

# Behavioural differences between breeding and nonbreeding pairs of protandry monogamous false clown anemonefish *Amphiprion ocellaris*

Eri Iwata<sup>1,2,3</sup>  • Tasuku Yoshida<sup>1</sup> • Tetsuya Abe<sup>1</sup> • Kou Takahata<sup>1</sup> • Koji Masuda<sup>4</sup> 

<sup>1</sup>Department of Science and Technology, Iwaki Meisei University, 5-5-1 Chuoudai, Ihino, Iwaki, Fukushima 970-8551, Japan

<sup>2</sup>Faculty of Veterinary Medicine, Okayama University of Science, 1-3, Ikoinooka, Imabari City, Ehime, 794-8555, Japan

<sup>3</sup>Institute of Osaka Marine Research, 19-34, Shimodachou, Nishi Ku, Sakai City, Osaka, 593-8329, Japan

<sup>4</sup>Department of Human and Animal-Plant Relationships, Tokyo University of Agriculture, 1737 Funako, Atsugi, Kanagawa 243-0034, Japan

## Correspondence

Eri Iwata; Faculty of Veterinary Medicine, Okayama University of Science, 1-3, Ikoinooka, Imabari City, Ehime, 794-8555, Japan

 e-iwata@vet.ous.ac.jp

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## Abstract

Anemonefishes are some of the most popular marine ornamental fishes. Due to the adverse impacts of commercial fishing on the wild populations of anemonefishes, a more suitable and efficient captive breeding programme must be promoted. In the wild, anemonefishes are protandrous sex-changing fish, but when two immature individuals with ambisexual gonads are raised together in captivity, the two differentiate directly into a male and a female, to form a breeding pair. However, not all the formed pairs spawn, explaining the great care required in captive breeding. This behaviour appears to be counter-adaptive, as anemonefish social groups form randomly in the wild, and such phenomenon would disturb their breeding success. This study evaluated the behavioural and physiological differences between breeding and nonbreeding pairs of false clown anemonefish *Amphiprion ocellaris* under captive conditions. Behavioural observations revealed that nonbreeding females monopolized the shelter provided, whereas breeding females allowed breeding males to use the shelter and the pair frequently stayed together in the shelter. Both nonbreeding and breeding females possessed mature ovarian tissue and similar level of plasma estradiol concentrations, but nonbreeding males had a smaller amount of testicular tissue and lower plasma 11-ketotestosterone levels compared to breeding males.

**Keywords:** Anemonefish; *Amphiprion ocellaris*; ornamental fish; social behaviour; breeding; sexual maturation

## 1 | INTRODUCTION

The false clown anemonefish *Amphiprion ocellaris* was one of the top ten most traded fish species between 1997 and 2002 (Wabnitz *et al.* 2003). Anemonefishes, genus *Amphiprion* (including *A. ocellaris*), live symbiotically with

sea anemones in the wild and have small home ranges (Hattori 1994; Moyer 1980), hence they can adjust quite well to small aquarium tanks (Fautin and Allen 1994a). In fact, on the “Aquarium suitability index” of the famous marine aquarium guide, *A. ocellaris* was rated “4” or

“generally durable and hardy, with most individuals acclimatizing to the home aquarium” (Michael 2001).

More than 90% of the freshwater ornamental fish are bred in captivity; in contrast, most marine ornamental aquaculture remains comparatively problematic (Monticini 2010). The Pomacentridae family, including the genus *Amphiprion*, accounts for about half of the catch, with the green damselfish *Chromis vividis* and *A. ocellaris* being the most marketable (Monticini 2010). Actually, several studies reported that *A. ocellaris* densities, total body length and group size became small (Maddupa *et al.* 2014) and genetic diversity of *A. ocellaris* is reduced (Maddupa *et al.* 2018).

