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Growth response of juvenile clownfish *Amphiprion sebae* Bleeker, 1853 fed with graded levels of dietary protein

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ABSTRACT

Clown fishes are the most popular marine ornamental species owing to their unique behaviour and symbiotic association with sea anemones. Captive propagation of these fishes is relatively easy; however, the lack of suitable diets influences their survival, growth and colouration. In this study, growth response of juvenile sebae clownfish *Amphiprion sebae* Bleeker, 1853 was evaluated through a feeding trial. Six iso-caloric experimental diets with graded dietary protein levels of 33.06 (D_{33}), 36.54 (D_{36}), 39.19 (D_{39}), 41.88 (D_{42}), 45.17 (D_{45}) and 47.94% (D_{48}) of dry matter were prepared. Lipid content of approximately 6% was maintained for all dietary treatments. The study was conducted for nine weeks in triplicate to evaluate survival, growth and feed utilisation. Fishes fed with diet D_{36} did not present any mortality; those fed diets $D_{39,}$ D_{45} and D_{48} had a lower survival with 86.7%, without significant differences among the treatments. However, weight gain, specific growth rate (SGR) and feed conversion efficiencies showed a significant difference among the treatments ($p<0.05$). Weight gain and SGR of sebae clownfish increased gradually among tested diets and peaked at D_{45} (697.28 mg and 3.84). A sharp decline in weight gain and SGR (486 mg and 3.39) was observed with the replicates fed with D_{48}. Feed conversion ratio (FCR) and protein efficiency ratio (PER) were superior with diets D_{42} (1.40 and 1.71) and D_{45} (1.60 and 1.38). The results of the present study revealed higher growth when fed with dietary protein level of 45%. The second-order polynomial regression on SGR and weight gain suggested an optimal dietary inclusion at 44.9 - 46.2%. Based on these results, it can be inferred that juvenile sebae clownfish has a higher dietary protein requirement of about 45%. However, the study also suggested the efficacy of diets having 36% protein and 6% lipid for healthy aquarium upkeep of sebae clownfish. These findings will be beneficial for the commercial propagation and maintenance of clownfishes.

Keywords: Anemone fish, Dietary protein requirement, Feed efficiency, Growth, Marine ornamental fish

Introduction

Marine ornamental fish keeping depends heavily on wild-caught species from coral reefs, which led to overexploitation and destruction of habitats (Calado, 2017; Palmtag, 2017; Dee *et al.,* 2019; Pouil *et al.,* 2020). Developing sustainable aquaculture practices can contribute to the protection and conservation of these vulnerable reef inhabitants. World trade in marine ornamental species involves 20-24 million fishes per annum; about 50% is contributed by clownfishes and damselfishes of family Pomacentridae (Wabnitz *et al.,* 2003). Coastal waters surrounding India were known to harbour 15 of the 29 species of clownfishes (Froese and Pauly, 2019). The sebae clownfish *Amphiprion sebae* Bleeker, 1853, dominates the Indian mainland coast and the other species are mostly found in the reefs of the Andaman and Nicobar and Lakshadweep islands. Among marine ornamentals, clownfishes are the most popular in trade and hobby (Olivotto and Geffroy, 2017) and constitute about 70% of the marine ornamental fishes from aquaculture (Olivier, 2001). This popularity gained further momentum with the release of the animation movie “Finding Nemo” in 2003, which further increased their demand (Yong *et al.,* 2011). Captive production techniques have been developed and standardised for most of the clownfish species of commercial importance.

Captive-bred clownfishes readily accept fresh, frozen and dry feeds; they are less demanding than other marine ornamental fishes (Olivotto and Geffroy, 2017). Clownfishes are usually reared using live feeds like rotifers, artemia and copepods during larval stages and are then weaned towards prepared feeds (Holt, 2003; Olivotto *et al*., 2008; Varghese *et al*., 2013). Sustainable aquaculture and maintenance of clownfish in production systems, hatcheries and in aquaria require proper diets. Many pioneering commercial production ventures turned unviable, predominantly owing to slow growth of juveniles due to lack of appropriate feeds (Hoff, 1996). Ornamental fish research got comparatively much lower attention than their food fish counterparts and even though research and development works are meagre, markets are flooded with expensive and specialised ornamental fish feeds (Craig *et al*., 2017).

