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Research article

Sexual differentiation and growth of clownfish (*Amphiprion ocellaris*) using commercial and experimental diets

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Abstract
The objective of this study was to evaluate the effect of different diets on sexual differentiation and growth of mature couples of clownfish (*Amphiprion ocellaris*). Three commercial diets and one experimental diet (in triplicate) were used: OTH (Otohime®); ALC (Alcon®); NRD (INVE-NRD®); and ED (experimental diet). To determine sexual differentiation, the larger individual was considered female and the smaller male. Once the diet was provided, biometric parameters (weight and length) of the formed couples were evaluated daily, to determine the duration of sexual differentiation. The duration of sexual differentiation was significantly longer in clownfish fed the NRD diet compared to the OTH and ALC diets ($P<0.05$). In conclusion, the duration of sexual differentiation of clownfish was influenced by diet.

Introduction

The clownfish (*Amphiprion ocellaris* Cuvier, 1830) is a protandrous hermaphrodite, whereby females are larger than males (Hattori 2012). Position in the social hierarchy is directly influenced by an individual's sex, size and growth rate, relative to those around them; a well-defined size difference with respect to individuals is important to maintain social rank (Buston 2003). For example, in a social group the female is largest (rank 1), the male is second largest (rank 2), and the non-breeders get progressively smaller as the hierarchy is descended (e.g. ranks 3–6) (Schuster et al. 2000). Thus, sexual differentiation be determined by the size of individuals. The clownfish has received attention in global aquaria due to its market demand, early life cycle, ease in forming couples (induced pairs), continuous spawning throughout the year and acceptance of different diet formulations (Varghese et al. 2009).

According to Kuwamura and Nakashima (1998), the formation of a couple seems to occur at random. However, Hattori (2012) demonstrated with a mathematical model that the formation of a couple in clownfish is non-linear, where a larger body size indicates a greater chance of success. As the quality and quantity of diet influences body size, consequently, it also influences the success of forming a couple (Dhaneesh et al. 2011), which can increase or decrease the time required for sexual differentiation.

The marine aquarium fish industry has been growing rapidly (Olivotto et al. 2011), but still heavily relies on wild-caught individuals (Mies et al. 2014). Maximised exploitation of clownfish exemplars and improved cultivation protocols for commercial-scale production are essential for greater economic gain in aquaculture of ornamental marine fish. According to Sales and Janssens (2003), the development of manufactured food can be considered one of the preponderant factors for the commercial expansion of aquaculture. However,

Table 1. Formulation of the experimental diet used for clownfish.

Ingredients	%
Squid meal	67.0
Fish meal	16.08
Mussel meal	6.0
Cassava starch	3.0
Amaranth flour	1.0
Quinoa flour	1.0
Spirulina	1.0
Chlorella	0.4
Paprika	0.4
Soy lecithin	0.4
Dextim antioxidant*	0.4
Vitamins/minerals premix**	0.3
L-Lisina	1.0
DL-Metionina	2.0
BHT antioxidant	0.02
Total	100%

*Benzeneethanamine, N,a-dimethyl-, hydrochloride (1:1),(aS)
**Folic acid 1,200 mg kg-1; nicotinic acid 20 g kg-1; pantothenic acid 10,000 mg kg-1; BHT 5,000 mg kg-1; biotin 200 mg kg-1; cobalt 80 mg kg-1; copper 3,500 mg kg-1; choline 100 g kg-1; iron 20 g kg-1; iodine 160 mg kg-1; inositol 25 g kg-1; manganese 10,000 mg kg-1; selenium 100 mg kg-1; zinc 24 mg kg-1 and vitamins A 2,400,000 UI kg-1; B1 4,000 mg kg-1; B2 4,000 mg kg-1; B6 3,500 mg kg-1; B12 8,000 mcg kg-1; C 60 g kg-1; D3 600,000 UI kg-1; E 30,000 UI kg-1; and K3 3,000 mg kg-1.

Table 2. Centesimal composition of commercial diets Otohime® (OTH), INVE-NRD® (NRD) and Alcon® (ALC) and experimental diet (ED) used for clownfish.

Centesimal composition	OTH	ALC	NRD	ED
Humidity (%)	7.18	5.75	7.39	6.97
Dry matter (%)	92.41	92.02	91.43	91.67
Crude protein (%)	53.36	46.28	55.73	60.73
Ethereal extract (%)	15.47	5.09	12.54	14.08
Carbohydrate (%)	24.71	43.14	26.51	23.96
Brute fibre (%)	6.46	5.51	5.22	1.24
Ashes (min)	16.00	15.07	14.15	15.19
Digestible energy (kcal kg-1)	5763	5112	5644	5801
Granulometry (μm)	600-1400	800-1200	800-1200	800-1200