In an online survey of marine aquarium hobbyists in 2009, 76% of respondents revealed that they would preferentially purchase cultured animals, while a further 21% said this would depend on the price difference (Murray and Watson 2014). *Amphiprion ocellaris* breed under captive conditions relatively easily compared with other marine fish (Wilkerson 2003) and are successfully produced by several hatcheries. However, captive-bred *A. ocellaris* cost more to purchase than wild-caught individuals, resulting in more than 600,000 wild-caught *A. ocellaris* being imported into the US between 2008 and 2011 (Rhyne *et al.* 2017). Furthermore, *A. ocellaris* broodstocks are produced from breeding pairs collected in the wild rather than an F1 generation (Abol-Munafi *et al.* 2011), possibly because breeding pair formation relies on the complex social structure of this species and so may be difficult to achieve under captive conditions. Therefore, a more efficient breeding technique is needed to protect *A. ocellaris* in the wild.

In the anemonefishes, the genus *Amphiprion*, is protandrous fish that possess the ability to change sex in response to social cues (Fricke and Fricke 1977; Moyer and Nakazono 1978). In the wild, anemonefishes form social groups consisting of a monogamous breeding pair and several subadult nonbreeders or juveniles. Females are the largest and dominant members of these social groups, displaying frequent dominant behaviour toward subordinate group members. The second-ranked individuals become males, displaying less aggression, and others remain as nonreproductive individuals, displaying little aggression (Fricke and Fricke 1977; Iwata *et al.* 2008; Iwata and Manbo 2013). If a female disappears from the social unit, the male changes sex into a female and the largest of the nonbreeders becomes a functional male (Fricke and Fricke 1977; Moyer and Nakazono 1978). However, under certain conditions immature anemonefishes with ambisexual gonads will differentiate directly into males or females. For instance, nonbreeding Clark’s anemonefish, *Amphiprion clarkii*, can directly differentiate into females in temperate waters, without passing through the func-

tional male stage (Hattori and Yanagisawa 1991a). In captivity, when two immature anemonefish with ambisexual gonads are raised together, the two differentiate directly into a male and a female and form a breeding pair (Goldstein 1989; Wilkerson 2003).

In the captive breeding of *A. ocellaris*, pair formation is developed by forming a social unit consisting of five to six individuals and choosing the dominant and second-ranked individual (Madhu *et al.* 2006; Rohini Krishna *et al.* 2015), which requires great care and time. Moreover, not all formed pairs spawn, some pairs remain bonded without spawning even though the pairs are raised under the same conditions. Our previous study indicated that some pairs of *A. ocellaris* did not exhibit sex differentiation after 450 days of pairing, with one pair pairing for 3 years without spawning (Iwata *et al.* 2010a). This phenomenon appears to be counter-adaptive, as social groups of anemonefish normally form randomly to breed.

In the wild, the dominant female and second-ranked male form a breeding pair, and females lay their eggs on a bare rock adjacent to the host sea anemone (Fautin and Allen 1994b; Wilkerson 2003). Hatching occurs 6 to 8 days after the eggs are laid. Larvae are planktonic but settle at the bottom of the sea after 8 to 12 days (Wellington and Victor 1989) to find a suitable host sea anemone (Fautin and Allen 1994b). In a study on saddleback anemonefish, *Amphiprion polymnus*, many offspring settled remarkably close to home, but none returned to the same anemone as their parents, suggesting that direct kin relationships between individuals in a sea anemone are rare (Jones *et al.* 2005; Buston *et al.* 2007). Thus, the social groups of anemonefish form randomly, and which individuals contribute to breeding in each group is determined only by their social rank. Furthermore, this protandrous breeding strategy indicates that breeding individuals are replaceable, and studies have shown that members of anemonefish social groups remain relatively stable for years in a wild population (Moyer 1986). Hence, if the reproductive pair of a social group does not spawn, the entire group might lose the chance to reproduce for years, which would decrease their breeding success.

In this study, we evaluated the differences between breeding and nonbreeding pairs of false clown anemonefish *A. ocellaris* under captive conditions, considering behavioural traits, physical parameters, plasma steroid concentrations, and gonadal histology to seek solutions for the problem of breeding pair formation in captivity. It is well known among oceanarium keepers that if an individual from a nonbreeding pair of anemonefish is replaced another, the newly-formed pair will occasionally start to breed (E. Iwata, personal observation). To confirm that the replacement of individuals induces spawning, a replacement test was also conducted.