Gordon *et al*. (2000) reported on ideal weaning age, survival and growth of the percula clownfish *Amphiprion percula*. In another study, Vargas-Abundez *et al*. (2019) probed the utility of insect meal-based diets on growth, analysing at the molecular level in ocellaris clown *Amphiprion ocellaris*. There are studies on the use of dietary oleoresins on the growth and skin colouration (Ebeneezar *et al.*, 2020) and carotenoid enriched feed effect on the pigmentation of *A. ocellaris* (Yasir and Qin, 2010). The relation between stocking density and diet on the growth of *A. percula* was also reported (Chambel *et al*., 2015). They are also considered an excellent model for essential fatty acid nutrition in marine larval stages (Olivotto and Geffroy, 2017).

Dietary protein plays an essential role in the growth, development and reproduction of fishes (Teles *et al*., 2020). It also has a decisive role in determining feed quality and cost-efficacy. Hence, it is crucial to know the optimal dietary inclusion level for the proper growth of fish and lower or higher inclusion levels affect viability. So, information on dietary protein is critical in the successful rearing of a new species in aquaculture. Though the macronutrient requirements of some freshwater ornamental fishes are known (Sales and Janssens, 2003: Velasco-Santamaría and Corredor-Santamaría, 2011), information about their marine counterparts, including clownfishes are scarce (Tacon and Haring, 1999; Olivotto and Geffroy, 2017; Díaz-Jimenez *et al.*, 2019). It has been recently reported that juvenile *A. ocellaris* performed better with a diet containing 43% protein and 10% lipid (Diaz-Jimenez *et al*., 2019). Research and development in nutritional aspects of clownfishes are indispensable in sustaining captive production, thereby releasing pressure on the reef ecosystem. The present study attempts to find optimum dietary protein needed for juvenile sebae clownfish.

Materials and methods

Juvenile clownfish were fed for nine weeks on feeds containing the same level of energy and lipid but with varying levels of dietary protein at 33.06% (D_{33}), 36.54% (D_{36}), 39.19% (D_{39}), 41.88% (D_{42}), 45.17% (D_{45}) and 47.94% (D_{48}) levels and their survival, growth and feed efficiency were assessed.

Experimental procedure

Sebae clownfish juveniles used in the study were obtained from the clownfish production facility at the Marine Aquarium and Hatchery of Vizhinjam Research Centre of ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI). The broodstocks were collected from the Rameswaram area of Tamil Nadu by traditional skin divers using scoop nets. Early juveniles had 14.7±0.5 mm total length and 62.6±5.9 mg live weight and were obtained from the same spawning. Under captive conditions, they achieved the size within 3-4 weeks. They were further reared for nine weeks under experimental conditions wherein they attained saleable size.

The juveniles were randomly distributed into 70 l plastic circular containers with aeration. Since sebae clownfish juveniles were reasonably aggressive under captivity, the experiment was conducted with fifteen juveniles in each replicate. Fishes were acclimatised to experimental conditions for a week before the commencement of the investigation. Tanks were not provided with substratum or the host sea anemones to reduce territorial aggression.

Seawater was passed through a 5 µm cartridge filter and water quality parameters were regularly monitored. Spectrophotometric methods (Genesys 10 UV, Thermospectronic) were used for the determination of NH_3-N (Strickland and Parsons, 1972), NO_2-N and NO_3-N (AOAC, 1980). Partial water changes of about 25% were done to compensate for losses due to waste removal and evaporation. The mean water parameters observed during the experimental period were: salinity (33.3±1.6 ppt), temperature (28.8±1.7°C), pH (8.09±0.1), NH_3-N (<0.003 mg l^{-1}) NO_2-N (<0.003 mg l^{-1}) and NO_3-N (<0.096 mg l^{-1}).

