charateristics, and fish physiology and behaviour (Elliott & Mariscal 1996).

1.5 Background to the experimental approach.

1.5.1 Care of anemones in the laboratory

Sea anemones are fascinating creatures that are sought out by marine aquarium hobbyists. They are also among the most difficult of reef inhabitants to keep in captivity. Although different anemone species need to be supported by specific requirements, most species can be cultured under similar conditions of water quality, water flow, lighting and compatibility of anemones with their fish. The conditions and methods that were developed for the experimental approach described in this thesis are as below. The methods were optimized for the six anemone species *Entacmaea quadricolor*; *Macrodactyla doreensis*; *Stichodactyla gigantea*; *S. haddoni*; *Heteractis crispa* and *Heteractis malu*.

a. Water quality

The water quality reflects the maturity of the aquarium. For keeping anemones, it should be very high water quality, clean and free from accumulated debris. Water changes of 10% of the aquarium volume were made once each week and achieved optimal environment parameters in this study as follows:

- The dissolved oxygen levels ranged from 8.12-8.67mg/L and pH from 8.17-8.25.
- The temperature range for anemones was 26-28°C, and the salinity range 33–35 psu. Levels of phosphate, ammonia and nitrite were kept close to 0 and nitrate was no more than 2 ppm. Alkalinity levels were 10.0 to 11.0, Calcium levels were 380-450 ppm, while Strontium levels and Iodine levels were 5.0 – 15.0 and 0.03 – 0.06 respectively.

b. Water movement and flow

It is important to achieve good water movement and flow so that the anemones can obtain oxygen directly and may also gather food from the water. Without sufficient water movement, the anemones will not be supported with the oxygen and food that they need for survival. In contrast, too much flow will make it hard for anemones to bury their foot into the substrate, and they will move until they find a place with the appropriate amount of flow. The anemones become ill and die if they cannot find a suitable place to anchor themselves (Nguyen, H-T T. observations). Most anemone species do well with low to moderate flow regimes. The flow rate for the six anemone species was kept at 60 - 90 L/min, using Jebao RW8, electrically powered wave-makers.

c. Lighting requirement

Anemones need suitable lighting to support their symbiotic zooxanthellae that provide anemones with energy and exchange for carbon and nitrogen. Different anemone species have different lighting needs. In this study, LED (Maxspect – Razor 130W) lights with ambient and long wave ultraviolet fluorescent light provided illumination for the tanks. Six levels of light intensity made up a daily light cycle, with the maximum light (100%) at noon (12:00), and the minimum (1%) at 06:00 and 18:00. The light intensity at maximum was about 16,000 lux at the water surface.

d. Compatible anemonefish species

It is not necessary to keep anemones together with anemonefish in the tanks, but anemones show enhanced expansion, growth and survival in the presence of cohabiting anemonefish (Balamurgan et al. 2014, Porat & Chadwick-Furman 2004; Porat & Chadwick-Furman 2005). Therefore, it is considered advantageous to keep anemonefish with their prefered host anemones as shown in Table 1. Throughout this study the anemones were maintained together with *A. clarkii* – the generalist fish species. The fish were removed from the tanks at least three weeks before anemones were used in the experiments.

1.5.2 Spawning and larval rearing of *Amphiprion ocellaris* in captivity.

The false clownfish, *A. ocellaris*, is one of the most popular clownfish for display. It is a specialist symbiont, normally found with the natural anemone host species either with *Heteractis magnifica*, *Stichodactyla gigantea* or *S. mertensii. A. ocellaris* broodstock can develop and spawn in laboratory conditions with or without host anemones. The first step of breeding was **pair formation**. Five males and five females of different sizes were stocked together, without an anemone. The largest male and female formed a spawning pair after about three months. Spawning pairs were transferred to separate 70L glass breeding aquaria, in which a terracotta pot was placed for the fish to reside. The breeding fish were fed with wet feeds that included mussel meat and shrimp, clam meat, fish egg mass, as well as formulated feeds enriched with vitamins, minerals and algae power. They were also supplied with live *Artemia* nauplii, given daily at midday.

Parameters in the breeding tanks were maintained as followed: temperature (26-30°C), dissolved oxygen (4.8 to 6.3ml/l); pH (8.1 to 8.25); salinity (32- 36psu). The water was re-circulated to ensure water movement and provide good water quality, with an exchange rate of 25% per week to avoid stress for the fish. Clay pots were provided in each tank for refuge and for egg deposition.

After five months in these conditions, a broodstock pair usually started breeding. A few days before **spawning**, the male selected a site in the pot for laying eggs, which was carefully cleaned by both the male and the female a few hours before they spawned. The spawning lasted for 1 – 1.5 hours, and happened between 10:00 – 17:00. The spawning cycle was at intervals of 12 – 15 days, depending on the size and the spawning history of the fish.

From spawning to **hatching**, the eggs were taken care of by both the male and the female fish. The male appeared to spend a higher percentage of his time at the nest than the female. There are two basic egg care activities: fanning with pectoral fins and mouthing to remove dead or weak eggs or dust particles. The egg incubation lasted for 6 - 8 days, depending on the temperature of the tank water. On the day of hatching, the eyes of the developing larvae inside the egg capsule were clearly visible, and the eggs had a silvery appearance. Hatching occurred at the water temperatures of 28 - 29°C, and took place shortly after sunset.

All of the fish in the experimental work in this thesis came from eggs produced by captive breeding pairs in such conditions. For each experiment, eggs were transferred from the breeding tanks to nursery tanks on the expected day of hatching, two hours before sunset. Air bubbles were used to supply oxygen to the eggs. Hatching occurred soon after sunset, in complete darkness. The newly hatched larvae were about 3-4 mm in length, had a transparent body, large eyes, and visible mouth. The larvae started to feed on micro algae, and then from the fourth day post hatching (dph) they were fed with small strain rotifers *Brachionus* sp. at concentration of 5 to 7 prey/ml. From 5 dph the larvae were weaned onto newly hatched *Artemia* nauplii (provided at 4 to 6 prey/ml) along with rotifers and mixed culture of micro algae. From 10 dph, larvae were fed with newly hatched *Artemia* nauplii only. At 30 dph, juvenile fish were transferred to 160L aquarium tanks without anemones and fed with *Artemia* twice daily. The range of environment parameters maintained in the nursery tanks was: salinity (32 to 35ppt), temperature (24-28ºC), dissolved oxygen (5.3 to 6.8ml/l) and pH (8.1 – 8.9).

1.5.3 Preliminary experiments

To establish captive populations of anemones and anemonefish, sampling was conducted from November 2014 to April, 2017 in Nha Trang Bay, Khanh Hoa province and Phu Quy Beach, Ninh Thuan province, Vietnam. Specimens of eight anemone species (representing two families Stichodactylidea and Actiniidea), and six anemonefish species (genus *Amphiprion*) were collected. All specimens were identified using relevant taxonomic references; for anemones (Dunn 1981; Fautin &Allen, 1997; Fautin 2008) and for anemonefish (Allen 1975; Allen et al. 2005). The anemone *Cryptodendrum adhaesivum* was collected only once, in March 2015, and it was not possible to sequence all three mitochondrial markers with the limited amount of tissue for this species. *Heteractis magnifica* was never found during the collection time, although Astakhov et al (2016) reported the presence of this species in reefs of Phu Quy, Vietnam in 2010.

The pilot experiment of anemonefish and anemones associations was first conducted in April 2015, which confirmed the host preference of the anemonefish. Further experiments were conducted over an 18 month period (from January, 2016 to November, 2017) to refine this symbiont-host system to support an experimental approach to host choice and acclimation. In addition, the system could be used for future studies of resilience to environmental conditions such as warming or ocean acidification.

Newly settling juveniles of *Amphiprion ocellaris* (about 12 dph), bred in the laboratory without anemones, were subject to testing the ability of the juveniles to recognize their natural host in host choice experiment. After training for 24 hours in the water flow condition of the experimental tanks, fish were exposed in two sets of 200 l tanks, with three replicates in each set: experimental tanks containing six species of anemones and control tanks containing six artificial rubber anemones. The whole experiment was run once as a preliminary trial and a second time with full data collection. In general, mortality was high for fish at this developmental stage, both in the preliminary and in the experimental run (reported in **Paper II**).

Acclimation experiments were conducted on post settlement juveniles (60 dph) bred in the laboratory without anemones. The experiment aimed to test whether fish could go through acclimation behaviour to establish themselves with their host anemones, and tested the ability of fish to adapt to unnatural hosts in the absence of their favorite host. The experiment was conducted in triplicate, in three sets of 200L aquaria. Fish in the experimental tanks were offered six anemone species, including one natural host species (*Stichodactyla gigantea*) and five unnatural host species (*Entacmaea quadricolor*, *Macrodactyla doreensis*, *Stichodactyla haddoni*, *Heteractis crispa*, *Heteractis malu*). The experiment lasted 32 days, divided into three intervals as described in detail in **Paper III**. After the first interval, the anemone host that attracted the highest number of anemonefish was removed from the experimental tanks. The fish were allowed to acclimate to the remaining anemones. At the end of interval II the removal of the anemone host with the highest number of associated anemonefish was repeated. These steps were repeated until the fish did not associate with any remaining anemones, at interval III.

A negative control group (NC group) was established in triplicate using artificial rubber anemones instead of living anemones; while in duplicate positive control tanks (PC group), six species of anemones were placed in the same way as in the experimental group, but the anemones were not removed at each interval.

The acclimation behavior was video recorded and observed directly; the growth and the activities of anemonefish were monitored over intervals. Fish skin mucus was also collected and the proteomic responses to the changes of host were measured.

The acclimation experiment was repeated several times. The mortality analysis of the fish living with different anemones was conducted based on two runs of the experiments.

Definitions

Acclimation behaviour: the actions of anemonefish, including a series of stereotypic behaviors of the fish toward anemones as their hosts. The acclimation process begins with the fish nosing or nibbling their host tentacles, and then touching the tentacles with their fins. The acclimation is completed when the fish penetrates and nestles among the tentacles without any defensive response from the anemones.

Natural host: the anemone species that an anemonefish species most commonly associates with in the natural environment.

Unnatural host: an anemone species that an anemonefish species does not associate with in the natural environment.

Symbiont: a fish that has established an association with one (or more) anemone (s).

Host: an anemone where one (or more) anemonefish resides or an anemone occupied by one (or more) anemonefish.

Naïve fish: a fish that has never encountered an anemone.

Experienced fish: a fish that has encountered or established an association with a host anemone (natural/unnatural host).

Generalist(s) (fish): an anemonefish species that has been found associated with many different anemone species in the natural environment (e.g *Amphiprion clarkii*).

Specialist(s) (fish): an anemonefish species that has been found associated with only a few anemone species in the natural environment (e.g *Amphiprion ocellaris*).

Extreme specialist(s) (fish): an anemonefish species that has been found associated with only one anemone species in the natural environment (e.g *Amphiprion frenatus*).

Newly settling juveniles: a transitional developmental stage when anemonefish move from the plankton to search for hosts.

Post settlement juveniles: developmental stage when anemonefish have completed their transition from the plankton, spending the majority of their time on the bottom or with a host.

2. OBJECTIVES

The work in this thesis is focussed on understanding the adaptation and mechanism of anemonefish in the anemone – anemonefish relationship, through four main research questions: (1) Does mutualism lead to coevolution of anemonefish and anemones? (2) Can *Amphiprion ocellaris* juveniles acclimate to live with unnatural host anemones? (3) Is there any change in fish skin mucus as the fish acclimate to unnatural host anemones? (4) Can anemonefish benefit (in terms of growth) from acclimating to unnatural anemone hosts? To answer the first question a cophylogenetic approach was used to test the coevolution of anemonefish and host anemones (**Paper I**). Genetic data based on mitochondrial markers in anemonefish (CO1, 16S rRNA and Cytb) and anemones (CO1, 16S rRNA and 12S rRNA) were generated from specimens of six species of anemonefish and eight species of anemone collected in Khanh Hoa and Ca Na beaches from 2014 to 2016. Together with GenBank data, the phylogenetic trees of both anemonefish and anemones were built and tested for historical coevolution using statistical and event-based methods.

Host-recognition experiments were conducted with newly settling juveniles and post settling juveniles of *A. ocellaris* to answer the second research question (**Papers II & III**), in which naïve juveniles were exposed to six species of anemones, including one natural host species and five unnatural host species. By successive removal of the "preferred" anemone at 9-day intervals, host-switching was induced. The fish acclimation behaviour (**Paper III**) was recorded and the growth rate and the activity of the fish were also monitored to evaluate the fitness of the fish living with different hosts (**Paper II**).

Skin mucus of naïve and experienced *A. ocellaris* during their acclimation to natural and unnatural hosts was collected and profiled to investigate any functional proteins contributing to the anemonefish's defence when the fish switched hosts. Proteins of fish's skin mucus were also investigated for the presence of anemone proteins to see if the fish produce their own protecting mucous coating, or if the fish acquire anemone’s mucus during their acclimation.