## 2 | METHODOLOGY

### 2.1 Experimental fish

Sixteen pairs of *A. ocellaris* were obtained from Dr. T. Sunobe, Tateyama Station, Field Science Center, Tokyo University of Marine Science and Technology, Tateyama, Japan. These pairs originated from individuals that had been purchased from a commercial ornamental fish store and then randomly paired. The fish were of unknown age but had mean total lengths of  $72.2 \pm 2.6$  mm (range: 51–88 mm) for the females and  $53.2 \pm 1.6$  mm (range: 43–67 mm) for the males on 5 September 2012. The fish were introduced to our laboratory on 15 June 2013, which was more than 18 months after the pairs had been formed. We divided the pairs into a breeding group ( $n = 9$  pairs) and a nonbreeding group ( $n = 7$  pairs) according to whether they had previously spawned. We then used five pairs from each group for behavioural observations and the remaining four breeding and two nonbreeding pairs for the replacement test.

Throughout the experiment, each pair was held in a 26-L experimental tank with a closed recirculating seawater system at  $25 - 26$  °C under natural light conditions. An unglazed ceramic flowerpot (15 cm diameter) was placed at the centre of each experimental tank as a substitute for a host sea anemone and a spawning surface. The fish were fed commercial pellets (Omega One Marine Flakes, Omega Sea Ltd., Painesville, OH, USA) daily. The experimental protocols followed Iwaki Meisei University's "Policies Governing the Use of Live Vertebrate Animals" and the Japan Ethological Society's "Guidelines for Research on Animal Behaviour".

### 2.2 Behavioural observations

Behavioural observations were conducted from 26 October 2013, after four months of acclimation. The usual behaviours of each pair were evaluated by recording the whole view of each experimental tank for twelve 5-min observation periods (*i.e.* a total of 60 min) during the light phase (at 0800, 0830, 1000, 1030, 1200, 1230, 1400, 1430, 1600, 1630, 1800 and 1830 h) on a single day using a digital video camera (DCR-HC62, Sony, Tokyo, Japan). Since anemonefish are diurnal, this schedule is suitable for providing an overview of their daily activities (Iwata *et al.* 2008, 2012, 2019). The camera was set in front of (but more than 1.5 m away from) the tank. The recordings were controlled by a timer and entry to the laboratory was restricted during recording to avoid disturbance.

We observed seven behavioural elements in the pairs: duration of shelter occupation by the female only, by the male only and by the breeding pair; duration of swimming as a pair; frequency of lunging (typical threatening behaviour of dominant anemonefish); frequency of trembling (typical appeasing behaviour of subordinate anemonefish); and frequency of solo trembling (typical behaviour

of anemonefish shown when fish is irritable or conflicted). Descriptions of these behaviours are provided in Table 1 and follow previously accepted definitions (Iwata *et al.* 2008, 2010b; Iwata and Mambo 2013). The behavioural observations of breeding pairs were conducted at all times except during the breeding phase (*i.e.* at the timing of courtship, spawning or rearing).

**TABLE 1** Description of the behaviours of *Amphiprion ocellaris* breeding and nonbreeding pairs.

Behaviour	Description
Occupying the shelter	Whole body of focal individual was in the shelter
Swimming in pair	A male and a female are swimming close in parallel
Lunging	Rapid, directed swimming movement while approaching others (typical threatening behaviour by dominant anemonefish)
Trembling	Focal individual twitters its whole body in front of its partner (typical ameliorative behaviour by subordinate anemonefish, or often seen as a male courtship behaviour)
Solo trembling	Focal individual twitters its whole body while away from other individuals (typical frustrating behaviour of anemonefish)

### 2.3 Sample collection and measurements

After the behavioural observations ended, fish were captured and euthanized with 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan). All resident fish of a tank were captured at once using a hand net and then moved to a smaller tank with the dissolved anesthetic chemical. The anesthesia was introduced within 3 min of the start of capture to minimize stress for the fish. Total body length, body weight, and gonadal weight were measured. Gonadosomatic index (GSI) and the male or female ratios of total length and body weight were calculated for each pair. Blood samples were collected from the caudal vessel of each fish using a heparinised syringe and centrifuged immediately. The plasma was removed and stored at  $-20^{\circ}\text{C}$  in a plastic tube until assay. The gonad was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for histological observation.

