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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

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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. Dixon *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 times 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 terms 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 act 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 becomes 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*, *Macroactyla doreensis*, *Heteractis crista* 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 ($\text{cm s}^{-1} L_T^{-1} \text{cm fish}$). 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
Interval II	68	Swimming activity recorded	D7		
	69	Photo and Weighing	Rest fish		Removal of <i>S. gigantea</i>
	70	Introduced to anemones			
	71	Swimming activity recorded	D1		
	73	Swimming activity recorded	D3		
Interval III	75	Swimming activity recorded	D5		Activity data analysed
	77	Swimming activity recorded	D7		
	78	Photo and Weighing	Rest fish		Removal of <i>S. haddoni</i>
	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. crispa*, 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

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

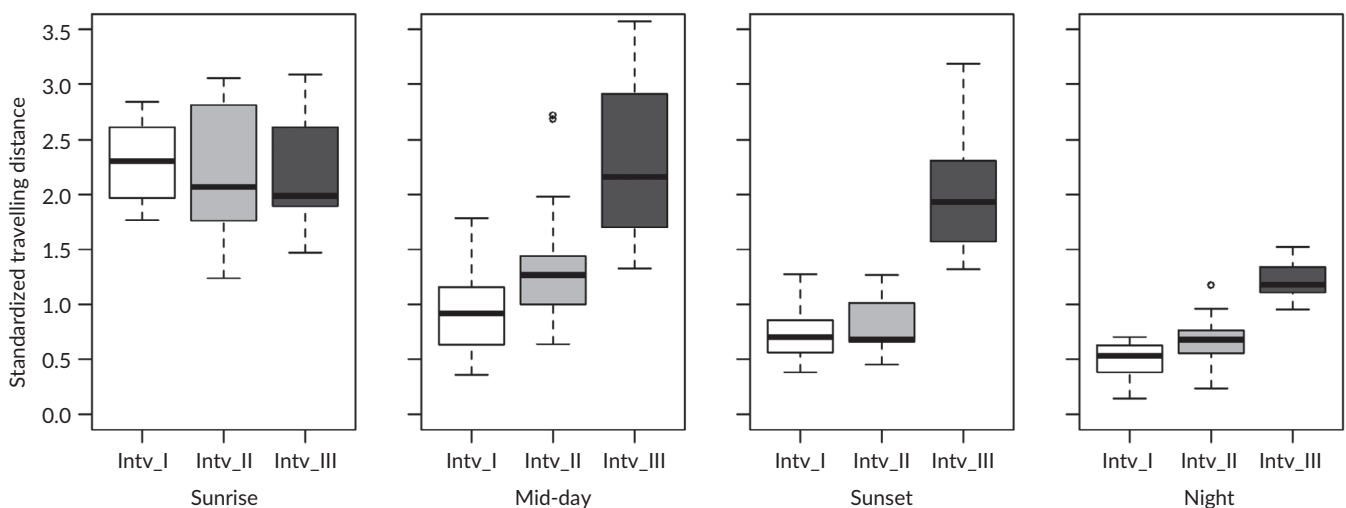


FIGURE 1 Box plots (—, median; □, 25th and 75th percentile; T 95% range; ○, outliers) of the diurnal changes in standardized travelling distance of *Amphiprion ocellaris* during each of three experiment intervals: Intv_I, interval I, when fish were introduced to six anemone species and associated with 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

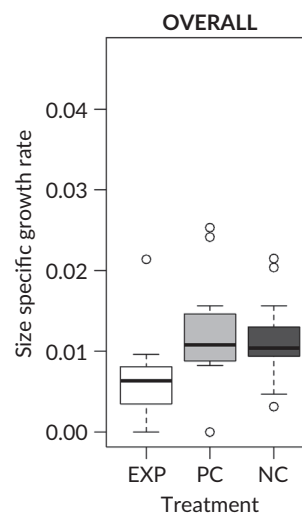


FIGURE 3 Box plots (—, median; □, 25th and 75th percentile; T 95% range; ○, outliers) of the growth of post-settlement *Amphiprion ocellaris* living with different host anemones, measured as change in size specific growth rate in the overall time of the experiment. EXP, experimental tanks, where fish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

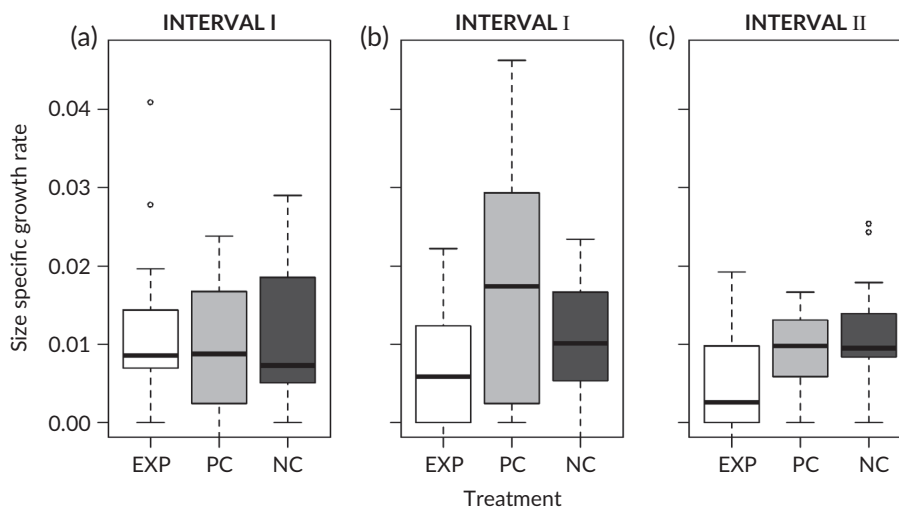


FIGURE 2 Box plots (—, median; □, 25th and 75th percentile; T 95% range; ○, outliers) of the growth of post-settlement *Amphiprion ocellaris* living with different host anemones, measured as change in size specific growth rate during each of three experiment intervals: (a) Interval I, when fish were 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