3. SUMMARY OF THE PAPERS

Paper I

Cophylogenetic analysis of the relationship between anemonefish *Amphiprion* (Perciformes: Pomacentridae) and their symbiotic host anemones (Anthozoa: Actiniaria).

Nguyen, H-T.T, Dang TB, Glenner H., Geffen, AJ.

Manuscript resubmitted.

Twenty-eight species of anemonefish of *Amphiprion* (Perciformes: Pomacentridae) are in obligatory association with ten species of anemones (Actiniidae, Stichodactylidea and Thalassianthidae) in coral reef systems. This living together has been suggested to be the key innovation leading to anemonefish expansion into new habitats. However, the coevolution history has not been thoroughly tested. Using a cophylogenetic approach (distance-based methods and event-based methods), we examine whether the symbiotic relationship has led to coevolution of anemone and anemonefish. Phylogenetic trees were reconstructed based on the mitochondrial marker sequences of anemones (16S, CO1 and 12S) and anemonefish (16S, CO1 and Cytb). Neither distance-based tests nor event-based tests showed significant global coevolution of anemones and anemonefish. However, some significant individual links indicated the dependence of the symbionts upon the hosts at some levels. These findings suggest that, at the molecular level, the symbiosis of anemone – anemonefish appears to be an example of commensalism rather than mutualism.

Paper II:

Nguyen, H.-T.T, Tran A-NT, Ha LTL, Ngo DN, Dang TB, Geffen, AJ. (2019): "Host choice and fitness of anemonefish *Amphiprion ocellaris* (Perciformes:

Pomacentridae) living with host anemones (Anthozoa: Actiniaria) in captive conditions", *J Fish Biol.* 2019;1–11.

Anemonefish are a group of 28 species of coral reef fish, characterized by their association with sea anemones. The fish can reside among the stinging tentacles of their host without being harmed. Host utilization varies among anemonefish species; some are specialists living with only one or two anemone host species, others are generalists living with more than one anemone host species. In this study, we investigated the host choice of naïve fish *Amphiprion ocellaris*, a specialist, at two different stages of development (newly settling juveniles, and post-settlement juveniles). Their fitness was assessed through their growth rate and activities when living with natural and unnatural host anemones in laboratory. Newly settling juveniles did not show the preference towards the natural host *S. gigantea*. On the other hand, the post settled juveniles immediately made symbiosis with the natural host, and chose *S. haddoni*, the congeneric species, as the second choice when the natural host was removed. The increasing activities and lower growth rate were observed when the fish switched to the unnatural host or failed to establish a new symbiosis association. Host specificity in the field may be a trade-off between immunity to anemone toxicity and better growth.

Paper III:

Sea anemone –anemonefish symbiosis: Behaviour and mucus protein profiling.

Nguyen, H-T.T, Zhao M., Wang T., Dang TB, Geffen, AJ., Cummins S.F.

Manuscript submitted, under review.

The symbiosis of anemonefish and anemone is the iconic example of mutualism in coral reefs. Fish of the genera *Amphiprion* and *Premnas* live essentially unharmed and well protected from predators among the tentacles of anemones. Fish skin mucus has been suggested to be the major role in preventing the discharge of nematocysts upon contact. However, the question of whether the fish produce their own mucus or the fish coat themselves with anemone mucus is still under debate. This study investigated the behavior during anemone – anemonefish interactions and the accompanying chemical changes in the fish skin mucus. The successful acclimation of *Amphiprion ocellaris* juveniles was observed when the fish were in contact with their natural host anemone *S. gigantea*, and then the unnatural host *S. haddoni* after the removal of *S. gigantea*. Some fish was observed to establish symbiosis for *Heteractis crispa*. No symbiotic association was established between the fish and the anemones *Entacmaea quadricolor*, *Macrodactyla doreensis* and *Heteractis malu*, which are unnatural host and the non-Stichodactyla species. The proteins in the fish skin mucus, both of control (the negative and positive control groups) and experienced groups (the experimental group) during their acclimation to natural and unnatural host anemones was also reported. Those proteins are involved in immunologic defense, molecular transport, stress response, and signal transduction. Ribosomal-type proteins increased in the fish, which were in contact with the anemones. There was also a presence of anemone protein in the skin mucus of the fish who established symbiosis. The findings suggest that fish might acquire anemone mucus (camouflage) on their skin mucus during their acclimation, which gives them the access to the anemones.

4. SYNTHESIS AND GENERAL DISCUSSION

By approaching mutualism of anemone-anemonefish in multiple ways, this thesis assessed the adaptations of anemonefish towards their hosts at different levels: (1) molecular, or gene level; (2) organ (skin mucus) – functional level; and (3) whole organism level – growth and behaviour. Cophylogenetic analysis showed no coevolution in the history of the anemone-anemonefish relationship (**Paper I**). In the presence of six potential host species, newly hatched naïve *A. ocellaris* did not recognize their parent's favourite host species. However, naïve post-settled juveniles (60dph) had a clear preference toward their natural host *S. gigantea*. After the removal of *S. gigantea*, the fish adapted to the unnatural host *S. haddoni*, which is a congener of the natural host. After the removal of *S. haddoni*, *A. ocellaris* was not attracted to the four remaining unnatural hosts *E. quadricolor, M. doreensis, H. crispa* and *H. malu*, except for some fish attracted to *H. crispa*. The activity level of the fish increased, and their growth decreased in response to the absence of a suitable host (**Paper II**). Protein profiles in the skin mucus of the naïve *A. ocellaris* juveniles are different from those of experienced fish. The uncertainty of whether the skin mucus acquires a form of 'camouflage' or is altered through the contact with the host is partly resolved in this thesis (**Paper III**). These findings also help to predict the future of the anemone-anemonefish relationship under extreme environment conditions, such as climate change that could decrease diversity of anemones and could also shift their distribution.

4.1 The evolution of anemone and anemonefish symbiosis (**Paper I**)

In this thesis, the phylogenetic trees of both anemones and anemonefish were reconstructed based on new gene sequences identified during this work and archival gene sequences accessed from GenBank. These data were used to test explicitly the hypothesis of co-diversification between anemonefish and anemones.

The phylogeny of anemones was recovered based on eight new combined sequences (12S rRNA+16S rRNA+CO1) obtained in this study and 63 sequences from GenBank. The tree recovered Stichodactylidae as a polyphyletic group, and Actiniidae as a paraphyletic group in the superfamily Actinioidea (Figure 1). Thalassianthidae was placed as a clade of *Stichodactyla* in Stichodactylidae. This topology agrees with the larger anemone phylogeny published by Titus et al. (2019), and also identifies the same significant taxonomic problems, at both family and genus levels, when comparing molecular to current morphological taxonomy (**Paper I**). At the species level, it was particularly difficult to interpret the relationships between specimens of species in the *Heteractis* and *Stichodactyla* genera (**Paper I).**

Based on combination of cytochrome oxidase subunit 1 (COI), 16S rRNA and Cytochrome b (COI+16S rRNA+Cytb), the recovered anemonefish tree topology considered the subfamily Amphiprioninae as a monophyletic group with early divergence of the *Actinicola* subgenera from the rest of the anemonefish (Figure 2). *Premnas biaculeatus* was not reconstructed as a basal group of all the other anemonefish, but became a clade within the *Amphiprion* genus. The tree suggests that anemonefish might have evolved from specialist ancestors (Elliott et al. 1999) (**Paper I**).

In this thesis, statistical - based methods (Parafit, PACo) and event - based methods (Jane4, Core-PA) of cophylogenetic analysis were used to test the congruence of anemones and anemonefish phylogenies, both for global and for individual contributions. There was no evidence for coevolution between anemones and anemonefish, with no global congruence between the two phylogenies (Parafit, PACo analyses). Jane4 showed that coevolution scenarios depended on the cost assignment, whereas none of the reconstructed solutions from Core-PA, which chose the best cost values for each co-evolution event, indicated significant co-speciation (**Paper I**).

Anemonefish are rather sedentary coral fish, but the group is widespread across temperate to tropical marine habitats (Jonathan 2009). The success of anemonefish has been explained by their ability to expand into untapped ecological niches due to

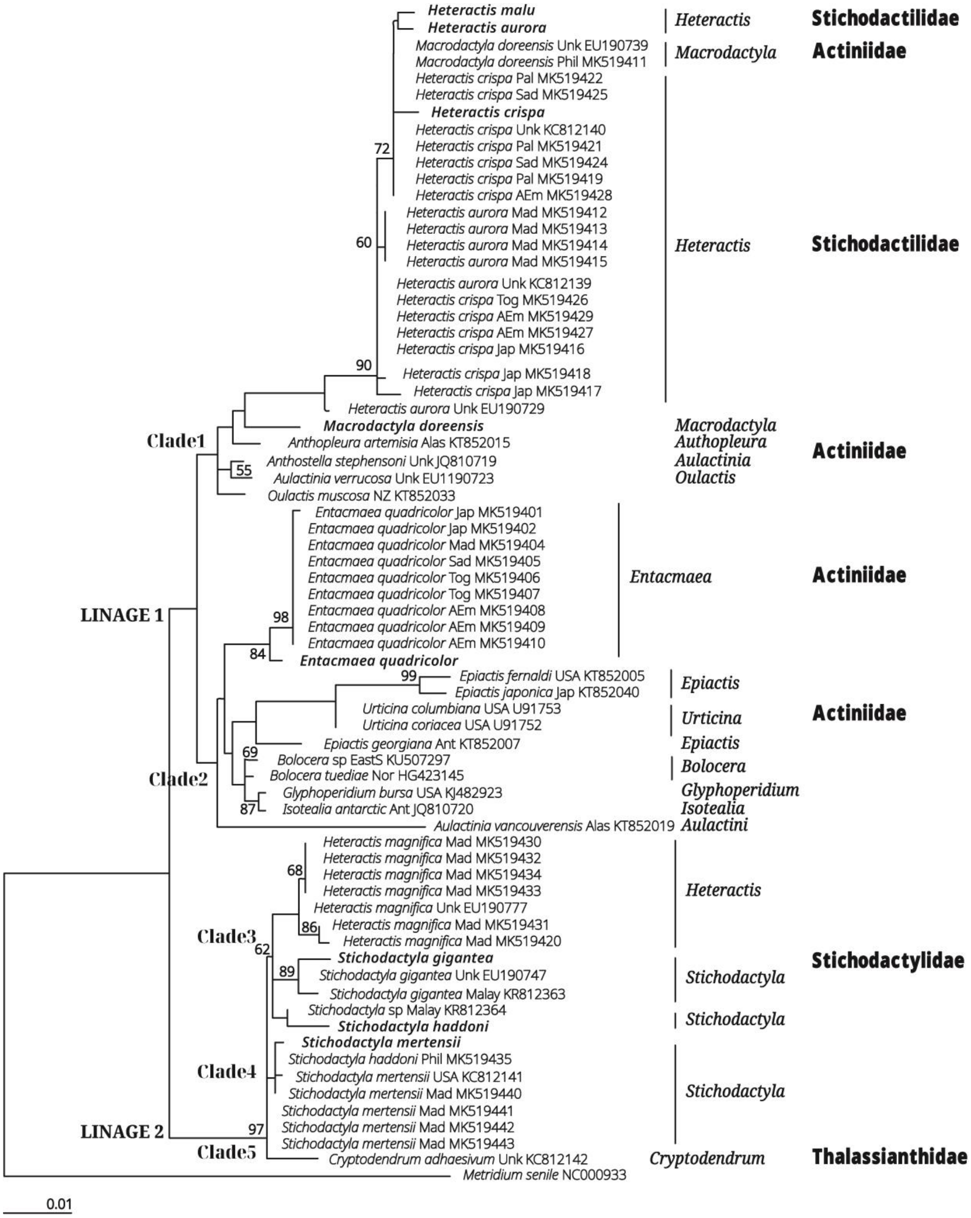

Figure 1. Anemone phylogenetic tree from combined mitochondrial genes dataset (COI mtDNA, 16S rDNA and 12S rDNA) based on 71 sequences of anemones, of which eight sequences are from the current study. The tree corresponds to the best ML tree assuming TrN+G+I model chosen from ModelTest available in Ape package. Node supports are indicated by bootstrap values (when >50%). Sequences from this study were bold. Sequences from GenBank go along with locations and ID. Metridium senile was used as outgroup. Abbreviations: Unk = Unknown; Phil = Philippine; Pal

=Palau; Sad = Saudi Arabia; AEm= United Arab Emirates; Mad=Maldives ; Tog = Tonga; Jap= Japan; Alas= Alaska; Malay = Malaysia.