### 2.4 Hormone assays

We extracted 5  $\mu\text{L}$  of each plasma sample using 2 mL diethyl ether and resuspended it in 500  $\mu\text{L}$  of enzyme immunoassay buffer (Cayman Chemical, Ann Arbor, MI, USA). The concentrations of 11-ketotestosterone (11-KT), estradiol (E2), and cortisol were then measured with an enzyme immunoassay kit (Cayman Chemical) following the manufacturer's instructions. All concentrations were measured in triplicate. The inter- and intra-assay coefficients of variation were 8.2% and 8.3% for 11-KT, 14.7% and 7.3% for E2, and 7.6% and 8.4% for cortisol, respectively.

## 2.5 Histology

Gonads were dehydrated and embedded in paraffin. A standard microtome was used to cut 6- $\mu$ m sections from the central region of the glands, which were stained with hematoxylin and eosin and examined under a light microscope (magnification x400). Digitized images of 10 sections of each male's gonadal tissues were captured, while the area of testicular tissue and the whole gonadal tissue were determined using the public-domain software ImageJ 1.47v (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The ratio of testicular tissue area to whole gonad was calculated.

## 2.6 Replacement test

From 8 April 2014, the individuals were replaced in combinations of breeding female and nonbreeding male ( $n = 3$ ), nonbreeding female and breeding male ( $n = 2$ ), breeding female and breeding male ( $n = 2$ ), and nonbreeding female and nonbreeding male ( $n = 1$ ). The spawning behaviour and the presence of laid eggs were checked daily until 120 days after the replacement.

## 2.7 Statistical analysis

Statistical analyses were performed using the StatView+Graphics 5.0J software (Abacus Concepts, Inc., Berkeley, CA, USA). All parameters met the assumptions for normal distribution, as assessed by the Bartlett test ( $P > 0.05$  for each parameter). We used a two-way Analysis of Variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) test to compare behavioural durations between categories of different elements and breeding groups; and to compare behavioural frequencies between categories of sex and breeding group. To compare body parameters between categories of sex and breeding group, we used two-way ANOVA followed by Fisher's PLSD. Because sufficient blood samples could not be obtained from several individuals, we used two-way ANOVA followed by a Turkey-Kramer multiple comparison test to compare plasma steroid profiles. The ratios of male/female body parameters and testicular/whole gonadal tissue were compared using an unpaired  $t$ -test. A probability level of  $P > 0.05$  was considered to indicate statistical significance.

## 3 | RESULTS

### 3.1 Behavioural observations

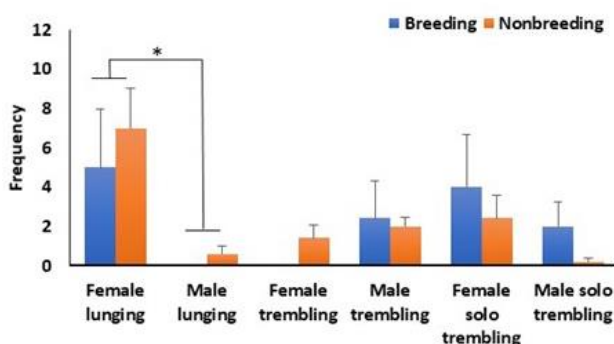
With regard to the duration spent in the shelter by females, males, and pairs and swimming in pairs, there were significant differences in the duration of behavioural elements (ANOVA:  $F_{3,32} = 5.406$ ,  $P = 0.004$ ) and the behavioural elements  $\times$  breeding group interaction was significant (ANOVA:  $F_{3,32} = 11.176$ ,  $P < 0.001$ ). However, the breeding group was not significant ( $P = 0.223$ ). Post-hoc analysis revealed that females in nonbreeding pairs spent

most of their time in the shelter alone, whereas breeding pairs tended to stay in the shelter together approximately as long as breeding females. Breeding males also stayed in the shelter alone compared to nonbreeding males (Figure 1).



**FIGURE 1** Duration of *Amphiprion ocellaris* occupation of the shelter by male only, female only, and the pair, and of swimming together in a pair outside the shelter. Mean  $\pm$  SE; \*,  $P < 0.05$  (two-way ANOVA and Fisher's PLSD).