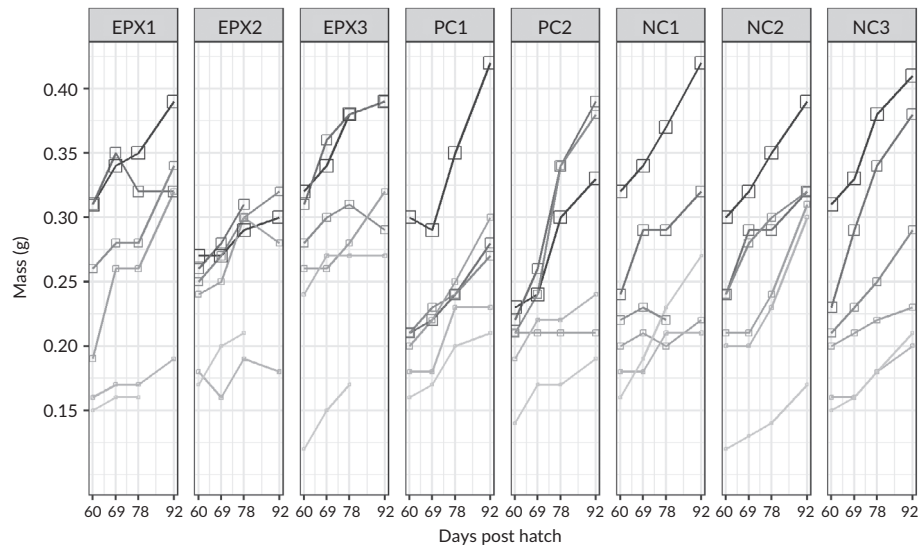


FIGURE 4 Growth of post-settlement *Amphiprion ocellaris* living with different host anemones, measured as change in individual fish mass from 60 days post hatch (dph) to 92 dph. Each panel represents the fish in each replicate tank (1–3) in each treatment: EXP, experimental tanks, where fish were living 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

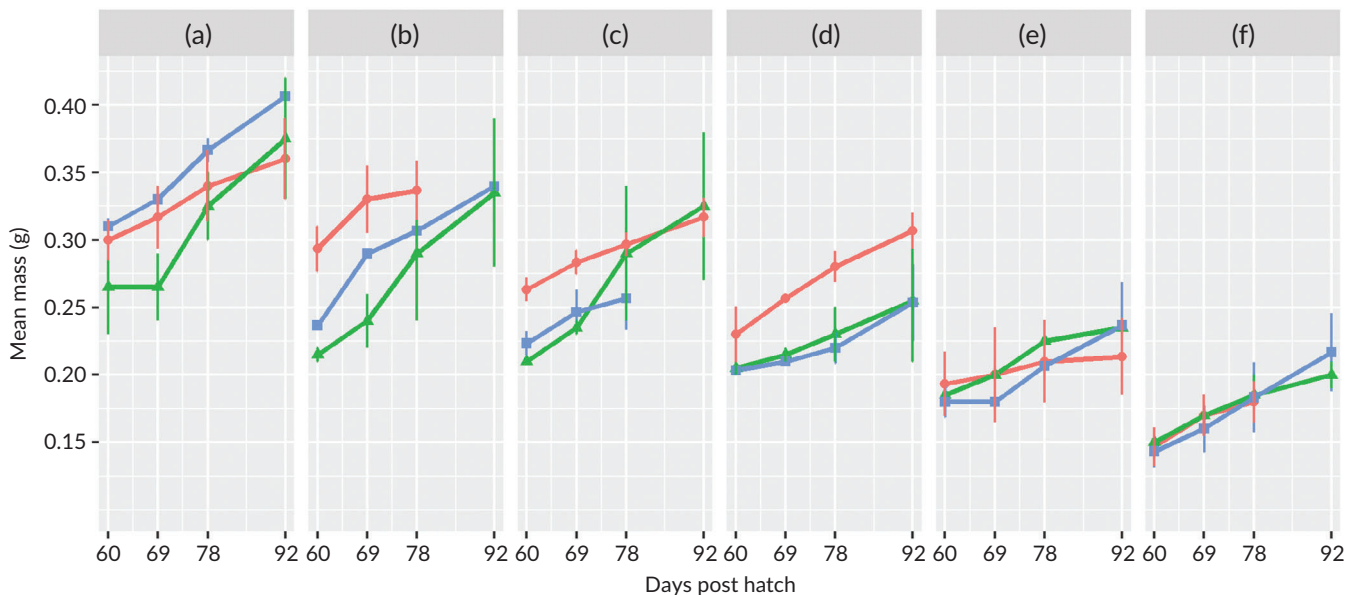


FIGURE 5 Growth of post-settlement *Amphiprion ocellaris* living with different host anemones, measured as the change in the mean (\pm SE) of fish mass in treatment tanks from 60 days post hatch (dph) to 92 dph. Each panel represents fish in the same rank, with different symbols indicating treatments. 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 (Ricklefs & 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; Colley *et al.*, 2009; Colley 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; Colley & 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. dorensis*, *H. crisper* 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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