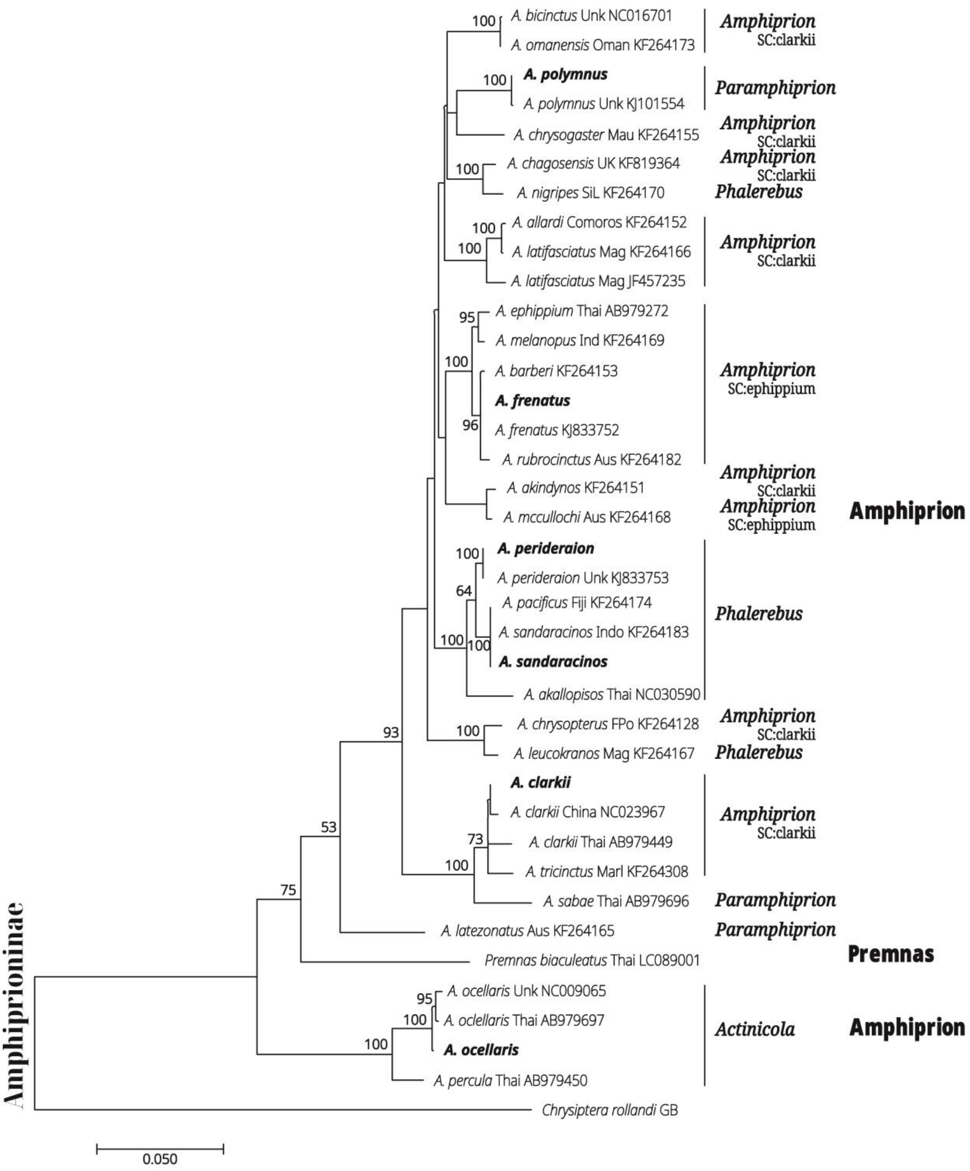

Figure 2: Anemonefish phylogeny tree from combined mitochondrial genes dataset (COI mtDNA, 16S rDNA and Cytb) representing 12 out of 28 species of anemonefish, of which six sequences are from the current study; The tree corresponds to the best ML tree assuming GTR+G+I model chosen from ModelTest available in ape package. Node supports are indicated by bootstrap values (when >50%). *Chrysiptera rollandi* was used as outgroup.

their association with anemone hosts (Litsios et al. 2012, 2014a). Their rate of evolution has also been likely to be more rapid than that of the other related species of damselfish (Litsios et al. 2012). In contrast, anemones can also be a host to hermit crabs, or live without (macro)symbionts (Gusmão & Daly 2010). This could explain the dependence, at some level, of anemonefish on anemone hosts as identified in the PACo analysis, but not vice versa, as identified in the Parafit analysis (**Paper I**). In a recent publication, Titus et al. 2019 also found that anemonefish and some anemone hosts have different biogeographic origins. The finding implies that the anemonefish and anemones apparently have not co-evolved (**Paper I**).

Fautin (1991) proposed the idea of three-way symbiosis in the anemone-anemonefish relationship, which should include zooxanthellae, a symbiont alga. Since then, a number of experimental studies have demonstrated the intimate relationship between parties (Murata et al. 1986; Elliott et al. 1999; Porat & Chadwick-Furman 2004, 2005). A large amount of wastes released by the fish, such as dissolved ammonia and phosphorus, are assimilated by zooxanthellae in photosynthesis, which increases the symbiont algae populations and the growth of anemones (Porat & Chadwick-Furman 2005). Anemones also grow bigger in response to the protection and nutrients provided by the symbiotic fish (Elliott et al. 1999; Porat & Chadwick-Furman 2004). The expansion of symbiotic anemones also promotes the light surfaces for zooxanthellae (Porat & Chadwick-Furman 2004). Murata (1986) also indicated that symbiotic zooxanthellae altered anemonefish movement pattern through their production of aplysinopsins (Murata et al. 1986). Evidence from recent studies suggests that the coevolution in the symbiotic systems actually involves a third species, rather than just the two usual species pairing (Filipiak et al. 2016; Nelson et al. 2014). The coevolutionary effects from the non-interacting species - the third party - were as strong as the effects from the directly interacting species in mutualistic networks (Guimarães et al. 2017).

4.2 Host choice in anemonefish (**Paper II & Paper III**)

Juvenile anemonefish are able to recognize and locate their host anemones by chemical cues and can also return to their natal location (Elliott et al. 1995; Gerlach et al. 2007; Miyagawa 1989). However, in the brief pelagic stage, larvae are subject to strong water currents and could travel far away from their parents' host and encounter several unnatural host species for the first time. Long-term observations of anemonefish in interaction with their hosts during the settlement transition offer new insights into the host selection process. Host choice can influence the future growth and behaviour of anemonefish. Therefore, understanding host choice of the anemonefish can help improve their survival and growth in captive conditions, and also help with the conservation of wild populations of anemonefish. (**Paper II**).

In our study, the naïve newly settling larvae were not able to identify the natural anemone host species and randomly approached any host anemone when introduced into the tanks. The morphological characteristics of the hosts were the first basis for attraction. The 12 dph fish approached the anemones which have long tentacles at first, rather than the natural host anemone, which has short tentacles. The fish were vulnerable to attack by all of the anemone species, but at the end of the experiment some fish successfully associated with *E. quadricolor*, the unnatural host species with long tentacles. Therefore, in an aquarium, it is possible to train newly settling *A. ocellaris* to live with unnatural host anemones if the natural host is not available (**Paper II**).

The post settlement *A. ocellaris* at 60 dph, on the other hand, appear to depend on a different mechanism to recognise and locate their hosts. The fish quickly recognised and established their symbiotic association with their natural host *S. gigantea*. They all switched to the unnatural host *S. haddoni*, the closely related species, when the natural host was removed. The fish inspected the anemone species with long tentacles (*E. quadricolor*, *M. doreensis*, *H. malu*) at first, but none of them settled in any of those unnatural hosts, including the long tentacle anemone *E. quadricolor* that was initially attractive to younger ones. The behaviour of the post settlement juveniles suggests that chemical cues appear to be more attractive in this stage, which helps the fish associate with suitable hosts and survive (**Paper II**).

4.3 The acclimation behavior and the change in the skin mucus protein components of the fish acclimated to their hosts (**Paper III**).

Anemonefish are not innately protected from anemone venom but acquire protection through a specific process of acclimation (Nedosyko et al. 2014; Burke & Nedosyko 2016). Naïve post juveniles in our study displayed acclimation behaviour upon contact, regardless of the anemone species. The time and type of behaviours varied depending on the anemone species (**Paper III**).

The anemonefish *A. ocellaris* showed no hesitation in contacting the natural host *S. gigantea* once they located the anemone. On the other hand, the fish took about one hour to establish an association with the related, but unnatural host species *S. haddoni* after the removal of the natural host *S. gigantea* (Figure 3A). The fish slowly approached the unnatural host *S. haddoni*, then hovered above the anemone's oral disc while nosing the tentacles repeatedly (Figure 3B). One of the fish was captured and eventually killed, but the remaining fish finally settled in the tentacles of *S. haddoni*. During the 1-hour process of acclimation, the fish actively nibbled, cleaned, and massaged the anemone's body. Some fish turned back and forth to the rock where *S. gigantea* had been located. The fish were not interested in the presence of the other four unnatural hosts after the removal of both *S. gigantea* and *S. haddoni*. In one of the tanks, five fish were in contact with *H. crispa* after about three hours of acclimation, but three of them subsequently died. The pairings of anemonefish with the unnatural hosts and the acclimation behaviours in this study are similar to those described in several previous studies (Sabol 1992; Balamuragan et al. 2014) (**Paper III**). The fish showed "schooling" behaviour while searching for an anemone host (Figure 3A).

The acclimation behaviors have been shown to provide further protection for the anemonefish in contact with the stinging tentacles of anemones as suggested by Elliot, (1994). *A. perideraion* in Mebs's experiment (1994) could live with *H. magnifica*, but was killed when exposed to the dissolved toxin of this anemone. The acclimation behaviors likely play an important role in allowing the fish to acquire protection from the anemone sting.

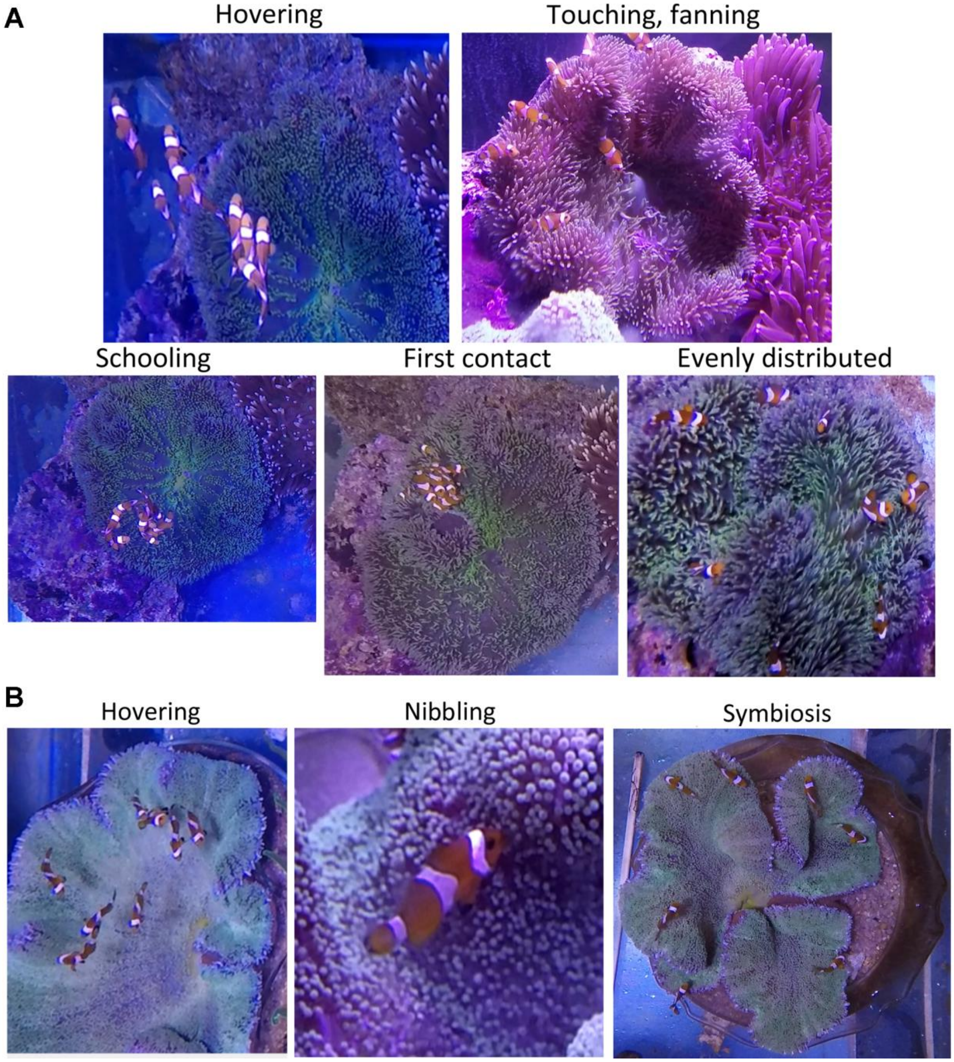

Figure 3: Summary of *A. ocellaris* behaviours in presence of anemones. (**A**) Behaviour of the contact with *S. gigantea*. (**B**) Behaviour of the contact with *S. haddoni*.