With regard to the frequency of behaviours displayed by focal individuals, there was a significant difference in the frequency of lunging between males and females (ANOVA:  $F_{1,16} = 9.951$ ,  $P = 0.006$ ), but not between breeding groups or in the sex  $\times$  breeding group interaction (both  $P > 0.05$ ). Females lunged more frequently than males regardless of the breeding groups, while trembling was higher in males regardless of the breeding groups, but showed no significant differences between the sexes, the breeding groups or in the sex  $\times$  breeding group interaction (all  $P > 0.05$ ). Solo trembling was also showed not significantly different between sexes, between breeding groups or in the sex  $\times$  breeding group interaction (all  $P > 0.05$ ; Figure 2).



**FIGURE 2** Frequencies of behavioural elements of *Amphiprion ocellaris* pairs. Mean  $\pm$  SE; \*,  $P < 0.05$  (two-way ANOVA).

### 3.2 Body parameters

Total length (ANOVA:  $F_{1,15} = 12.443$ ,  $P = 0.003$ ) and body weight (ANOVA:  $F_{1,15} = 25.987$ ,  $P < 0.001$ ) were significantly different between males and females, but not between breeding groups for length and weight (both  $P >$



0.05). The interactions of breeding group  $\times$  sex were not significant for total length and body weight (both  $P > 0.05$ ). Females were longer and heavier than males, regardless of the breeding groups. There were no significant differences in the GSI of males and females and between the breeding groups (both  $P > 0.05$ ). The interaction of breeding group  $\times$  sex was also not significant ( $P = 0.487$ ; Table 2). The male/female ratios in total length and body weight also showed no significant differences ( $t$ -test: both  $P > 0.05$ ; Table 3).

**TABLE 2** Total length, body weight, and gonadosomatic index (GSI) of *Amphiprion ocellaris* individuals in breeding and nonbreeding pairs. Values are means  $\pm$  SE. Different letters in superscript indicate significant differences ( $P < 0.05$ ; two-way ANOVA and Fisher's PLSD).

Measure-ments	Male		Female	
	Breeding	Non-breeding	Breeding	Non-breeding
Total length (mm)	54.14 $\pm$ 2.23 <sup>a</sup>	53.60 $\pm$ 2.05 <sup>a</sup>	69.60 $\pm$ 7.80 <sup>b</sup>	70.15 $\pm$ 5.14 <sup>b</sup>
Body weight (g)	3.47 $\pm$ 0.44 <sup>a</sup>	3.47 $\pm$ 0.52 <sup>a</sup>	11.14 $\pm$ 1.91 <sup>b</sup>	8.96 $\pm$ 1.76 <sup>b</sup>
GSI (%)	0.42 $\pm$ 0.12 <sup>a</sup>	0.40 $\pm$ 0.20 <sup>a</sup>	1.93 $\pm$ 1.71 <sup>a</sup>	0.97 $\pm$ 0.25 <sup>a</sup>

**TABLE 3** Male/female total length (TL) and body weight (BW) ratios in breeding and nonbreeding pairs of *Amphiprion ocellaris*. Values are means  $\pm$  SE. No differences were significant ( $P > 0.05$ ; unpaired  $t$ -test).

Ratio	Male / Female Ratio	
	Breeding	Nonbreeding
TL ratio	0.78 $\pm$ 0.06	0.77 $\pm$ 0.05
BW ratio	0.37 $\pm$ 0.03	0.43 $\pm$ 0.07

### 3.3 Hormone assays

Plasma concentrations of  $E_2$  showed significant differences between the sexes (ANOVA:  $F_{1,14} = 5.281$ ,  $P = 0.038$ ), but not between the breeding groups or in the sex  $\times$  breeding group interaction (both  $P > 0.05$ ). However,  $E_2$  levels in nonbreeding males showed similar levels to those of breeding and nonbreeding females. Plasma concentrations of 11-KT showed significant differences between the sexes (ANOVA:  $F_{1,14} = 13.845$ ,  $P = 0.003$ ), between breeding groups (ANOVA:  $F_{1,14} = 5.139$ ,  $P = 0.045$ ), and in the sex  $\times$  breeding group interaction (ANOVA:  $F_{1,14} = 10.971$ ,  $P = 0.007$ ). Post-hoc analysis revealed that plasma 11-KT concentration was highest in the breeding males. The nonbreeding males tended to show levels similar to those of breeding and nonbreeding females. Plasma cortisol concentration showed no significant differences between the sexes, the breeding groups, or in the sex  $\times$  breeding group interaction (all  $P > 0.05$ ; Table 4).