Experimental diets and feeding

Six diets with similar levels of gross energy and lipid content were prepared with varying dietary protein from 33 to 48% (D_{33}, D_{36}, D_{39}, D_{42}, D_{45} and D_{48}). The above range was opted as a preliminary study elicited a better growth response between 30 to 50% dietary protein (unpublished data). The ingredient inclusion levels and proximate composition of the experimental diets are given in Table 1. The raw ingredients were procured from the fish market, *i.e.,* dried anchovies (fish meal), dried *Metapenaeus* spp. (shrimp meal) and fresh *Loligo* sp. (squid meal). The ingredients were cleaned and cut into suitable sizes before oven drying at 50°C. They were then pulverised using a hammer mill and stored in airtight containers. Proximate composition of each ingredient was determined and mixed to form a basal mix in the ratio of 15:4:1 (fishmeal: shrimp meal: squid meal), as this ratio had a similar amino acid profile to that of sebae clownfish. The vitamin mix and mineral mix were also prepared separately in the laboratory by procuring chemicals and mixing them properly in an inert filler. The feed formulation was done through simultaneous equations in spreadsheets, and the prepared feeds were analysed for proximate composition to ascertain the nutrient levels. Cod-liver oil was used as the lipid source, dextrin to balance the energy level and

Table 1. Ingredient inclusion levels and proximate composition of experimental diets

	Dietary protein levels (%)					
	D_{33}	D_{36}	D_{39}	D_{42}	D_{45}	D_{48}
Ingredients (g kg^{-1})						
Fish meal	337.5	367.5	397.5	435	465	502.5
Shrimp meal	90.0	98.0	106.0	116.0	124.0	134.0
Squid meal	22.5	24.5	26.5	29.0	31.0	33.5
Wheat flour	150.0	150.0	150.0	150.0	150.0	150.0
Dextrin	214.2	178.0	141.0	93.0	56.0	9.0
Cod-liver oil	25.8	22.0	19.0	17.0	14.0	11.0
Vitamin mix[a]	20.0	20.0	20.0	20.0	20.0	20.0
Mineral mix[b]	40.0	40.0	40.0	40.0	40.0	40.0
Carboxy methyl cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Proximate composition of the diet (% dry matter basis)						
Crude protein	33.06	36.54	39.19	41.88	45.17	47.94
Crude lipid	6.04	5.93	5.90	6.03	6.00	6.03
Crude ash	10.85	11.67	12.49	13.51	14.33	15.35
Nitrogen free extract	32.75	29.31	25.79	21.23	17.71	13.24
Gross energy[c]	15.74	15.92	15.93	15.82	15.97	15.87
P/E ratio[d]	21.00	22.95	24.60	26.47	28.28	30.21

[a]Mineral mix (g kg^{-1} mix): $MgSO_4.7H_2O$ - 90.0; $MnSO_4.H_2O$ - 1.3; KI - 0.3; $NaH_2PO_4.2H_2O$ - 80; $NaHSeO_3$, - 0.01; $CoCl_2.6H_2O$, - 0.8; $ZnSO_4.7H_2O$, - 3.0; NaCl- 25; $CuSO_4.5H_2O$, - 0.5; KH_2PO_4. $2H_2O$, - 100; $FeSO_4.7H_2O$, - 12.5; $CaHPO_4$, - 50.

[b]Vitamin (mg or IU kg^{-1} in diet): Vit. A - 5000 IU; Cholecalciferol - 3000 IU; Tocopherol acetate - 250 IU; Menadione - 10; Ascorbic acid - 250; Thiamine hydrochloride - 25; Riboflavin - 15; Calcium pantothenate - 50; Pyridoxine hydrochloride, 20; Folic acid, 6; Nicotinic acid, 60; Biotin, 0.5; Cyanocobalamin, 0.04; Choline chloride, 900; Inositol, 200.

[c]Gross energy (kJ g^{-1}) was calculated based on 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.2 kJ g^{-1} for carbohydrate.

[d]P/E ratio = Protein to Energy ratio.

cellulose as an inert filler. Feed granules were prepared using specially designed aluminum scraping plates having a 2 mm dia. The feed granule size obtained varied with the speed of manual scraping. It was then oven-dried and passed through standard sieves of required mesh sizes. Fishes were fed to satiation and the feeding was done three times a day (09:00, 13:00 and 17:00 hrs) for the first three weeks of rearing with granules of 500-1000 μm. Later the feeding was done with granules of 1-2 mm. The total length and wet weight of fishes were taken before and after the experiment. As fish were found sensitive to frequent handling, intermediate measurements were avoided.