Specialist anemonefish species seem to adapt and have a better resistance to the toxins that are intrinsic to their natural hosts, regardless of the toxicity level of host's venom, as indicated by *Amphiprion ocellaris* in this study (**Paper III**). This resistance may be related to changes in the fish mucus during acclimation (Mebs 2009). The work presented in this thesis tested this idea by analyzing the skin mucus of *A. ocellaris* after manipulation of host availability. The fish were kept over several weeks with latex anemones (the negative control group), with access to their natural hosts (the positive control group), and with a decreasing choice of host anemone species (experimental group). Their behaviour and skin mucus was sampled and analyzed during three separate experimental intervals. The experiment is described in detail in **Paper III**.

A total of 348 non-redundant proteins were identified in all samples, of which the majority was found in the positive control group (Figure 4A; for more details see File S1a - **Paper III**). Sixty-three proteins were shared in common in all experimental and control samples, which provides a reference list of anemonefish proteins, regardless whether the fish are in contact with hosts or not. Gene ontology analysis of those proteins for biological function level indicated a prominence of cellular and metabolic processes involved (Figure 4C), while the major function at the molecular function level involved ion binding and DNA binding processes (Figure 4D). The list of novel proteins with their functions also provides a reference for proteins of anemonefish skin mucus (for more details, see Supplementary File S2b **- Paper III**).

The analysis of skin mucus proteins from the experimental group found a progressive increase in protein variability during each interval of the experiment. Twenty-three proteins are present in all biological replicates, most of which are identified in the total 63 conserved proteins. One unique protein was found after the first interval (as the natural host was removed), while samples from the last interval contained an abundance of histone and ribosomal proteins that was absent from the other interval samples (Figure 5, for more details on proteins, see Supplementary File S2c – **Paper III**).

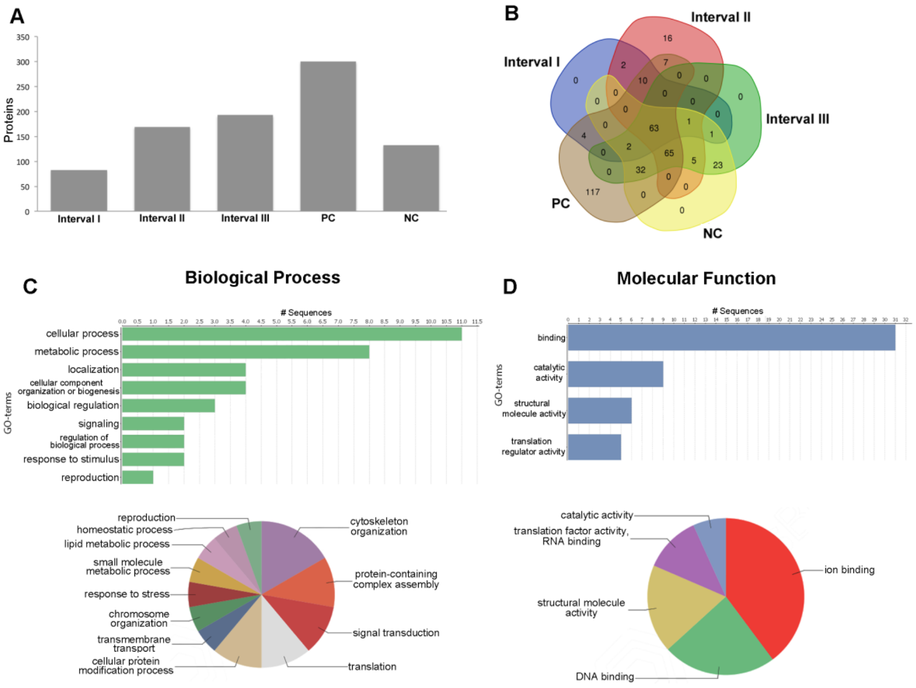

Figure 4: Summary of *A. ocellaris* skin mucus proteins. (**A**) Graph of total proteins identified within each sample. (**B**) Venn diagram showing distribution of identified proteins in fish skin mucus samples. (**C**) Graphs showing biological process categories for common proteins identified. (**D**) Graphs showing molecular function categories for common proteins identified.

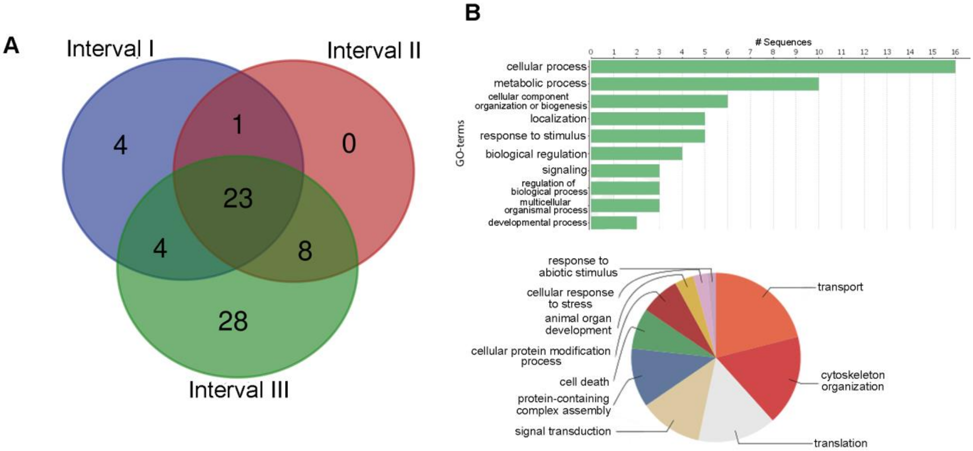

Figure 5: Summary of A. ocellaris skin mucus proteins within experimental groups at intervals I-III. (**A**) Venn diagram showing distribution of identified proteins in fish skin mucus samples at intervals I-III. (**B**) Graphs showing biological process categories for all proteins identified.

The positive control groups had more unique proteins by the end of the experiment, compared with the negative control groups. Gene ontology analysis of those proteins for biological function showed that the majority are involved in cellular and metabolic processes, translation, small molecule metabolic process and response to stress.

The protective mucus coat could act as camouflage so that anemones recognize anemonefish as "self" and this may be responsible for not eliciting nematocyst discharge (Jem & Brooks 1984; Miyagawa 1989; Elliott et al. 1994; Elliott & Mariscal 1997). Proteins from the fish skin mucus were compared to those in the anemone mucus to test the hypothesis that fish acquire a form of 'camouflage' through contact and accumulation of anemone particles during acclimation (see Table S2 in **Paper III** for more details). A *S. haddoni* transcriptome-derived protein database was assembled in this study and compared to the fish samples. Thirty-nine proteins could be confidently (e-value >10-3) designated as non-fish, of which seven were anemone-like. None of these anemone-like proteins were found in the skin samples of the negative control fish, those who had never been in contact with anemones (Table 6 – **Paper III**). These observations, along with the changes observed during the host manipulation, suggest that these proteins could help during the establishment of symbiosis. Further research should be more precise in identifying anemone skin mucus if a *S. gigantea* protein database were available. A fully quantitative Liquid chromatography/Mass spectrometry protein analysis was beyond the scope of this study, but it would be needed in order to evaluate whether the amount of those proteins found are sufficient to be able to prevent the discharge of anemone's nematocyst as suggested previously (Lubbock 1980). Therefore, quantifying the skin mucus compounds from fish and anemones, with a focus on the anemone-like proteins, would be the next step to understanding which compounds contribute to the flexibility of the chemical responses during acclimation.

4.4 The effect of acclimation on fish fitness (**Paper II**).

The growth rate and activity of the fish during and after acclimation to different hosts were measured as proxies for fitness (the growth rate) and energy expenditure (the activity). The fish were ranked by weight, then individually tagged, and followed throughout the experiment. Fish were photographed and weighed at the beginning of the experiment, and again at the end of each interval. Fish growth was calculated as size - specific growth rate for each interval and for the entire length of the experiment. Fish behaviour was recorded by video, and the average total distance travelled by each individual was measured and used to express the swimming activity of the fish (**Paper II**).

4.4.1 Activity

The juvenile *A. ocellaris* showed the typical pattern of swimming activity of diurnal coral-reef fish, which is emerging from shelter at dawn, being active during the day, and returning to their shelter at dusk and hiding at night (Rickel & Genin 2005). There was no significant difference in the activity of fish at dawn, even when associated with different anemone hosts. However, at other times of the day, the behaviour of fish differed when associated with different host species, and also differed from normal reef-fish (**Paper II**).

The fish tended to be less active at noon, and equally active at dusk and at night when they were living with *S. gigantea* and *S. haddoni* (Figure 1 in **Paper II**), while reef fish feed actively at this time (Rickel & Genin 2005). The fish were inactive at night when they were not in symbiosis with anemone hosts. These differences might be explained by the interactions of anemonefish with their hosts, as well as the defence of their territories against intruders or the agonistic behaviour to maintain the social structure.

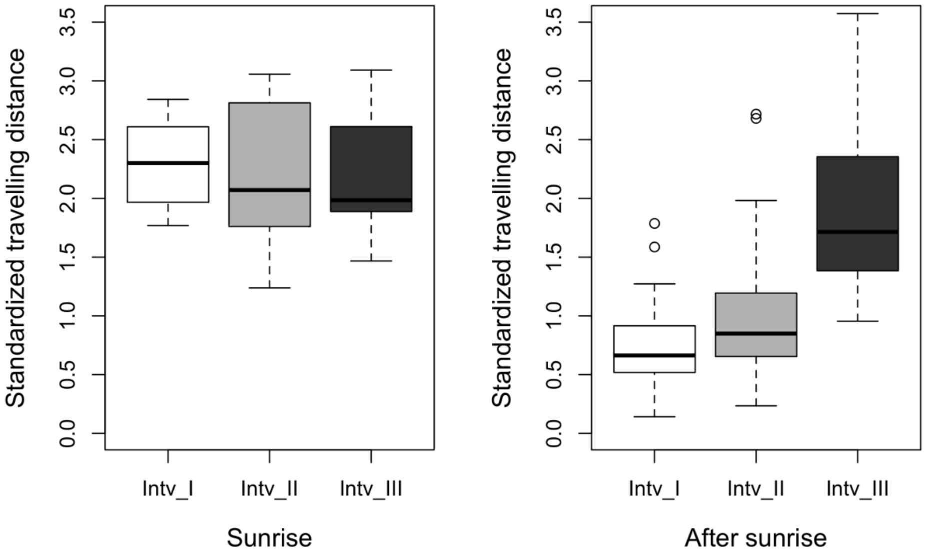

Figure 6. Activity of juvenile *A. ocellaris* living with different anemone hosts at dusk (Sunrise) and the rest of the day (after sunrise). Intv_I: fish living with the natural host anemone *S. gigantea*; Intv_II: Fish living with the unnatural host anemone *S. haddoni*; Intv_III: Fish were not living with any host.

A. ocellaris did not express those activities when not associated with an anemone host and the behaviour was more similar to diurnal coral-reef fish (**Paper II**).

The travelling distance of fish living with *S. haddoni* was greater than those living with *S. gigantea*, and significantly greater than those fish with no suitable host choice (Figure 6), reflecting the different quality of refuge provided by the anemones.

4.4.2 Growth

Growth of juvenile *A. ocellaris* also responded to the changes in host availability. Fish grew fastest when living with the natural host anemone *S. gigantea*, and growth rate decreased when the fish were forced to switch to the unnatural host *S. haddoni.* Growth rate decrease even more when the fish lost their access to the two *Stichodactyla* species. The size – specific growth rate of juvenile *A. ocellaris* of the same rank did not change over time, suggesting that the social structure remained constant even through the acclimation to a new host.

It is likely that the juvenile *A. ocellaris* can adapt to live with an unnatural anemone host, but experience reduced fitness (a lower growth rate and increased swimming activity) as a consequence of stress and vulnerability when losing access to an anemone host (**Paper III**). Several immune defense- and stress- related proteins were identified in the skin mucus of fish after removal of their host anemones, while those proteins were absent from the skin mucus of fish living in symbiosis.