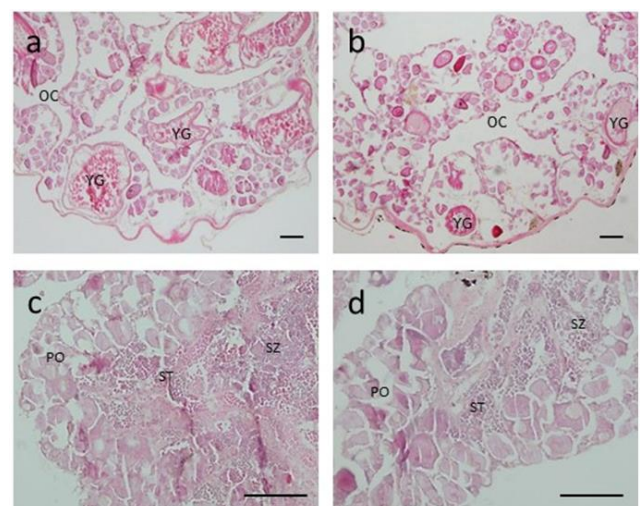
**TABLE 4** Steroid profiles of *Amphiprion ocellaris* individuals in breeding and nonbreeding pairs. Values are means  $\pm$  SE with

the numbers of samples shown in parentheses. \*,  $P < 0.05$  (two-way ANOVA and Turkey-Kramer multiple comparison test).

Compari-sons	Male		Female	
	Breeding	Non-breeding	Breeding	Non-breeding
$E_2$ (ng/ml)	0.38 $\pm$ 0.16 (4)	0.71 $\pm$ 0.07 (5)	0.78 $\pm$ 0.01 (4)	0.75 $\pm$ 0.08 (5)
11-KT (ng/ml)	0.26 $\pm$ 0.09* (3)	0.11 $\pm$ 0.02 (5)	0.06 $\pm$ 0.01 (3)	0.09 $\pm$ 0.01 (5)
Cortisol (ng/ml)	4.46 $\pm$ 2.16 (4)	4.96 $\pm$ 1.50 (5)	6.13 $\pm$ 3.32 (4)	6.04 $\pm$ 2.00 (5)

### 3.4 Histology

Both nonbreeding and breeding females possessed fully mature ovarian tissue with vitellogenic oocytes and ovarian cavity (Figure 3, a and b), and nonbreeding and breeding males had developed testicular tissue with spermatozoa at the centre of the gonads (Figure 3, c and d). However, the proportion of testicular tissue in the gonads was smaller in nonbreeding males than in breeding males (unpaired  $t$ -test:  $t_6 = 5.441$ ,  $P = 0.002$ ; Figure 4).

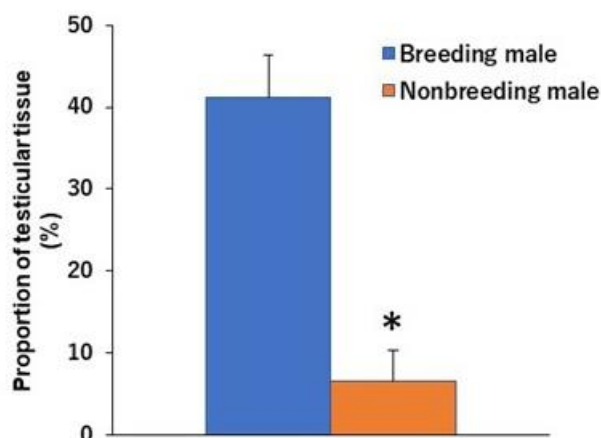


**FIGURE 3** Light micrographs showing the gonadal structure of *Amphiprion ocellaris* individuals; sections were stained with hematoxylin and eosin: (a) breeding female, (b) nonbreeding female, (c) breeding male, and (d) nonbreeding male. OC, ovarian cavity; YG, yolk globule stage; PO, perinucleolus stage; ST, spermatid; SZ, spermatozoa. Scale bar = 100  $\mu$ m.

