



Effect of Supplemented Algal Carotenoid Diets on Skin Color of Tomato Clownfish, *Amphiprion frenatus*

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ABSTRACT

This study analyzes the effect of *Nannochloropsis oculata* and *Porphyridium cruentum* as natural pigment sources on skin color of tomato clownfish, *Amphiprion frenatus*. Two groups, each of 6 fish were fed on feed containing (69.8±9.158 mg.g⁻¹) *N. oculata* (Group A), and 67.21±7.068 mg.g⁻¹ of *P. cruentum* (Group B). The third group (Group C) was fed on control basal diet (34.93±29.07 mg.g⁻¹). Total carotenoid content of fish skin was determined at 30-day intervals. At the end of the experiment the highest weight gain was found to be 1.73±0.37g in Group B, whereas the lowest performance (1.29±0.38 g) was received in Group C. The best feed conversion ratio was found in at Group B. The total carotenoid content of skin of fish were found to be 0.77±0.61 µg.g⁻¹ on the initial day in the experimental group. As a result of the measurements performed on the 120th day, the pigment values were determined as 30.39±0.39 µg.g⁻¹ in Group A, 39.07±1.12 µg.g⁻¹ in Group B and 35.68±10.69 µg.g⁻¹ in the Group C. Group B pigment source is more effective on the color of tomato clownfish *A. frenatus*.

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Authors' Contributions

MAH, KF and YD conceived and designed the study. MAH executed the experimental work. MAH and KF statistically analyzed the data, looked after fish, analyzed the fish colour and wrote the article. AK helped in fish feed preparation. CS analyzed the fish feed. SS helped in statistical analysis.

Key words

Ornamental fish, Carotenoid, *Amphiprion frenatus*, *Porphyridium cruentum*, *Nannochloropsis oculata*.

INTRODUCTION

The tomato clownfish, *Amphiprion frenatus*, is an important ornamental fish for the aquarium industry because of its color. The tomato clown fish is characterized by a white band just behind the eyes, being dominated by colors orange/red in the natural environment. The tomato clown fish also has distinctive differences from the fish being collected from the nature, in terms of color tone, brightness and saturation. In this context, it is required to impose carotenoids, which give color to the fish, from the outside in order to increase the visual effect (Yasir and Qin, 2010).

The coloration of the fish skin is dependent on absorption and deposition of carotenoid pigments from the diet, since fish, like other vertebrates, are unable to synthesize carotenoids de novo (Goodwin, 1984). Carotenoids are biosynthesized by plants, algae, and certain yeast and bacteria (Ong and Tee, 1992). Carotenoids are responsible for the red, orange, and yellow colors of fish and crustaceans (Packer, 1992). Dietary carotenoids play an important role in regulating fish color because fish,

like other animals, are unable to synthesize carotenoids and their skin color is highly dependent on carotenoids from the diet (Torrissen *et al.*, 1989). Upon intake, fish can modify alimentary carotenoids and store them in the integument and other tissues (Ha *et al.*, 1993).

In this study, the unicellular red alga *Porphyridium cruentum*, which is a member of the Rhodophyta, and *Nannochloropsis oculata*, a marine unicellular alga belonging to the Eustigmatophyceae family, were used as natural pigment sources. *P. cruentum*'s carotene has Phycoerythrin. Pigment composition of algae from the genus *Nannochloropsis* is characterized by chlorophyll a (not chlorophyll b or chlorophyll c), carotene, violaxanthin and vaucherixanthin as major pigments. These algae also contain some minor carotenoids among which canthaxanthin and astaxanthin, both with ketonic groups; stand out (Whittle and Casselton, 1975; Karlson *et al.*, 1996).

Although there are studies on the effects of pigment materials, being mixed in feed for a number of saltwater fish and other clown fish species, upon color changes on skin (Yasir and Qin, 2009), studies on *A. frontalis* only concentrate on the spawning management (Nakamura *et al.*, 1994) micro habitat (Hattori, 2005) and diseases (Silphaduang *et al.*, 2000). There is not any study about the effect of coloration on the tomato clown fish; *A. frenatus*.