The repeated observation that *A. ocellaris* juveniles can establish symbiosis with the unnatural anemone species *S. haddoni* in captivity means that toxicity is not always the main driver of the anemonefish-anemone species pairing. *S. haddoni* is more toxic, and one individual fish was stung during its first encounter. Fish activity did increase when living with this species, and growth rate declined as a consequence. Therefore, in the field, beside species competition and compatible geographic distribution (Fautin 1991), host choice of anemonefish might be trade-off between better immunity (reduced activities) and better growth. Anemonefish will choose the anemone host that provides them the highest quality of refuge and lowest cost in term of physiological expenditure (Burke & Nedosyko 2016). This might be the reason we do not find *A. ocellaris* living with *S. haddoni* in the natural environment.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The work described in this thesis partly resolves questions about the evolutionary aspects and physiological responses underpinning the adaptation of anemonefish toward their anemone hosts. The results represent the first co-phylogeny analysis of the anemonefish-anemone association, demonstrating that the mutual relationship does not lead to permanent adaptation of the anemonefish symbionts to their host anemones through coevolution. However, there is the dependence of anemonefish evolution on anemones at some level. *Amphiprion ocellaris* juveniles can adapt to unnatural host anemones, in the absence of their favourite host, but can experience a lower fitness, with lower growth and increased activity. The fish expressed acclimation behaviours toward all of the anemone species, but the time and type of behaviours varied, depending on anemone species. For those anemonefish who established symbiosis, there is a clear increase in ribosomal-type protein, which relates to immune function. This study also provides evidence for the “camouflage” hypothesis, since anemone-like proteins are present only in the skin mucus of individuals that have established symbiosis. The fish are likely to suffer from stress and to be vulnerable when they fail to establish an association with the unnatural host anemones *E. quadricolor*, *M. doreensis* and *H. malu*. These results contribute to understanding of the mechanisms that enable the anemone-anemonefish relationship.

Future research effort should be directed to some of the unanswered questions about the adaptation of anemonefish toward anemones.

1. The samples analyzed for this thesis were limited to six of the 28 species of anemonefish, and eight of the 10 species of anemonefish hosting anemones. None of the anemone samples collected is a non-symbiotic species. Therefore, extending the number of anemonefish and anemones species, including non-symbiotic anemone species, would increase the representativeness and precision of the test. On the other

hand, Anthozoan mtDNA is largely uninformative due to its slow rate of evolution. Therefore, the using of mitochondrial genes (COI + 16S rRNA +12S rRNA) of anemones in attempts to resolve species-level of anemones is limited. High resolution genomic methods, such as targeted capture and enrichment approaches for ultra-conserved elements would likely improve the phylogenetic resolution.

2. Significant changes in the protein components of the skin mucus were detected in the experimental group, after the fish switched to new hosts. However, there is still uncertainty about which proteins are involved. Quantitative LC-MS analysis of both anemones and anemonefish mucus would help to determine which proteins contribute most to the changes of the fish skin mucus.

3. It has not been determined why protection of the fish toward their hosts only lasts for 24 hours after fish leave the anemone hosts. Is it the flexibility of the fish to be able to adapt to many anemone hosts they encounter? Tracking the changes of anemonefish skin mucus and anemones mucus by quantitative LC-MS analysis, before and after fish leave their hosts, would help to identify the key factor mediates the fish protection.

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7. PAPERS

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SYMPOSIUM SPECIAL ISSUE REGULAR PAPER

Host choice and fitness of anemonefish *Amphiprion ocellaris* (Perciformes: Pomacentridae) living with host anemones (Anthozoa: Actiniaria) in captive conditions

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In this study, we investigated the host choice of naïve *Amphiprion ocellaris*, a specialist, at two different stages of development (newly settling juveniles and post-settlement juveniles). The fish were exposed to their natural and unnatural host species in the laboratory and their fitness was assessed in terms of activity and growth rate. Newly settling juveniles exhibited little host preference, while post-settlement juveniles immediately associated with their most common host in the wild. The analysis of fish activity confirmed that *A. ocellaris* is diurnal; they are most active in the morning, less at midday and barely move at night. The average travelling distance of juveniles was shorter in the groups living with their natural host, increasing in the groups living with an unnatural host and was highest in groups that did not become associated with any other unnatural host species. Post-settlement juveniles living with the natural host species grew better than those living with unnatural hosts or without anemone contact. These results suggest that the welfare of *A. ocellaris* in captivity will be optimized by keeping them with their natural anemone host species, although more generalist *Amphiprion* species may survive in association with other hosts.

KEYWORDS
Amphiprion, clownfish, coral reef fish, growth rate, mutualism, swimming activity

1 | INTRODUCTION

1.1 | The anemonefish and anemone association

The association between anemones and anemonefishes (family Pomacentridae, subfamily Amphiprioninae) in coral reefs is a classic example of a mutualistic interaction, in which both organisms benefit from living together. The bright colours of anemonefishes and their behaviour in association with anemones, make them a popular target for the ornamental aquarium trade. To ensure their welfare and survival in captivity, it is important to understand the basis for the association between the fish and their anemone hosts.

There are *c.* 1200 species of sea anemone (Actiniaria), but 10 species (families Actiniidae, Stichodactylidae and Thalassianthidae) are found in association with fish symbionts. These symbionts comprise 28 species of anemonefish in the genus *Amphiprion* Bloch & Schneider 1801, belonging to the subfamily Amphiprioninae (Burke & Nedosyko, 2016). Host utilization varies among anemonefish species, ranging from specialists, such as the tomato clownfish *Amphiprion frenatus* Brevoort 1856 that are found only on one anemone host species, to generalists, such as the yellowtail clownfish *Amphiprion clarkii* (Bennett 1830) which may live with any one of a number of anemone host species (Fautin & Allen, 1992). Under normal conditions, anemone fishes establish an association with one anemone and do not switch to another, irrespective of species. The anemone species that the fishes are most commonly associated with in the natural environment are characterised as natural hosts and the anemone species that fishes do not associate with in nature are characterized as unnatural hosts (Elliott *et al.*, 1995). In the field, the anemonefish *Amphiprion ocellaris* Cuvier 1830 has been found associated with three natural host anemones: *Stichodactyla gigantea*, *Stichodactyla mertensii* and *Heterastis magnifica*. The host specificity influences both the host choice of anemonefishes during their settling period and can potentially influence their future growth. Therefore, understanding host specificity is important in order to provide the best condition for fish growth in captivity. This can enhance survival for anemonefishes that are captured from the wild or are bred in captivity for the aquarium trade.

After the demersal eggs of anemonefishes are hatched, the larvae have a pelagic stage of 11 to 15 days, where they can be dispersed by ocean currents over large spatial scales (Elliott *et al*., 1995; Gerlach *et al*., 2007). After the pelagic stage, they return to benthic habitat and search for a host anemone to settle. During this transition, the larvae are subject to a strong selective pressure to find and associate with a suitable anemone host. Newly settling juveniles may detect their host anemone by smelling chemical cues and return to the habitat where their conspecifics have already settled (Elliott *et al*., 1995; Gerlach *et al*., 2007; Miyagawa, 1989). Chemical cues are apparently more important for this stage than visual cues that may be used at later stages. However, laboratory and field studies have shown contradictory results for the role of chemotaxis in settlement. Laboratory results emphasised the role of imprinting on host habitat recognition of settling larvae (Miyagawa, 1989; Arvedlund & Nielsen, 1996). Miyagawa (1989) studied 12 anemonefish–anemone species combinations and found that the anemonefishes were only attracted to their natural host species (the anemone species found in symbiosis with that anemonefish species in the wild), but not to unnatural hosts. Elliott & Mariscal (1997) found that juvenile *A. ocellaris* that had been previously exposed as embryos to their natural anemone host *H. magnifica* showed a strong attraction toward this host, while fish that had been never been in contact with the host ignored their natural host. However, in a host selection experiment conducted in the field, Elliott *et al*. (1995) showed that the attraction response of settling larvae to the host anemones varied. Some anemonefishes were not attracted to their natural hosts, while others were attracted toward unnatural hosts. Dixson *et al*. (2014) used genetic parentage analysis to demonstrate that juvenile anemonefishes did not show a preference for returning to their natal site. Therefore, larvae could be just simply and randomly settling on the host anemones as they encounter them (Burke & Nedosyko, 2016). Long-term observations of anemonefishes in interaction with their hosts during the settlement transition could offer new insights into the selection process.

In the laboratory, individual anemonefishes have been observed to display particular acclimation behaviours, which allow them to live among the stinging tentacles of a host anemone, even an unnatural host species (Elliott & Mariscal, 1997). If the particular anemonefish can acquire protection against the toxins of the anemone and live with them, there must be some reason why do not we find them associating in the field.

1.2 | Benefits of anemonefish living with their host anemone

Primary benefits that anemonefishes receive from their host anemones include protection from potential predators (Fautin, 1991), removal of external parasites (Allen, 1972), additional nutrients from tentacles and increase in reproductive fitness (through egg protection; Allen, 1972; Berumen *et al*., 2012). Anemonefishes can have a lifespan of more than 30 years, which is twice as long as other pomacentrid species and up to six time longer than other marine fishes of a comparable size (Holbrook & Schmitt, 2005). Clearly, association with the anemone is a highly advantageous strategy for resident anemonefishes. There is little information available to indicate whether anemonefishes acclimated to live with unnatural hosts obtain the same benefits. Fitness may play a role in host choice if the anemonefish maximize fitness by choosing anemone hosts that provide them with the highest quality of refuge and lowest cost in term of physiological expenditure (Burke & Nedosyko, 2016; Nedosyko *et al*., 2014).

1.3 | Growth as an indicator of fish fitness

Fitness can be defined in many different ways, however, a general consensus considers fitness as an ability of individuals, or populations or species, to survive and reproduce in the environment where they find themselves (Barker, 2009). In a given habitat, there are three approaches to measure fitness of population, individuals, or species: (a) directly assessing fitness among genotypes, (b) growth rate of the population and individuals, and (c) individuals traits (fitness-related traits) as measure of performance (Kawecki & Ebert, 2004). Growth, either alone or in combination with other fitness-related traits, has been used as an indicator for fitness in previous research (Ivan & Miguel, 2007; Kaltz *et al*., 1999; Sato, 2006). In marine fish, measurements such as body size at age, condition factor and growth rate can be proxies for fitness (Marshall *et al*., 2003). These variables also can be used as relative indicators of the underlying quality of the habitat (Magnhagen, 2008). In the anemone–anemonefish relationship, anemones acts indirectly as a microhabitat for the anemonefishes. In this study, we measured the growth (body mass and rate of increase in mass) as a proxy of fish fitness during acclimation to different anemone hosts.

In addition to growth, swimming activity is an important variable related to energy expenditure (Crossin *et al*., 2014). The energetic cost of swimming contributes to the overall metabolic load, therefore affecting the potential growth response and eventually fecundity (Arnott *et al*, 2006). Roach *Rutilus rutilus* (L. 1758) has been shown to reduce locomotor activity to compensate for producing gonadal tissue during a reproductive season (Koch & Wieser, 1983). There is also a metabolic trade-off between growth and other fundamental demands such as swimming performance, by which metabolic allocation for growth will decrease its availability for swimming (Kawecki & Ebert, 2004; Arnott *et al*, 2006). This may be explained by oxygen limitation, because growth may compete with other activities, including swimming (Pauli *et al*., 2017).

1.4 | Size hierarchies in anemonefish groups

In the wild, anemonefishes inhabit sea anemones in groups of up to six individuals with a well-defined size hierarchy based on their roles: the largest fish is a dominant female, the second largest is a breeding male, followed by up to four non-breeding individuals with progressively smaller sizes (Buston, 2003; Iwata *et al*., 2008). If the dominant female dies, then the male changes sex and become the female and the largest subordinate becomes the breeding male. Removal of a fish of any rank in an anemonefish group leads to faster growth of lower rank fish to ascend in rank. In order to maintain her dominance, the female displays frequent aggressive behaviour toward other members in the group. Subordinates, on the other hand, receive charges and show submissive responses (Chen & Hsieh, 2016; Iwata *et al*., 2008). This size-based dominance hierarchy seems also to be regulated by acoustic behaviour, in which aggressive sounds in conjunction with threat postures produced by the winners make the losers emit submissive sounds and submissive postures (*e.g*., head shaking movement) at the same time (Colleye & Parmentier, 2012). The precise size regulation in anemonefish groups has been proposed as a strategy for maintaining relative stability and resolving group membership conflicts by reducing the threat to dominants from their subordinates (Buston, 2003).

1.5 | Aims

Our study of anemonefish activity and growth in the presence of natural and unnatural hosts focused on four research questions: (a) is there any difference between naïve newly settling juvenile and post-settlement juvenile *Amphiprion ocellaris* in recognizing their natural anemone hosts and unnatural anemone hosts; (b) can naïve juvenile *Amphiprion ocellaris* acclimate to live with unnatural hosts; (c) do anemonefishes benefit in terms of growth by associating with unnatural anemone hosts; and (d) does association with unnatural anemone hosts alter the growth and size hierarchy between individuals in an anemonefish group?