Sample collection and analysis

The feed moisture, crude protein, and crude fibre were determined following AOAC (1995). Crude lipid was estimated by soxhlet extraction with petroleum ether and the ash content was determined from the residue remaining after the incineration of the samples at 550^0C in a muffle furnace. The gross energy was calculated using values of 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipids and 17.2 kJ g^{-1} for carbohydrates (Blaxter, 1989).

Response variables

The effect of the diets on the survival (%), weight gain (WG), condition factor (CF), specific growth rate (SGR), feed conversion ratio (FCR) and the protein efficiency ratio (PER) of the fishes were established after completing the experiment.

Statistical analysis

Statistical analyses were performed and response variables were statistically analysed by ANOVA and Duncan's multiple range test using the statistical package SPSS (SPSS Inc., Chicago, IL, USA). Probability at 5% level was used to determine the significant difference among the treatment means ($p<0.05$). The dietary protein requirement was established by a second-order polynomial regression analysis (Robbins *et al.,* 1979), as the growth data exhibited a curvilinear pattern.

Results

The mean growth response and feed performance data derived from the experiment are given in Table 2. Except for survival, other parameters showed significant ($p<0.05$) differences between the tested dietary treatments. The weight gain in sebae clownfish exhibited an increasing trend up to 45% dietary protein inclusion; however, a drastic decline was observed above the optimal level. The diet with 33% protein (D_{33}) led to least weight gain, which was less than one-third of the growth response compared to diet D_{45} with 45% protein.

Table 2. Growth performance, survival and feed utilisation of juvenile clownfish fed diets with varying protein content

Parameters	Dietary protein levels (%)					
	D_{33}	D_{36}	D_{39}	D_{42}	D_{45}	D_{48}
Weight gain (mg)	190±38^{a}	270±10^{a}	390±72^{b}	560±23^{c}	700±91^{d}	490±37^{c}
Length gain (mm)	11.3±0.1^{a}	11.1±0.9^{a}	14.6±0.9^{b}	21.1±1.5^{c}	20.4±0.9^{c}	17.8±1.7^{d}
Specific growth rate	2.44±0.17^{a}	2.68±0.01^{a}	3.06±0.23^{b}	3.63±0.17^{c,d}	3.84±0.32^{d}	3.39±0.18^{b,c}
Condition factor	1.52±0.19^{a,b}	1.92±0.18^{c}	1.78±0.21^{b,c}	1.40±0.13^{a}	1.67±0.12^{a,b,c}	1.61±0.15^{a,b}
Weight gain (%)	360±47^{a}	440±03^{a,b}	590±96^{b,c}	880±107^{d,e}	1020±221^{e}	740±92^{c,d}
Survival (%)	93.3±11.5	100	86.7±11.5	93.3±11.5	86.7±11.5	86.7±23
FCR	2.41±0.06^{a}	2.04±0.09^{b}	2.16±0.03^{c}	1.40±0.01^{d}	1.60±0.02^{e}	2.19±0.10^{c}
PER	1.26±0.01^{a}	1.34±0.07^{b}	1.18±0.11^{c}	1.71±0.02^{d}	1.38±0.05^{c}	0.95±0.08^{e}

Mean values with different superscripts in the row are significantly different from each other ($p<0.05$)
D_{33} to D_{48} - Feeds with 33 % to 48% dietary protein
Specific growth rate (SGR) = {[ln (final weight) - ln (initial weight)]/ No. of days} x 100
Condition factor (CF) = (Final weight / Final length^3) x 100
Weight gain (%) (WG) = [(Final weight – Initial weight) / Initial weight] x 100
Survival (%) = (Final fish number / Initial fish number) x 100
Feed conversion ratio (FCR) = Feed intake (g dry weight) / Fish weight gain (g wet weight)
Protein efficiency ratio (PER) - Fish wet weight gain (g) / Protein intake (g)

The specific growth rate (SGR) ranged between 2.44 and 3.84 and improved with increasing dietary protein level up to 45% level and further, decreased at 48%. The highest gain in length was obtained with diet D_{42} and was closely followed by D_{45}. The condition factor was significantly higher in diet D_{36} with 36% protein and observed differences in the condition factor between D_{39}, D_{45} and D_{48} were insignificant.