### 3.5 Replacement test

The results of the replacement test are shown in Table 5. One out of three pairs consisting of breeding females and nonbreeding males spawned 61 days after the replacement; one pair bonded but did not spawn. In another pair, the female evicted the newly introduced male by fierce aggression, and the observation was terminated. None of the two pairs consisting of nonbreeding females and breeding males spawned. One of the two pairs con-

sisting of breeding females and breeding males spawned 12 days after the replacement, while in the other pair the female also evicted the male. In the one pair consisting of a nonbreeding female and a nonbreeding male, the female also evicted the male. When such cases of eviction occurred, the experiment was terminated immediately, as the males were almost fatally injured.



**FIGURE 4** Per cent testicular tissue in ambisexual gonads of male *Amphiprion ocellaris*. Mean  $\pm$  SE%; \*,  $P < 0.05$  (unpaired t-test).

**TABLE 5** Frequency of cases in which replacement pairs spawned, did not spawn or in which the male was evicted.

Construction	N	Spawning	No spawning	Eviction
Breeding female $\times$ nonbreeding male	3	1	1	1
Nonbreeding female $\times$ breeding male	2	0	2	0
Breeding female $\times$ breeding male	2	1	0	1
Nonbreeding female $\times$ nonbreeding male	1	0	0	1

#### 4 | DISCUSSION

In this study, we confirmed that some pairs of *A. ocellaris* may not spawn after long-term pairing. The nonbreeding pairs had clearly reached maturation size, as there were no significant differences in total length or body weight between the breeding and nonbreeding individuals. At the start of the experiment, more than 22 months had passed since the pairs were formed. Our previous 36-month observation showed that spawning never occurred if a pair had not begun to spawn by 18 months after the pair formation. Madhu *et al.* (2012) reported that, in laboratory conditions, spawning also normally occurred within 18 months of hatching. These findings support the existence of *A. ocellaris* pair that never spawn. A possible explanation for this might be that some physical disorders exist in either females or males; however, the proportion

of nonbreeding pairs was too high (7/16 pairs) and the result of the replacement test revealed that nonbreeding individuals started to spawn if their partners changed occasionally.

Recent studies of anemonefishes have revealed that they might have a much more flexible life history than previously thought, although the social group remain relatively stable once the members a particular group are fixed. The protandry and social system of anemonefishes have been regarded as adaptations to the extreme difficulty of moving between hosts due to the low population density of sea anemones and the related predation pressures in subtropical and tropical waters (Fricke and Fricke 1977; Hattori 1991; Ross 1978). Under such circumstances, dominant individuals occasionally evict or kill subordinates (Buston 2003a), whereas lower ranked individuals may reduce their effects on the breeding success of the adult pair to avoid eviction or death (Buston 2004). In this study, several males used in replacement tests were forcefully evicted by females, which may reflect the confined space situation in captive conditions. In contrast, the migration of anemonefishes in temperate waters is common (Ochi 1989a, 1989b), resulting from low predation pressure, a high density of available sea anemones, and the small population sizes of anemonefishes in temperate waters. Under such circumstances, if a female and a male are incompatible, either of a pair could move between host sea anemones in search of new mates. As females are dominant over males, the males are normally the ones to undertake this migratory behaviour.

In this study, breeding females appeared to allow partner males to use the shelter, whereas nonbreeding females tended to monopolize the shelter. Previous studies showed that in a group of sexually immature *A. ocellaris*, the dominant individual spent significantly more time in the shelter, while other individuals swam around the shelter but hardly entered it, because of aggressive behaviour from the dominant (female) fish (Iwata *et al.* 2008, 2010b). Interestingly, in this study, breeding males appeared to be able to stay in the shelter alone, as well as with females. In wild populations, it was reported that wild female *A. clarkii* normally initiate courtship (Moyer and Sawyers 1973), suggesting that in this study, breeding females may accept breeding males as their breeding partners, not in the case of nonbreeding pairs and females may take the initiative to spawn.