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Therefore, this study was undertaken for the first time to determine the effect of microalgae's pigment on skin of coloration of *A. frenatus*.

MATERIALS AND METHODS

Fish and experimental design

The tomato clown fish, which have been produced in a commercial aquaculture facility in Akvatek Company (Turkey), was used in the research. 45 days old 180 clown fish were used for experiment. Their average wet-weight were 0.26 ± 0.02 g and average total length was 1.29 ± 0.01 cm. This study was conducted in rectangular glass aquaria in 50 L working volume (47 x 37 x 29 cm) with 20 fish in each. The aquaria were supplied with seawater through a recirculating system treated with biofilter and mechanical filter. Oxygen was maintained at 6.8 ± 0.2 mgL⁻¹ with air stone. The environmental variables were maintained at 25.5 ± 0.4 °C, 35 ± 0.2 ‰ salinity and light was supplied by fluorescent lamps: with a power of 1200 lx at water surface and photoperiod applied 16 h light: 8 h dark. During the experiment the major physical and chemical parameters were maintained at stable conditions.

Algal culture

N. oculata and *P. cruentum* were cultured in laboratory in 10-liter balloon as a batch culture at 18°C at the Plankton Laboratory at Ege University (Izmir, Turkey). The alga was cultured in enriched seawater with F/2 medium. The culture was kept illuminated with fluorescent lamps at photon flux density of $116 \mu\text{Em}^{-2} \text{s}^{-1}$. Culture was continuously aerated by the air. Harvesting of the culture was done by centrifugation. The pellet obtained was dried at 65°C in the drying oven for used pellet feeds.

Preparation of feed and application

The experimental diets were formulated to meet the nutritional requirements of the tomato clownfish and prepared with using laboratory type pellet machine in the fish nutrition and fish feed technology laboratory at Ege University (Turkey). The experimental feeds were prepared with basic ingredients such as fish meal, soybean meal, wheat meal, corn gluten, and fish oil. The two natural carotenoid sources such as powdered *Nannochloropsis oculata* (Group A) and powdered *Porphyridium cruentum* (Group B) used in the experimental diet and the control diet (Group C), were not included any algae powder as a carotenoid source. So, only the pigment sources show differences in the feed, which were prepared as 3 groups.

Three replicates were used for each of the three experimental diets. Fish in each treatment only received one diet type twice a day (morning and evening) ad

libitum. The feeds being used throughout the study were stored at +4°C.

Calculations and analyses

At the beginning and the end of the experiment, fish were measured and weighed. During the study the specific growth rate (SGR), whose formulation is given below, was used to determine growth rate of fish (Jensen, 1985):

$$SGR = \frac{\ln \text{Final weight} - \ln \text{Initial weight}}{\text{Day}} \times 100$$

Daily growth rate (DGR) was calculated according to Guillaume *et al.* (1999):

$$DGR (\%) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

Feed Conversion Ratio (FCR) was calculated as below formula:

$$FCR = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$$

Assuming a complete of diets, the carotenoid retention rate were calculated by the following equation (Ingle De La Mora *et al.*, 2006):

$$\text{Retention Rate (\%)} = \frac{\text{mg of carotenoid of skin + flesh}}{\text{mg of carotenoid in diet}} \times 100$$

The crude protein, crude fat and ash in diets were determined with method of Padmore (1990).