The feed utilisation parameters like FCR and PER also showed a significant difference between treatments. The lower and higher dietary protein levels (D_{33} and D_{48}) resulted in high FCR and among the diets, the most efficient FCR was observed with diet D_{42}. The PER was high at D_{42} followed by D_{36}, D_{45}, D_{33}, D_{39} and D_{48}. The least protein efficiency was obtained with higher protein inclusion.

Survival was found to be high and did not vary significantly between the treatments. The results indicated that the tested dietary protein levels did not affect survival and produced measurable growth. Survival ranged from 86.7 to 100% and no mortality occurred in treatment D_{36},. The least value of 86.7% was observed in D_{39}, D_{45} and D_{48} and was intermediate in D_{33} and D_{42} with 93.3%.

ANOVA suggested the best growth performance of juveniles at 45% dietary protein among the tested diets. Since the growth showed a curvilinear pattern, a second-order polynomial regression was fitted. Second-order polynomial regression analysis of weight gain ($y = -2.5451x^2 + 235.25x - 4861.1$; $r^2 = 0.90$) exhibited maximum weight gain at 46.2% (Fig. 1) dietary protein inclusion. However, the same on SGR ($y = -0.0091x^2 + 0.8162x - 14.767$; $r^2 = 0.9$) indicated optimum value at 44.9% crude protein. Based on the second-order polynomial regression analysis, the optimum dietary protein required for the maximum growth of juvenile sebae clownfish was estimated to be 44.9-46.2%.

Discussion

Information on dietary protein is the primary need for developing diets for any species for successful aquaculture. Though several studies were done on the biology, broodstock management, breeding and larval rearing of clownfishes (Hoff, 1996; Varghese *et al*., 2009; Olivotto and Geffroy, 2017), those on its nutrient requirements were lacking. Most ornamental fish feeds are formulated to have a dietary protein content of 32-40% and requirements are higher in smaller-sized fishes (Craig *et al*., 2017). Since published information on the nutrient requirement of marine ornamental fishes

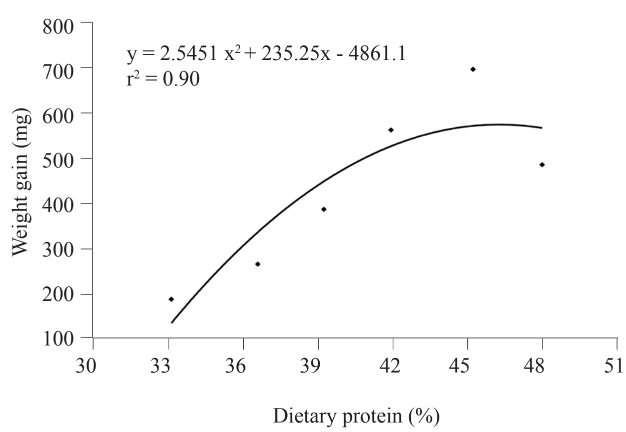

Fig. 1. Second-order polynomial regression analysis of weight gain on dietary protein for *A. sebae*

is not available, the data were compared with those of freshwater counterparts and other marine fishes.

The feeding trial resulted in significant growth variation in juvenile sebae clownfish. Results indicate the dietary protein requirement of clownfish at 44.9-46.2%. Similar higher requirements were observed in other ornamental fishes like discus, *Symphysodon aequifasciata* (Chong *et al*., 2000) and neon tetra, *Paracheirodon innesi* (Sealey *et al*., 2009). Higher protein requirement was also observed for edible fishes such as European seabass, mangrove red snapper, rainbow trout, olive flounder and cobia (Teles *et al*., 2020). The dietary protein level of 44.9 - 46.2% recorded in the present study is in line with that observed for Asian seabass (Sakaras *et al*., 1989), European seabass (Perez *et al*., 1997) and Japanese flounder (Lee *et al*., 2002). However, the estimated dietary protein requirement for sebae clownfish was substantially higher than that of most aquarium fishes (Sales and Janssons, 2003), such as dwarf gourami (Shim *et al*., 1989), goldfish (Lochmann and Philipps, 1994) and tinfoil barb (Elangovan and Shim, 1997).