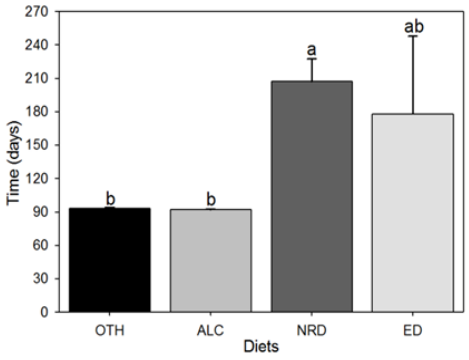

Figure 1. Sexual differentiation (mean±SEM) of clownfish couples subjected to different diets for 240 days (n=36). OTH=Otohime®. NRD=INVE-NRD®. ALC=Alcon®. ED=Experimental diet. Different letters indicate a significant difference between the different diets by one-way ANOVA followed by Tukey's post hoc test (P<0.05).

as a high diversity of ornamental marine fish are often kept together in aquaria, it is difficult to provide diets adapted to the specific requirements of each species (Pannevis and Earle 1994).

A diet suitable for clownfish should provide the nutrients needed for its growth and metabolism and can influence sexual precocity and sexual differentiation. Thus, the objective of this study was to evaluate the effect of different diets not only on sexual differentiation, but also on the growth of differentiated couples of clownfish.

Methods

Clownfish exemplars (6 months old with weight 1.79±0.14 g and length 37.61±1.39 mm) were purchased from Laboratório de Larvicultura Marinha, Salvador-BA, Brazil, where the experiments were carried out. During acclimatisation, the fish were provided a diet of commercial feed with 46.28% crude protein and 5,112 kcal kg-1 digestible energy. After feeding, the aquaria were siphoned for the removal of faeces and food residues. The individuals were fasted for a period of 24 h prior to the experiments.

The system used was composed of 12 tanks (19.6 L), interconnected to a sump of 170.24 L, with continuous recirculation with the aid of a submersible pump with a specified flow rate of 4,000 L h-1. The filtration system was composed of physical filtration and sterilisation by ultraviolet radiation (UV-15 W), chemical filtration (aeration by protein skimmer for separation of foam, coupled to activated carbon matrices) and biological filtration (bio-ball and super-concentrated Biodigest-Prodibio®). Synthetic sea salt was used (Aqua One®).

During the experiments, water quality parameters were measured and maintained thus: temperature (25.92±0.72ºC); dissolved oxygen (6.18±0.02 mg L-1 O2), measured with an oxygen meter (YSI 55/12FT®); pH (8.23±0.02), measured with a pH meter (ML1010-Misuraline®); salinity (30.2±0.31 ppm), measured with a

Table 3. Biometric parameters (mean±SEM) of clownfish (females and males) subjected to different diets for 60 days (n=24). Otohime®=OTH; INVE-NRD®=NRD; Alcon®=ALC; ED=experimental diet; IW=initial weight; FW=final weight; WG=weight gain; ITL=initial total length; FTL=final total length; TLG=total length gain; SGR=specific growth rate.

Parameters	OTH	ALC	NRD	ED
		Females		
IW (g)	3.49±0.31	3.11±0.34	3.44±0.33	3.16±0.44
FW (g)	3.53±0.42	3.33±0.30	3.51±0.38	3.61±0.54
WG (g)	0.04±0.03	0.22±0.05	0.08±0.05	0.45±0.44
ITL (mm)	56.57±2.27	56.40±0.10	56.57±1.94	56.42±1.86
TLG (mm)	57.63±2.53	56.85±0.16	57.21±2.06	56.71±1.92
GCT (mm)	1.06±0.61	0.45±0.18	0.64±0.12	0.29±0.27
SGR (%)	0.02±0.02	0.12±0.04	0.03±0.03	0.21±0.21
		Males		
IW (g)	2.19 ± 0.26	2.24 ± 0.14	2.13 ± 0.39	2.03 ± 0.21
FW (g)	2.55 ± 0.48	2.28 ± 0.14	2.39 ± 0.41	2.32 ± 0.19
WG (g)	0.36 ± 0.35	0.04 ± 0.01	0.26 ± 0.13	0.29 ± 0.03
ITL (mm)	48.16 ± 1.44	49.33 ± 1.19	47.14 ± 2.77	45.93 ± 1.35
TLG (mm)	48.77 ± 1.72	50.10 ± 1.17	48.08 ± 2.85	47.11 ± 1.76
GCT (mm)	0.61 ± 0.29	0.77 ± 0.23	0.94 ± 0.19	1.18 ± 0.58
SGR (%)	0.21 ± 0.20	0.03 ± 0.01	0.19 ± 0.08	0.24 ± 0.04

refractometer (RTS-101ATC-Instrutherm); alkalinity (113.27±7.55 mg L-1 CaCO3); total ammonia (0.48±0.04 mg L-1 N-NH3); and nitrite (0.15±0.03 mg L-1 N-NO2) using an Alfatecnoquímica kit (Florianópolis, SC, Brazil).