Total β -carotene content of the fish was determined spectrophotometrically after extraction with acetone (Choubert and Storebakken, 1989). Three fish were randomly sampled from each aquarium. Fish were then anesthetized with MS-222 (70 ppm). The anaesthetized fish were put down with a needle right in the brain and the skin was dissected immediately from body area for carotenoid content determination. Skin samples were collected from both sides between the abdominal and dorsal region of the fish. 10 mg skin sample passed through the homogenization procedure with the addition of 5 ml acetone, they passed through the centrifuge procedure for 10 min at 3500 rpm. After that these samples were read in 475 nm wavelength on the spectrophotometer (Jenway 6305 model). A calibration curve was made using the absorbance values in 5 ml acetone solution which has 0.16, 1.63, 2.04, 3.27 and 4.09 mg g⁻¹ β -carotene to determine the quantity of β -carotene.

Statistical analysis consisted nonparametric test of Kruskal Wallis, using the probability level of 0.05 for rejection of the null hypothesis. After ANOVA, significant

differences among means were determined by Mann-Whitney U test. All statistical analysis were performed using SPSS 15.0 for Windows.

RESULTS

The crude protein, lipid content of all experimental diets did not vary in different experimental diets from each other (Table I). The protein content from Group A, Group B and Group C were 42.94%, 42.70% and 42.79%, respectively. The lipid value and crude cellulose from experimental diets groups were 7.90%, 7.05% 6.79 and%, 3.07%, 2.80%, 3.02%, respectively. Total β -carotenoid amount of experimental diets were determined as 69.8 ± 9.158 , 67.21 ± 7.068 and 34.93 ± 29.07 mg g^{-1} , respectively.

Table I.- Feed composition and proximate chemical composition of diets.

Ingredient (g.100 g ⁻¹)	Group A	Group B ^b	Group C
Fish meal	35	35	35
Soybean meal	25	25	25
Wheat meal	19	16.8	20
Corn gluten	11	10.2	15
Fish oil	2	2	2
<i>Nannochloropsis oculata</i>	5		
<i>Porphyridium cruentum</i>	-	8	-
Mineral premix ^d	3	3	3
Total	100	100	100
Chemical Composition			
% Crude protein	42.94	42.70	42.79
% Crude lipids	7.90	7.05	6.79
% Crude cellulose	3.07	2.80	3.02
% Crude ash	8.49	9.5	8.15
Gross energy (MJ. Kg ⁻¹)	2540.3	2450.5	2604.7

^aGroup A, Diet *Nannochloropsis*. feed included *Nannochloropsis oculata*.

^bGroup B, Diet *Porphyridium*, feed included *Porphyridium cruentum*.

^cGroup C, Diet Control, feed not included Algae. ^dMineral premix, g Per g mixture (vitamin A 342 IU, vitamin D3 329 IU, vitamin E 0.0274 IU, vitamin K3 48 mg, vitamin B1 2.05 mg, vitamin B2 3.42 mg, vitamin B3 20.5 mg, vitamin B5 5.48 mg, vitamin B6 2.05 mg, vitamin B12 2.74 mgm vitamin C 24.0 mg, biotin 0.411 mg, folic acid 0.685 mg, Zn 12.3 mg, Mn 4.80 mg, Cu 1.64 mg, I 0.274 mg, Se 0.0274 mg. Ca: 125 mg, K 189 mg). Agromey Feed Mill Company, Izmir, Turkey.

All experiment diets were equally accepted by fish. In this study, no mortality was observed. Fish showed a homogeneous distribution within the aquarium in all groups throughout the experimentation. At the end of the 8th day, individuals were observed chasing each other, attacking their tails and fins or swimming after each other.

The fish generally displayed the behavior of grabbing feed on the water surface and rarely from the bottom, throughout the experimentation. At the end of the 20th day, the fish retreated in groups to certain areas of the aquarium.

At the end of the experimentation, there were no significant differences between the groups in terms of total length ($p > 0.05$). The highest weight of experimental groups was measured as 1.73 ± 0.37 g (Group B, Table II). Lower performance was measured as 1.29 ± 0.38 g in Group C. The weight of both groups (Group A, Group B) were significantly different from the Group C ($p < 0.05$). The best feed conversion ratio was measured at Group B. Similarly, the best SGR and growth values were measured at Group B (Table II).