2 | MATERIALS AND METHODS

Two sets of experiments were conducted to address our research questions. First, we observed the initial host selection and survival of newly-settling juveniles at 12 dph (days post hatching) when introduced to tanks with a choice of six different anemone host species, including the natural host species with which *A. ocellaris* is associated in the wild, *i.e*., *S. gigantea.* Second, we recorded the swimming activity and monitored the growth of post-settlement juveniles (60–92 dph) through a series of manipulations of host species availability. In both experiments the test fish were naïve, reared up to that point in tanks without contact with anemones.

2.1 | Anemonefish and anemone rearing

Breeding pairs of the anemonefish *A. ocellaris* were established in separate 70 l glass aquaria in a recirculating seawater system at Nha Trang University, Vietnam. There were no anemones in the breeding tanks and breeding pairs laid eggs inside a terracotta pot placed in the aquaria. Water temperature, salinity and oxygen were measured with portable probes twice daily. Temperature ranged 28–30°C and salinity 32–35. Nitrates, ammonia and phosphates were measured with a commercial water quality monitoring kit twice per week. The fish were held under a natural daylight cycle (12° 15′ N) of 10L:14D with natural daylight illumination throughout the different life stages.

Adult *A. ocellaris* were fed a mixture of shrimp, oyster, liver and commercial fish pellets, with vitamin supplements added. Larvae were fed with rotifers *Brachionus* sp. from 4 dph and *Artemia* sp. nauplii were gradually introduced until larvae were only fed with *Artemia* (5 nauplii per ml) by 10 dph. From 30 dph, juvenile fish were transferred to 160 l aquarium tanks without anemones and fed with *Artemia* twice daily. The juveniles in these tanks were considered naïve fish and used in the post-settlement host-choice experiment.

Six species of anemone, consisting of *S. gigantea*, a natural host for *A. ocellaris* and five unnatural hosts, *Stichodactyla haddoni*, *Entacmaea quadricolor*, *Macrodactyla doreensis*, *Heteratis crispa* and *Heteractis malu* (Fautin & Allen, 1992) were purchased from local tropical fish stores that obtained their animals from Ca Na Beach (11° 20′ 15′′ N 108° 52′ 46′′ E). Anemones were maintained in 200 l glass aquaria fitted with a recycling system, where water quality was monitored daily. Nitrates, ammonia, phosphates and total alkalinity were measured with a commercial water quality kit and salinity, temperature, pH and oxygen were measured with portable multiprobes. Water flows were adjusted as needed to maintain salinity 33–35, temperature at 26–28°C, pH at 8.17–8.25 and oxygen at 8.12–8.67 mg l^{-1}.

The anemones were fed with small pieces of prawn flesh once each week. The health of the anemones is a very important factor in host choice and acclimation, since if an anemone is in poor condition (signified by moving location, paleness in colour, or weak prey capture ability) then they appeared less attractive to potential anemonefish symbionts (H.-T. T. Nguyen pers. obs). Therefore, to be certain that cnida discharge was active, a non-symbiotic species, yellowtail damselfish *Chrysiptera parasema* (Fowler 1918), was used to test the discharging and capturing ability of an anemone. A net was made to cover a tested anemone in the tank and an individual fish was introduced into the tank at a height of approximately 10 cm above the anemone. If the anemone reacted to the presence of the fish, then that anemone was used for the host choice experiments (Elliott & Mariscal, 1997).

The experiments were conducted using 200 l glass aquaria, with recirculating flow systems and anemones were established in these tanks prior to any experimental work. Natural rocks were placed in the rearing aquaria for the anemone to attach. To avoid any influence of spatial cues, the rocks bearing the attached anemones were positioned to occupy the same position in each of the tanks. The same size and colour of anemones was set up to avoid any influence of colour variation in the host choice (H.-T. T. Nguyen pers. obs.). Anemones were maintained in these conditions for at least 3 months before beginning the experiments.

2.2 | Host recognition experiment of newly settling juveniles & post-settlement juveniles

2.2.1 | Newly settling juvenile host choice

Ten individual fish at 11 dph, showing the white barring colouration indicating that they were close to settling (Elliott *et al*., 1995), were chosen randomly from the offspring of a single parental pair. They were trained for 24 h in a flume giving the same water circulation conditions as in the 200 l experimental tanks with anemones. On the following day, the *A. ocellaris* were moved into the experimental tanks.

The experimental design consisted of two sets of 200 l tanks, with three replicates in each set: experimental tanks containing six species of anemones and control tanks containing six artificial rubber anemones. The environmental conditions were the same as described previously for the rearing tanks and the fish were fed daily with *Artemia* nauplii. The behaviour of the fishes was observed when they came into contact with the anemones and survival was measured over the following 10 days by counting the number of dead or missing fish. High mortality in two of the experiment replicates led to their early termination, after 24 h and qualitative results only are therefore presented for the remaining experimental tank.

2.3 | Acclimation behaviour experiment

2.3.1 | Post-settlement juveniles

Post-settlement fish with juvenile colouration, reared without contact with anemones, were introduced at 60 dph into the experimental tanks for a longer duration experiment where their behaviour was recorded and growth monitored. The experimental design consisted of three sets of 200 l tanks, with three replicates in each set: three replicates of experimental tanks containing six species of anemones, three replicates of positive control tanks where fish were also offered six species of anemones, but these were not manipulated, and three replicates of negative-control tanks containing six artificial rubber anemones. Daylight and water conditions were the same as in the rearing tanks and fish were fed once daily with *Artemia* nauplii (to avoid deterioration of water quality for the anemones from any excess food).

The *A. ocellaris* used in the experiments were divided into groups of six individuals, with an initial size hierarchy; they were selected based on length and mass, to form groups in which there were one big fish, one small fish and four medium-size fish. Results from a pilot experiment indicated that *A. ocellaris* formed size hierarchies from day 15 after hatching and we chose to include this aspect to evaluate any differential growth response during the exposure to different anemone hosts.

To form the size structured groups, the fish were anaesthetized with MS-222, photographed and their wet mass measured (Krejszeff *et al*., 2013). Each individual was then tagged with a visible implant elastomer (VIE) tag (Northwest Marine Technology; www.nmt.com) suitable for small sized fish (Hohn & Petrie-Hanson, 2013). The tag was injected using a small bore needle (gauge 30, *c*. 0.34 mm diameter). Orange, green and red elastomer dyes were chosen to mark the different individuals, injected just below the dermis, at either the caudal peduncle or below the dorsal fin. With the combination of colour and location the individual fish of different ranks could be followed throughout the experiment.

The duration of the experiment was 32 days and consisted of three intervals. Fish were photographed and weighed at the beginning of the experiment and again at 69, 78 and 92 dph (the end of the experiment). The fish were not fed on the day before each weighing. During the first interval (60–69 dph), there was no manipulation of conditions. At the beginning of the second interval (69–78 dph), the natural host (*Stichodactyla gigantea*) was removed from the experimental treatment tanks. At the beginning of the third interval (78–92 dph), the most popular of the five unnatural host species (*S. haddoni*) was removed from the experimental tank. No anemones were removed from the positive-control tanks (Table 1).

Fish growth was calculated for each interval and for the entire length of the experiment. Because of the individual size differences in the hierarchies, growth was expressed as size-specific growth rate: $(M_{t+1} - M_t)(\Delta t\, M_t)^{-1}$, where, M_t is fish mass (g) at the beginning of the interval and M_{t+1} is fish mass (g) at end of interval, Δt is the number of days in the interval.

Activity is a consistent behavioural trait in anemonefish symbionts and a useful measure to monitor acclimation (Wong *et al*., 2013). To characterise whether the behaviour pattern of *A. ocellaris* changed when they encountered different anemone hosts, the fish in each tank were videotaped on the third day after their introduction into the experimental system. *Amphiprion ocellaris* are normally diurnal, but activity was recorded over a 24 h cycle to detect any differences due to host changes. Activity was recorded using a Gopro Hero 5 Black (www.gopro.com) for 3 min, at 15 min intervals during 1 h in the morning (07:30 to 08:30); at mid-day (11:30 to 12:30), at sunset (17:00 to 18:00) and at night (21:00 to 22:00) on day 1, 3, 5 and 7 of the experiment (Table 1). For each video, 3000 frames were recorded. Video recording of *A. ocellaris* was made from above of the tanks. A ruler was taped to the bottom of the tank, which provided a length reference for subsequent distance measurements.

The recorded videos were analysed with Tracker software (www.cabrillo.edu/~dbrown/tracker), which is able to track individual fish against the colourful background of the sea anemone. Activity was expressed as the average total distance travelled by an individual, expressed as cm s^{-1}, standardized to fish length (cm s^{-1} L_T^{-1} cm fish). Only the recorded activity from day 5 of the experiment was used to analyse fish activity, because of time limitations for the video processing (Table 1).

2.4 | Statistical analysis

To test the effect of anemone host species on the growth of *A. ocellaris*, linear mixed effect models with repeated measurements, implemented in R (www.r-project.org), were used to estimate difference in means of three treatments. Treatment tank (replicate) was a random effect, with fish age, fish rank and treatment as fixed effects. Significant differences in these factors were identified by Tukey *post hoc* tests. For travelling distance, we also used a mixed-effects model to test for differences between treatments and host species (interval), taking into account the effects of time of day.

3 | RESULTS

3.1 | Host choice of naïve settling and post-settlement juvenile *Amphiprion ocellaris*

3.1.1 | Naïve settling larvae

Naïve settling larvae responded randomly when first introduced to potential hosts, but the response of fish was different among the three replicates (Table 2). Within the first 10 min after being introduced to the host anemones, between one and five individuals in each replicate tank had made sustained contact with an anemone. However, only one individual out of a total 10 in two of the replicate tanks had settled on the

TABLE 1 Experimental schedule for test of post-settlement *Amphiprion ocellaris* growth and swimming activity after introduction to natural and unnatural host anemone species from 60–92 days post hatch (dph)

Age of fish (dph)			**Handling**	
Interval I	60	Photo and Weighing	Rest fish	
	61	Introduced to anemones		
	62	Swimming activity recorded	D1	
	64	Swimming activity recorded	D3	
	66	Swimming activity recorded	D5	Activity data analysed
	68	Swimming activity recorded	D7	
Interval II	69	Photo and Weighing	Rest fish	Removal of *S. gigantea*
	70	Introduced to anemones		
	71	Swimming activity recorded	D1	
	73	Swimming activity recorded	D3	
	75	Swimming activity recorded	D5	Activity data analysed
	77	Swimming activity recorded	D7	
Interval III	78	Photo and Weighing	Rest fish	Removal of *S. haddoni*
	79	Introduced to anemones		
	80	Swimming activity recorded	D1	
	83	Swimming activity recorded	D3	
	85	Swimming activity recorded	D5	Activity data analysed
	87	Swimming activity recorded	D7	
	89	Swimming activity recorded	D9	
	91	Swimming activity recorded	D11	
	92	Photo and Weighing	End of the experiment	

Interval I: fish living with the natural host anemone *S. gigantea*; Interval II: fish living with the unnatural host anemone *S. haddoni*; Interval III: fish did not associate with remaining available host anemones.

natural host *S. gigantea*. By the end of 24 h after being introduced (Table 2), the naïve *A. ocellaris* were found in sustained contact with *S. gigantea*, *S. haddoni*, *E. quadricolor* (one individual in each of two out of three replicate tanks) and *M. doreensis* (one individual in one of three replicate tanks). In one of the replicate tanks, three *A. ocellaris* were attracted to and settled in the unnatural host *H. crispa*.

One day after being introduced into the tanks, 40% of the *A. ocellaris* were found to be contact with a host anemone. The remaining fish were either found dead or were missing and had probably been consumed. This high mortality in two of the replicate tanks led us to terminate these tanks after 24 h. At this point, no fish were associated either with their natural host *S. gigantea* or the closely related unnatural host *S. haddoni*. Instead, the fish spent most of the time associated with the unnatural hosts *E. quadricolor* and *H. cripsa*, which are characterised by long tentacles. In the remaining replicate tank, over the following days, four *A. ocellaris* died and the remaining fish associated primarily with *E. quadricolor*, with one or two individual fish changing between *E. quadricolor*, *H. crispa* and *M. doreensis*. At the end of the experiment, on day 10 (22 dph), all five surviving fish were found living *E. quadricolor*. In the control group, *c.* 20% of the fish were attracted to the artificial anemones, while *c.* 80% of the rest stayed hiding behind dark rocks.