In sebae clownfish, the weight gain (1020%) and specific growth rate (3.84) increased significantly up to 45% dietary protein inclusion and later declined at 48% level. These results are superior to the lower values obtained for *A. ocellaris* at 30.59% and 0.14, respectively, when fed with a diet containing 43% protein and 10% fat (Diaz-Jimenez *et al*., 2019).

Growth reduction with surplus dietary protein was reported in ornamental fishes like discus (Chong *et al*., 2000) and tin foil barb (Elangovan and Shim, 1997). The dietary protein level of 48% led to the decline in growth rate and this may be due to the utilisation of protein as the energy source for metabolic activities than protein synthesis, thereby depressing growth (Jauncey, 1982; Ye *et al*., 2016). A similar reduced growth rate with higher dietary protein was also reported in marine fishes (Teng *et al*., 1978; Lim *et al*., 1979; Jauncey, 1982; Vergara *et al*., 1996; Bai *et al*., 1999; Lee *et al*., 2002). Lower performance of a higher protein diet might also be due to the diversion of energy to other metabolic activities, thereby depressing growth. Mortality observed was independent of the dietary protein inclusion and was mainly due to aggression (Díaz-Jimenez *et al*., 2019).

Suboptimal nutrient availability causes increased feed consumption in fishes to meet their energy requirements (Su *et al*., 2019); it affects the feed conversion and viability of aquaculture operations. The feed efficiency improved with increasing dietary protein, 42% dietary protein giving better FCR (1.4) and PER (1.7), which was slightly lower in 45% dietary protein (1.6 and 1.4) wherein the highest growth was obtained. Feed conversion was better in diets with 42 and 45% protein, indicating better assimilation and conversion of the ingested nutrients. The higher FCR, when fed D_{48}, means growth depression and inadequate feed utilisation. The protein efficiency ratio decreased with an increase in dietary protein inclusion, except with D_{39} and was below 1 with $D_{48,}$ indicating inefficient protein utilisation. A similar decrease in PER with increased dietary protein above optimum was in agreement with other fishes (Siddiqui *et al*., 1988; Lee *et al*., 2002). However, the FCR and PER values obtained for sebae clownfish are much superior to the values reported for clownfish *A. ocellaris* at 25.9 and 0.09, respectively (Diaz-Jimenez *et al*., 2019). Gordon *et al*. (2000) reported an FCR of 10.35 and PER of 0.22 when percula clownfish were fed with a dry diet containing 43.8% protein and 8.4% lipid. These results indicated the feed quality, nutrient and energy balance of diets used in the present investigation.

Maintaining a proper protein to energy ratio (P/E) is essential in formulating a balanced diet (Meyer and Fracalossi, 2004). In the Nile tilapia, the P/E ratio significantly influences growth and feed efficiency (Kabir *et al*., 2019). In the present study, the P/E was between 21 to 30 mg kJ^{-1} and it gradually increased with dietary protein inclusion. Better growth performance was obtained at a dietary protein inclusion of 45% with a P/E ratio of 28.28 mg kJ^{-1}. The treatments are given lower dietary protein and higher non-protein energy sources led to a low P/E ratio, which reduced growth and feed utilisation. An important observation in the present study is the effectiveness of D_{36} having lower dietary protein. The diet not only sustained the highest survival but also provided measurable growth. Superior condition factor observed with 36% dietary protein inclusion may improve the visual appeal of clownfishes in aquaria. This suggests that a diet with 36% protein and 6% lipid prepared from high-quality ingredients can be effectively used for aquarium maintenance of sebae clownfish.

In summary, the present study indicated higher growth with the dietary inclusion of 45% crude protein for sebae clownfish. However, the regression analysis of growth parameters predicted an optimal dietary protein of 44.9 - 46.2%. The information generated can help to develop proper grow-out diets and supplementation of the formula with carotenoids can ensure the natural vibrancy of clownfishes under captivity. As clownfish are omnivores, replacing animal protein ingredients with plant substitutes can be explored in future research.

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