In this study, female lunging behaviour was infrequent, regardless of their breeding status. However, strong aggression behaviour can be observed when the social construction of groups is unstable (Iwata *et al.* 2008, 2012, 2019) explaining the forceful eviction of some replacement male observed in this study. There were no significant differences between breeding and nonbreeding individuals in other behaviours, except for the time spent

occupying the shelter. These results suggest that the pair bond of nonbreeding pairs in this study may be fully established and stable. The lower plasma concentrations of cortisol regardless of the breeding experience and sex also support this hypothesis. Buston (2003b) reported that male clown anemonefish *A. percula*, a species that is very similar to *A. ocellaris*, tended to retain a smaller body size than dominant females to avoid conflict and to maintain their social group in harmony. Therefore, our lack of a significant difference in male/female ratios of total length and body weight between breeding and nonbreeding males might indicate a lack of conflict among nonbreeding pairs. Taken together with our behavioural observations, under laboratory conditions, males that are completely unacceptable for females are evicted, and males that are acceptable, but not suitable as breeding partners, allow to stay without spawning.

In a previous study, similar male removal tests were conducted in wild populations of *A. clarkii* in temperate water (Yanagisawa and Ochi 1986). In 22 of 33 removals, foreign males entered the experimental territories, but in three removals, a male which had once settled in the territory was replaced another foreign male. In the 11 removals, no foreign males replaced the removed males. Because the number of females is small compared to males in temperate waters (Moyer 1980; Ochi 1989a), foreign males are supposed to enter the territories where the position of males is vacant. These results also indicate that females might select acceptable individuals and evict others. Recent studies revealed that individual personalities exist in fish, including anemonefishes (Schmiege *et al.* 2016; Wong *et al.* 2017). The behavioural parameters in our study showed some variation depending on the experimental tank, *i.e.* larger values of standard error. These also support the hypothesis that there might be various degrees of male acceptance by females in anemonefish.

Furthermore, masculinization of nonbreeding males tended to be suppressed compared to breeding males. Nonbreeding males possessed a lower plasma 11-KT level and smaller amounts of testicular tissue compared to breeding males. Plasma  $E_2$  levels in nonbreeding males also showed levels similar to those of breeding and nonbreeding females. The duration between the spawnings of breeding pairs is known to vary, yet no spawning occurred at the time of the experiment, so it might not attribute the suppression of masculinization to that the testis of nonbreeding males was in resting phase. These findings suggest that the process of masculinization is suppressed or delayed when males are unacceptable to females. If the suppression of male maturity by females is indeed occurring, how do the females achieve this? Although studies have shown that adult bluegill *Lepomis macrochirus* (Aday *et al.* 2003) and swordtail characin *Corynopoma riisei* (Bushman and Burns 1994) can con-

trol juvenile sexual maturity in members of the same sex, social inhibition of maturity by individuals of the opposite sex seems to be rare, as it might reduce the fitness of the species. However, anemonefishes are socially-controlled protandrous fishes, and it is common for such species to control individuals' sexual maturity through dominance (Devlin and Nagahama 2002; Guerrero-Estevez and Moreno-Mendoza 2010). Furthermore, it is possible that females might suppress masculinization when males are unacceptable to them, yet the exact mechanism through which they achieve this suppression remains unknown.

The suppression of masculinization in nonbreeding males in our study might be a result of social interactions between males and females of nonbreeding pairs. Anemonefishes are unique among socially-controlled sex-determined fish species, in that a long period of social interaction in the group is needed to induce sex determination or change. For example, sex determination in ambisexual fishes takes several months (Hattori and Yanagisawa 1991b; Iwata *et al.* 2008, 2010b), and it takes about 45 days for sex change to occur (Fricke 1979; Godwin and Thomas 1993). Thus, the time-course change of social interaction between dominant and second-ranked individuals must be evaluated to understand the masculinization mechanism of *A. ocellaris*.

This study indicated that some sort of compatibility may exist between *A. ocellaris* individuals and that the suppression of masculinization may occur when males were unacceptable to females. The exact characteristics of males which are unacceptable to females and the mechanisms underlying the suppression of male masculinization in *A. ocellaris* need to be further investigated.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author, EI.

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#### CONTRIBUTION OF THE AUTHORS

El data analysis and manuscript preparation;

TY, TA & KT primary data collection;

KM research supervision.



E Iwata <http://orcid.org/0000-0003-4178-7551>

K Masuda <https://orcid.org/0000-0003-2338-8450>