Clownfish exemplars were fed twice daily ad libitum. The treatments were three commercial diets and one experimental diet: (1) OTH - Otohime® S1 (Marubeni Nisshin Feed Co, Ltd.); (2) ALC - Alcon® (Alcon Marine Sticks); (3) INVE-NRD - NRD® (Inve Nutrição Animal Ltd.); and (4) ED - experimental diet (Tables 1 and 2). The experimental diet was manufactured in the Laboratório de Nutrição at the Universidade Federal do Recôncavo da Bahia, Cruz das Almas-BA, Brazil. A biomatological composition was determined according to AOAC (2000).

Initially, an experiment was conducted to evaluate sexual differentiation, where the four diets were tested separately in a completely randomised design with three individuals per aquarium (in a triplicate design; total n=36). The time for the sexual differentiation and formation of couples was evaluated through observation of agonistic interactions between the individuals, characterized by territorialism of the formed couple and physical aggressions to the individual segregated, where the individual segregated from each treatment was removed from the aquarium. The couple was evaluated with morphometric data (in which the larger individual was considered as female) and observation of cohort behaviour. The observation time was 240 days.

At the end of the sexual differentiation evaluation, the formed couples were maintained for 60 days in a second experiment, under the same treatments, for the evaluation of biometric parameters. To measure length (mm) and weight (mg), digital callipers (accuracy of 0.1 mm) and a digital balance (sensitivity of 0.1 mg) were used, respectively. The following biometric parameters were evaluated: final weight; final length; total weight gain (final weight−initial weight); total length gain (final length−initial length); and specific growth rate (SGR) (SGR = 100×[ln(final weight)−ln(initial weight)]/days).

All data are expressed as mean±SEM and were subjected to the Levene test to verify homogeneity of the variances. The evaluation of growth and sexual differentiation was performed by a one-way ANOVA followed by a Tukey post hoc test. Significance was set at a critical level of 95% ($P<0.05$).

Results

There was no mortality during the experimental procedures. All diets tested in this study resulted in a sexual differentiation time of less than 240 days, and the duration of sexual differentiation was significantly longer in clownfish fed the NRD diet compared to OTH and ALC diets ($P<0.05$), but not to the ED diet (Figure 1).

The specific growth rate varied between 0.02 and 0.24%, and the different diets did not significantly influence the biometric parameters for either mature females or males (Table 3).

Discussion

Protandry (a change from male to female) is expected to occur in species such as clownfish which have mating systems in which the expected reproductive success of males is less sensitive to body size, than that of females, which increases with increasing body size (Warner 1988). Because large fish use more resources, such as food and space, when such resources are limited growth of the dominant fish may retard the growth of subordinates (Buston 2003). Thus, the conversion from a functional male to a female or from an immature male to a mature male takes a few months to a few years (Hoff 1996). Additionally, because the cost of being expelled from a group is high, submissive and non-reproductive individuals avoid confrontation (Allen 1972) and do not develop. These characteristics allow the formation of captive couples and the production of clownfish throughout the year (Kodama et al. 2011).

All diets tested in this study resulted in a sexual differentiation time of less than 240 days, and only NRD treatment was less efficient when compared to OTH and ALC treatments. Adequate diets should provide the necessary nutrients for growth, maintenance of cellular tissues and reproduction in fish. For example, lipid and protein is known to significantly influence the process of reproduction in fish (Sales and Janssens 2003, Varghese et al. 2009).

The analysis of the centesimal composition of the diets was not shown to influence the biometric parameter. It is possible that such a difference occurred because of the diet ingredients; however, accurate information of the quantity of each ingredient used is patented and is thus not provided by the commercial feed manufacturers. In addition, other factors might influence sexual differentiation in fish, such as social, spatial and habitat factors, for example (Ruckstuhl 2007); furthermore, differentiation is dependent on ecological factors, size of individuals, hunger state and age (Romey and Wallace 2007).

Clownfish are sexually dimorphic, where the growth rate and size adopted by clownfish is a strategic response to resolving social conflicts (Buston 2003). In addition, the larger body size positively influences female fecundity and reproductive success (Hattori 2012). Thus, the patterns of precocity and sexual dimorphism determined by the diets tested in this study could also have influenced the development of clownfish. However, our data demonstrated that both mature females and males grew very little in the post-differentiation period. This is advantageous in choosing a post-differentiation clownfish diet, since it allows the diet to be selected by considering cost and not only its composition.

Understanding reproduction and growth is an essential part of the study of the biology of species and diets may influence sexual differentiation and growth. Our observations demonstrated that the duration of sexual differentiation of clownfish was influenced by diet. As a recommendation, we suggest conducting experiments that evaluate the effect of different dietary ingredients on the duration of sexual differentiation and the development of clownfish.

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