Table II.- Effect of feeding carotenoids supplements on total length, survival rate, growth and feed utilization parameters after 120 days of experiment.

		Group A	Group B	Group C
Total length (cm)	Initial	1.29 \pm 0.02	1.29 \pm 0.02	1.29 \pm 0.02
	Final	4.15 \pm 0.24	4.38 \pm 0.30	4.00 \pm 0.38
Total weight (gm)	Initial	0.26 \pm 0.03	0.26 \pm 0.03	0.26 \pm 0.03
	Final	1.59 \pm 0.35	1.73 \pm 0.37	1.29 \pm 0.36
Growth (DGR)		4.26	4.71	3.30
SGR ^c		1,59	1,68	1,39
FCR ^d		2,02	1.83	2,61
Survival rate (%)		100	100	100

Values are mean \pm S.D. of three groups per treatment. a, no significantly differences ($P > 0.05$) were observed among treatments means. For diet composition of group A, B and C, see Table I.

The total carotenoid values of skin of the fish in experimental groups (Group A, Group B and Group C) were determined as 0.77 ± 0.61 μ g g^{-1} on the initial day. After other analyses conducted on the 120th day, a rapid decrease was observed in the carotenoid amount and it was determined that these values were lower than the initial value (Table III). The data were not found statistically significant ($p > 0.05$).

Table III.- Total carotenoid amounts (mean \pm S.D.).

Initial days	Group A	Group B	Group C
30	130.91 \pm 118	140.05 \pm 67.71	140.33 \pm 136.49
60	78.77 \pm 44.131	85.84 \pm 46.45	57.68 \pm 21.81
90	29.88 \pm 14.52	50.88 \pm 18.57	50.67 \pm 6.85
120	30.39 \pm 0.39	39.07 \pm 1.12	35.69 \pm 10.69

As a result of the analyses performed on the 60th day, it was determined that the pigment accumulation of individuals in Group B did not differ from Group A and Group C; the acquired data were different from the initial value ($p < 0.05$). As a result of the measurements performed on the 120th day, the pigment value was determined as $30.39 \pm 0.39 \mu\text{g}\cdot\text{g}^{-1}$ in Group A, $39.07 \pm 1.12 \mu\text{g}\cdot\text{g}^{-1}$ in Group B and $35.68 \pm 10.69 \mu\text{g}\cdot\text{g}^{-1}$ in the Group C. In addition to this, Group B showed a greater pigment accumulation compared to other groups, on the 120th day and no difference was determined between Group B and others ($p > 0.05$).

DISCUSSION

The variation of colors and patterns of the fish enables the implementation of different functions such as warning, hiding and recognizing the species and sex. Coloration is controlled by the endocrine and nervous system. But dietary sources of pigment also play a role in determining color in fishes. Carotenoids are known to have a positive role in the intermediary metabolism of fish (Tacon, 1981; Segner *et al.*, 1989; Chatzifotis *et al.*, 2005) that could enhance nutrient utilization and may ultimately result in improved growth (Amar *et al.*, 2001). As long as carotenoids are added to feeds as an extra material, they may prevent skin darkening caused by stress and production in the culture conditions. Accordingly, studies on the effect of carotenoids upon the color of fish showed that it could change according to the species, pigment of the fish, as well as the duration of application (Chatzifotis *et al.*, 2005; Yasir and Qin, 2010).

Microalgae are significant organisms since they form the primary link in the food chain in seas. It is known that the success of any hatchery operation depends mainly on the availability of basic food such as microalgae and zooplanktons. *N. oculata* have been extensively utilized for mass production of zooplanktons such as rotifer, *artemia*, copepods etc. and are also used for generating "green water" in many hatcheries. *Rhodophyta* tends to have a simple pigment composition with b-carotene, zeaxanthin and chlorophyll as the predominant thylakoid pigments (Grabowski *et al.*, 2000). In addition to Beta carotene, the major xanthophylls pigment found in all *Nannochloropsis* species are vilaxanthin and a vaucherxanthin-like pigment (Karlsen *et al.*, 1996; Sukenik, 1999). Besides this, it was stated that pigments being added to the feeds of some saltwater fish and other fish had no effect upon growth (Chebbaki *et al.*, 2002; Gomes *et al.*, 2002; Nickell and Bomage, 1998).