TABLE 2 Number of newly settling *Amphiprion ocellaris* living with different anemone host species at 24 h after introducing to the experimental tanks, by replicate

		Number of fish living with anemone				
Anemone	**Host characteristics**	**Replicate1**	**Replicate 2**	**Replicate 3**	**Number of fish released**	**Total number of fish remaining in all tanks**
Stichodactylidae						
*Stichodactyla gigantea**	Natural short tentacles	0	1	1	10	2
Stichodactyla haddoni	Related to natural short tentacles	1	0	1	10	2
Heteractis crispa	Unnatural long tentacles	0	3	0	10	3
Heteractic malu	Unnatural long tentacles	0	0	0	10	0
Actiniidae						
Entacmanea quadricolor;	Unnatural longest tentacles	1	0	1	10	2
Macrodactyla doreensis	Unnatural long tentacles	0	1	0	10	1

Mortality of the newly settling juveniles was higher in the experimental tanks, with live anemones, compared with the control tanks, with artificial anemones (χ^2-test, $P < 0.05$). Of the initial 30 fish across all three experimental tanks, about 40% of the fish survived, 30% were found dead and 30% were missing. Of the initial 30 fish across all three control tanks, about 70% of the fish survived, about 17% were found dead and 13% were missing. The number of dead and missing fish was highest in the first 24 h after the fish were introduced to the experimental tanks, at which point two of the replicates were terminated. Fish were dead and missing also in the control tanks starting on day 2. Mortality continued until day 5 in the control tanks and until day 7 in the single replicate of the experimental tanks.

3.1.2 | Post-settlement juveniles

In contrast with newly settling juveniles, naïve *A. ocellaris* at the post-settlement stage showed an obvious pattern of preference toward their natural host and avoided the unnatural host species. After being introduced into the tanks, the fish used approximately 10 min to explore the tanks and then made contact with the natural host species *S. gigantea*. All of the fish became associated with their natural host, until *S. gigantea* was removed from the experiment tanks by 69 dph, the end of the first interval. By 70 dph, in the absence of the natural host, all of the *A. ocellaris* associated with an unnatural host anemone, *S. haddoni*, which is related to *S. gigantea*. During this period, the second interval, one *A. ocellaris* was attacked by *S. haddoni* and subsequently died. However, when *S. haddoni* was removed from the tanks on 78 dph, the fish were not attracted to any of the four remaining unnatural hosts. By the end of the experiment at 92 dph, there were no fish associated with any anemones and three fish were missing. One individual died in the negative control group, while there were no mortalities in the positive control groups.

3.2 | Activity of juveniles associated with natural and unnatural hosts

Because of the high mortality among the newly settling juveniles, long-term measurements of activity were only possible for the post-settlement juvenile experiment. Both time of day and host species had a significant effect on the travelling distance of fish and the interaction of these factors was also significant (mixed-effects model, $F_{6,131} = 5.27$, $P < 0.001$). The time of day effect was more important than the effect of host species (mixed-effects model, $F_{3,131} = 78.12$, $P < 0.001$ and $F_{2,131} = 37.35$, $P < 0.001$).

During the first interval, in all tanks, *A. ocellaris* were more active at sunrise than any other time of day (mixed-effects model, $F_{3,64} = 102.34$, $P < 0.001$). There were no significant differences between midday and sunset (Tukey *post hoc*, $Z = -1.58$, $P > 0.05$), or between sunset and night (Tukey *post hoc*, $Z = 2.24$, $P > 0.05$). Midday activity was significantly higher than night time activity (Tukey *post hoc*, $Z = -3.77$, $P < 0.001$). During the second interval, fish activity decreased significantly over the course of the day (mixed-effects model, $F_{3,54} = 33.74$, $P < 0.001$), except between sunset and night (Tukey *post hoc*, $Z = 1.14$, $P > 0.05$). During the third interval, *A. ocellaris* were significantly more active during the day than at night (mixed-effects model, $F_{3,24} = 13.92$, $P < 0.001$). Swimming activity increased, but not significantly, between sunrise and midday (Tukey *post hoc*, $Z = 2.29$, $P > 0.05$) and decreased, but not significantly, between midday and sunset (Tukey *post hoc*, $Z = -1.53$, $P > 0.1$).

We also compared the changes in swimming activity between intervals, for each period of the day separately (Figure 1). Activity at sunrise was not affected by the change in anemone host species (mixed-effects model, $F_{2,34} = 0.43$, $P > 0.05$). However, at midday, sunset and at night, the swimming activity was significantly affected by the host species availability (mixed-effects model, midday: $F_{2,38} = 16.12$, $P < 0.001$; sunset: $F_{2,38} = 42.35$, $P < 0.001$; night: $F_{2,30} = 20.75$, $P < 0.001$). The pattern of change was the same in each case; swimming activity increased slightly (but not significantly)

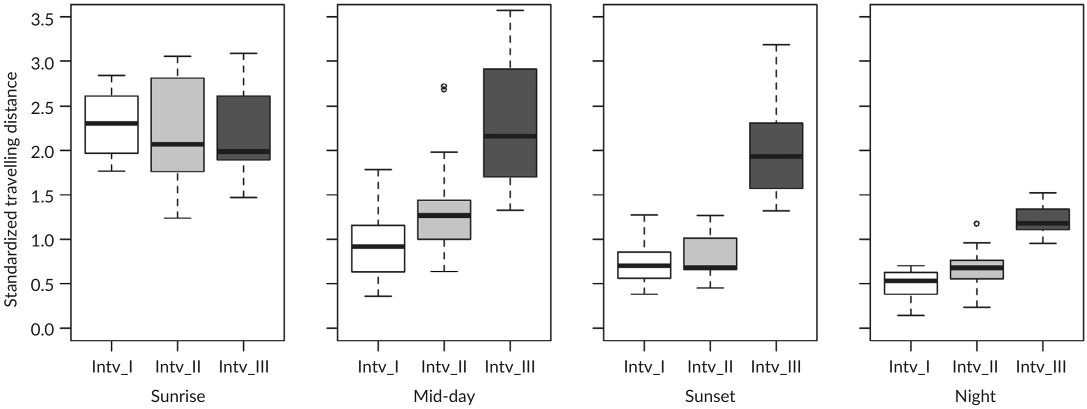

FIGURE 1 Box plots (——, median; □, 25th and 75th percentile; T 95% range; ○, outliers) of the diurnal changes in standardized travelling distance of *Amphiprion ocellaris* during each of three experiment intervals.: Intv_I, interval I, when fish were introduced to six anemone species and associated with the natural host *Stichodactyla gigantea*; Intv_II, interval II, when fish associated with *S. haddoni* after the natural host was removed; Intv_III, interval III when fish would not associate with any of the remaining available hosts after their congener *S. haddoni* was removed. L_T, Total length

between the first and second interval, when *S. gigantea* was removed from the tanks. However, when *S. haddoni* was removed, during the third interval, swimming activity increased significantly (Tukey *post hoc*, midday: $Z = 3.422$, $P < 0.001$; sunset: $Z = 7–76$, $P < 0.01$; night: $Z = 4.84$, $P < 0.001$).

3.3 | Growth of juvenile *A. ocellaris* associated with natural and unnatural hosts

Amphiprion ocellaris growth responded to the changes in host anemone availability. During first interval, the growth rate of fish in the experimental group appeared to be slightly higher than those fish in the positive control group, which only associated with the natural host and slightly lower than those in the negative control group that lived with artificial anemones (Figure 2a). However, when *A. ocellaris* switched to the unnatural host *S. haddoni* (second interval), the mean growth rate declined and seemed to be lower than the groups of fish that still had access to their natural host or even the rubber sea anemones (Figure 2b). During the third interval of the experiment, when fish in the experimental group had no suitable host anemones, the mean growth rate declined further and remained lower than those of the fish in the control groups (Figure 2c). Over the entire period, the fish in the experimental group grew more slowly than those in the positive control group (Tukey *post hoc* comparison, $Z = 2.65$, $P < 0.05$) and those of the fish in the negative control group (Tukey *post hoc* comparison, $Z = 2.34$, $P < 0.05$). There was no significant difference in growth rates between the positive and negative control fish (Tukey *post hoc* comparison, $Z = 0.525$, $P > 0.50$; Figure 3).

3.4 | Size hierarchies in *A. ocellaris* associated with natural and unnatural hosts

At the beginning of the experiment, at 60 dph, the size of *A. ocellaris* varied from 0.12 g to 0.32 g, reflecting the size hierarchy created

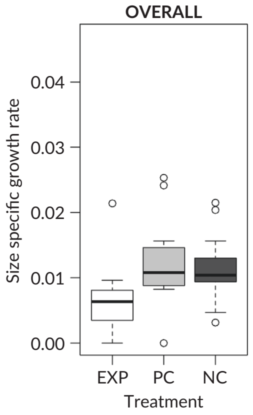

FIGURE 3 Box plots (——, median; □, 25th and 75th percentile; T 95% range; ○, outliers) of the growth of post-settlement *Amphiprion ocellaris* living with different host anemones, measured as change in size specific growth rate in the overall time of the experiment. EXP, experimental tanks, where fish were living with different hosts; PC, positive control tanks, where fish were living with only the natural host *S. gigantea*; NC, negative control tanks, where fish were living with artificial anemones

among individuals in each treatment (Figure 4). Fish mass increased significantly with age (mixed-effects model-repeated measures, $F_{1,149} = 141.87$, $P < 0.001$) and differed between fish of different ranks (mixed-effects model, $F_{5,149} = 117.69$, $P < 0.001$,) and treatments (mixed-effects model, $F_{2,149} = 7.75$, $P < 0.001$). Size hierarchy influenced the effects of host availability on fish mass, since there was a significant interaction between fish rank and treatment (mixed-effects model, $F_{10,149} = 3.71$, $P < 0.001$; Figure 5). There were no other significant interactions between the factor combination (age + rank: mixed-effects model, $F_{5,149} = 1.63$, $P > 0.05$; age + treatment:

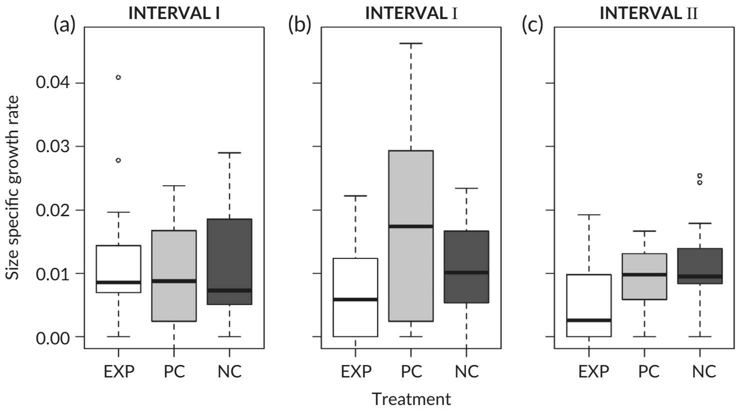

FIGURE 2 Box plots (——, median; □, 25th and 75th percentile; T 95% range; ○, outliers) of the growth of post-settlement *Amphiprion ocellaris* living with different host anemones, measured as change in size specific growth rate during each of three experiment intervals: (a) Interval I, when fish were introduced to six anemone species and associated with the natural host *Stichodactyla gigantea*; (b) Interval II, when fish associated with *S. haddoni* after the natural host was removed; (c) Interval III when fish would not associate with any of the remaining available hosts after their congener *S. haddoni* was removed. EXP, experimental tanks, where fish were living with different hosts; PC, positive control tanks, where fish were living with only the natural host *S. gigantea*; NC, negative control tanks, where fish were living with artificial anemones

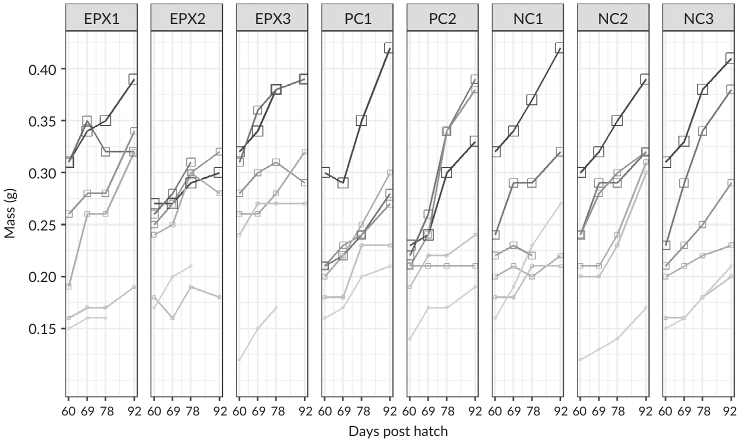

FIGURE 4 Growth of post-settlement *Amphiprion ocellaris* living with different host anemones, measured as change in individual fish mass from 60 days post hatch (dph) to 92 dph. Each panel represents the fish in each replicate tank (1–3) in each treatment: EXP, experimental tanks, where fish were living with different hosts; PC, positive control tanks, where fish were living with only the natural host *S. gigantea*; NC, negative control tanks, where fish were living with artificial anemones. Individual repeated mass measurements are indicated by marker symbol and line colour. FishCode (—□—) a, (—□—) b, (—□—) c, (—□—) d, (—□—) e, and (—□—) f

mixed-effects model, $F_{2,149}$ = 2.16, $P > 0.05$; age + rank + treatment: mixed-effects model, $F_{10,149}$ = 0.72, $P > 0.05$; Figure 5).

4 | DISCUSSION

4.1 | Host choice

Naïve newly-settling juvenile *A. ocellaris* were introduced to new anemone species, including species that they are not associated with in the wild (unnatural hosts). The purpose was to test whether there was an innate association with a particular host in this specialist anemonefish. As pelagic larvae, *A. ocellaris* can be dispersed far away from their natal reef and can encounter a range of anemone species during their settling. In this study, naïve newly settling juveniles were interested in several of the available anemone species, including both their natural and the unnatural host species. In our tanks, naïve *A. ocellaris* showed little preference among the available host species and seemed to survive best after contact with an unnatural host *E. quadricolor*. It was not possible to follow the individual fish movement due to the

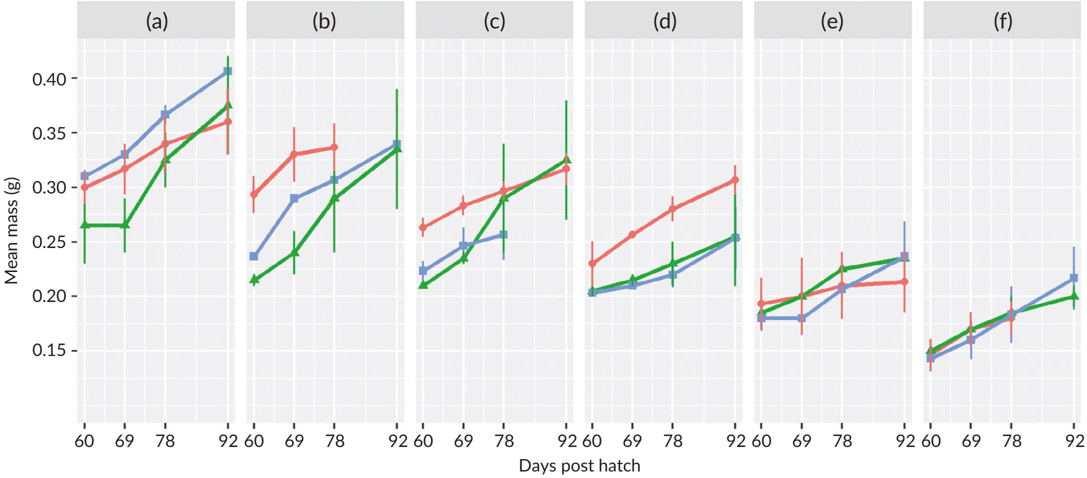

FIGURE 5 Growth of post-settlement *Amphiprion ocellaris* living with different host anemones, measured as the change in the mean (± SE) of fish mass in treatment tanks from 60 days post hatch (dph) to 92 dph. Each panel represents fish in the same rank, with different symbols indicating treatments. Fish ranks are a – f = largest to smallest size. EXP (—●—), experimental tanks, where fish were living with different hosts; PC (—▲—), positive control tanks, where fish were living with only the natural host *S. gigantea*; NC (—■—), negative control tanks, where fish were living with artificial anemones

small size of fish at this age and the large size of the experiment tank filled with anemones and rocks. We assumed that all of the dead and missing fish in the experimental tanks were the result of contact with (and consumption by) anemones, since naïve *A. ocellaris* are not innately protected from attack by all anemone species (Brooks & Mariscal, 1984; Elliott *et al*., 1994; Miyagawa, 1989). Mortality was very high in the first day of the experiment. There were some mortalities in the control group tanks as well, where no anemones were present, though these deaths occurred later and were probably the result of fish weakened by the recirculating system currents.

Our result demonstrated that naïve newly-settling larvae were interested in the anemone that were available but were not able to identify which one was the natural host.

Several studies have suggested that anemonefishes locate their hosts using chemical cues (Elliott *et al*., 1994, 1995; Miyagawa, 1989), but the question of whether or not the fish can distinguish between the smells of their natural hosts or unnatural hosts is controversial (Elliott *et al*., 1995; Elliott & Mariscal, 1997; Miyagawa, 1989). Our results suggest that newly settling *A. ocellaris* were not able to identify the smell of a natural anemone host species that they had never encountered previously and just randomly settled any host anemones when introduced into the tanks (Burke & Nedosyko, 2016). Moreover, by our observation, the fish initially chose unnatural host anemones with longer tentacles over the natural host anemone with short tentacles. The newly settling fish in our tanks may have been attracted to hosts based on the host morphology characteristics. Fautin (1991) also proposed that the extreme generalist *A. clarkii* probably uses visual cues to locate its host, including morphological features.

Newly settling juvenile *A. ocellaris* in our experiment were vulnerable to attack by all of the species in the tanks. However, by the end of the experiment, the surviving individuals had successfully associated with an unnatural anemone host. Thus, through early exposure, this specialist anemonefish can establish symbiosis with anemone species that they do not commonly live with in the wild (Burke & Nedosyko, 2016). Therefore, in captive conditions where that natural host may be unavailable, it could be possible to train *Amphiprion* spp. to live with unnatural hosts, if introduced during their early life stages.

The post-settlement *A. ocellaris*, on the other hand, apparently depend on a different mechanism to locate, identify and establish contact with their hosts. In all our tanks, these older juveniles recognised and quickly established their symbiotic association with their natural host *S. gigantea*. All of the fish switched to the unnatural host *S. haddoni*, which is a closely related species, as the second choice when their natural host was removed. Morphology may have played some role because the fish first briefly inspected species with long tentacles (*E. quadricolor*, *M. doreensis*, *H. crispa*). However, within a short time all of the fish shifted their attention to their natural anemone host with short tentacles, *S. gigantea*. Based on direct observations of these post-settlement fish, one individual would recognize and react to a potential host and then all the fish would move together (H.-T. T. Nguyen, pers. obs.). At the end of the experiment, none of the fish associated with any of the four other unnatural host species, even *E. quadricolor*, the species that younger *A. ocellaris* became associated with. At this stage, the chemical cues appear to be stronger than the visual cues, whereas older *A. ocellaris* were able to identify their natural host anemone, avoiding attack by unsuitable host species.

Amphiprion ocellaris can be considered as a specialist, a symbiont with up to three anemones species, as opposed to an extreme specialist which is a symbiont with only one species (Fautin, 1991). *Amphiprion ocellaris* is found in association with *S. gigantea*, *S. mertensii* and *H. magnifica* in the wild (Fautin & Allen, 1992) and it has been suggested that *A. ocellaris* may also approach *S. haddoni* without any hesitation (Elliott *et al*., 1995; Elliott & Mariscal, 1997). Elliott & Mariscal (1997) also indicated that naïve juvenile *A. ocellaris* were innately protected from the tentacles of *S. haddoni* and this appeared to be the case in our experiments. However, in a chemical stimuli test, Arvedlund & Nielsen (1996) compared host selection of *A. ocellaris* that had been imprinted from the egg stage with their natural host *H. magnifica* with *A. ocellaris* that had never been exposed to this host species. At 60–100 dph the imprinted fish quickly acclimated to *H. magnifica*, within 10 min of contact, while the fish that had never been exposed to this anemone host took 2 days to contact and associate with it (Arvedlund & Nielsen, 1996). Both the imprinted and non-imprinted fish ignored the presence of the unnatural host *S. haddoni*, the species that was the preferred alternative host in our experiments. Crossover to an alternate host may be easier when it is in the same genus.

Amphiprion ocellaris rejected four unnatural anemone species as hosts and there are several likely explanations for that behaviour. The fish could have been sensitive to the toxic tentacles of those species in a way that reduced contact (Fautin & Allen, 1992). The chemical signals secreted by those anemone species may not have been attractive to the fish. We did observe some individual fish approaching the remaining unnatural host species, but they did not follow through with acclimation behaviour, so it is possible that the fish were not attracted by chemical cues from these anemones.

4.2 | Activity

Activity was considered one of three consistent behavioural traits of *Amphiprion* spp. in symbiosis with anemones (Wong *et al*., 2013) and thus a useful indicator of fitness. *A. ocellaris* juveniles showed typical behaviour of diurnal reef fish, emerging from shelter at dawn, actively feeding during the day and retreating to shelter at dusk and hiding at night. Such a behaviour pattern is characterised by the trade-off between food intake and predation risk (Rickel & Genin, 2005). Anemonefishes might be expected to show different behaviour since they are protected from predation by the anemone. They spend much of their time swimming among tentacles of anemones and occasionally leave to feed on zooplankton or phytoplankton in the water column (Fautin & Allen 1992). There was a wide individual variation in the measured swimming variables in our experiments and this reflects the different ranks and roles of the individuals in each group (Buston, 2003; Chen & Hsieh, 2016; Iwata *et al*., 2008). In our study, *A. ocellaris* juveniles tended to be less active in the middle of the day, unlike reports of coral-reef fish that actively feed at this time. Moreover, when the fish lived with anemone hosts *S. gigantea* and *S. haddoni*, they were equally active at dusk and at night, while *A. ocellaris* that did not have access to these species were significantly less active at night. The activities of anemonefishes includes interacting with the host anemones (massaging, picking, *etc*), defending their territory against intruders, agonistic behavior by the dominant fishes and the submissive activities of the subordinates (Buston, 2003; Colleye *et al*., 2009; Colleye and Parmentier, 2012; Szczebak *et al*., 2013). When *A. ocellaris* in our experiments were not in symbiosis with any anemones, they did not express these interacting activities and the behaviour pattern was more similar to diurnal reef fish that are immobile at night.

4.3 | Growth

The association with a host anemone is classically considered to provide a safe home for anemonefishes, protecting them from potential predators. The symbiosis is also credited with increasing the longevity of anemonefishes and increase reproductive fitness. However, the benefits in terms of growth for anemonefishes are poorly documented. Given the result of this study, we showed that growth of *A. ocellaris* juveniles was different when they lived with different hosts. Fish grew fastest when living with the natural host, *S. gigantea*. When *A. ocellaris* were forced to switch to a new host *S. haddoni* in the experimental tanks, growth rates declined. When *S. haddoni* were in turn removed and the fish were not able to find new host species, this may have triggered a stress reaction that resulted in reduced growth. We found the presence of several stress-reducing proteins in the skin of fish that did not establish any new symbiosis during the last experiment interval, while there was the absence of those proteins in the skin of fish living with *S. haddoni* (H-T. T. Nguyen, pers. obs.). This suggests that the swimming activity and growth responses of *A. ocellaris* after removal of both *S. gigantea* and *S. haddoni* species were influenced by stress due to loss of access to an anemone host.

The growth rate of juvenile *A. ocellaris* living with their natural host did not differ significantly from those fish living with artificial anemones in the negative control group. This result is consistent with previous studies using a plastic insert as shelter for *A. ocellaris*, which also showed that there was no difference in oxygen consumption between fish living with and without the shelter and thus shelter on its own was unlikely to support any fitness in term of growth improvement (Kegler *et al*., 2013). Our results also show that the size-specific growth rate of *A. ocellaris* of the same rank did not change significantly over time among treatments, suggesting that the social ranks remained constant throughout of the experiment. Anemonefishes form a strict social hierarchy, in which each individual maintains their size based on rank and this social structure is robust to manipulation of the host association (Buston, 2003; Chen & Hsieh, 2016; Colleye & Parmentier, 2012).

In conclusion, the presence of six potential host species, including one natural host, naïve *A. ocellaris* juvenile at 60 dph indicated a clear preference toward their natural host *S. gigantea*. In the absence of *S. gigantea*, fish can acclimate to live with the unnatural host *S. haddoni*, which is a congeneric, as the second choice. *Amphiprion ocellaris* was not attracted to the four unnatural anemone hosts *E. quadricolor*, *M. doreensis*, *H. crispa* and *H. malu* after removal of *S. haddoni*. The increased level of activity and lower growth performance was a response to the lack of a suitable host for establishing a new symbiotic relationship. Living with unnatural host *S. haddoni*, resulted in lower fitness in term of growth than living with the natural host *S. gigantea*. According to these criteria *S. gigantea* provides a better quality of refuge than *S. haddoni*, possible due to having longer tentacles, providing better shelter for the fish when hiding or sleeping to avoid predators and having a moderate level of venom toxicity, which was proven to be better for anemonefish survival and reproductive (Nedosyko *et al*., 2014). These characteristics could explain why *A. ocellaris* is not found in symbiosis with *S. haddoni* in the wild. In captive conditions, anemonefishes should be kept with their natural host anemone in order to ensure the optimal welfare.

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Procedures conducted on the fish complied with local and national animal welfare laws.

Author contributions

H-T. T. N. conceived of and designed the experiment, generated the data, analysed the data and wrote the manuscript. A-N. T. T. helped to generate the data. L. T. L. H. advised on the care and breeding of the anemonefish and anemones and the experimental design. D. N. N. helped with experimental design and data analysis. B. T. D. helped with experimental design and manuscript preparation. A.J.G. helped with the experimental design, analysed the data and wrote the manuscript.

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