Supplementing algal biomass to the basal diet has not had measurable effects on alteration of growth or voluntary

feed intake by fresh water fish like rainbow trout (Gouveia *et al.*, 2003). The study determined that the group which had *N. oculata* and *P. cruentum* in their feeds had a higher weight development, which could be explained through the fact that the algae being added to the feed positively affect the feed evaluation. This condition was also reported in other fish species.

A. frenatus, which is originally from Pacific Ocean, can breed all year long in the tropics but only in the warmer months of temperate locations. It is stated that while the tomato clownfish being collected from the nature have a few white bands on the neck during youth, these bands decrease as they age (Hoff, 2009). It is also stated that the tomato clownfish juveniles that are produced under controlled conditions take the color and band features of adults on the 30th day (Madhu *et al.*, 2011). In the study, band changes were completed within a process of 4 months, which is thought to be caused by the broodstock management, environmental conditions and nurture regime differences.

The species being produced in the aquarium and marine aquarium sector, where the visual effect is in the forefront, have an utterly important pigmentation feature. In this context, it is inevitable to use materials involving synthetic and natural carotenoid as a feed additive. Although adding various synthetic materials into the feed increases the color intensity of the fish in a short time, a great majority of them are not suggested for use since they contain carcinogens. In addition to this, the most frequently used non-carcinogenic pigment materials are too expensive. Microalgae, on the other hand, are rich nutritional sources that are economically accessible, could be produced under convenient conditions and involve a rich carotenoid and nutritional value (Kop *et al.*, 2010). Carotenoids are lipid soluble and follow the same absorptive pathways as other dietary lipids. Carotenoids are used by fisheries mainly due to the existence and place of the double bond and hydroxyl group in their structures. Absorptions, metabolic cycles and accumulations of carotenoid pigments differ according to the fish species. Trouts and shrimps use carotenoid of 4-4' oxo that bear a double bond in their structures. Goldfish, on the other hand, use carotenoid of 3-3' hydroxy (Torrissen *et al.*, 1989). Trouts and some species such as shrimp only absorb linings and similar carotenoids and accumulate them in their tissues also as linings. Koi and goldfish, on the other hand, absorb carotenoids; however, they also metabolize and accumulate them in their tissues as linings.

Spirulina meal has also been used successfully to increase the skin coloration of red tilapia (Matsumo *et al.*, 1980), red sword tail (James *et al.*, 2006), blue gourami *Trichogaster trichopterus* (Alagappan *et al.*, 2004),

goldfish *Carassius auratus* (Gouveia *et al.*, 2003; James *et al.*, 2006) and yellow tail cichlid *Pseudotropheus acei* (Güroy *et al.*, 2012).

CONCLUSIONS

In conclusion it was observed in the study that the two algae species created no statistically significant difference in the pigmentation of the fish, but this condition could be due to the low level of carotenoids in the algae being added in the feed. In addition, this study has consequently shown that adding algae species involving natural pigment materials to the feeds increases the total amount of carotenoids in tomato clown fish skins. Besides that the study is also thought to be important in terms of the first application of *Porphyridium cruentum* and *Nannochloropsis oculatus*, which are among natural pigment resources, on this species. Although the results do not show a statistical difference, appearances of the fish are observed to differ according to the colors of the fish in the Group C. This condition is especially observed in the group that had *P. cruentum* in their feeds. In addition to this, it will definitely have a greater meaning together with the increase of the algae amount that will be added to the feed in next studies and consequently the total pigment amount that will be added to the feed.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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