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The effect of *Artemia urmiana*, Earthworm, Cow heart and concentrate as supplementary diets on skin color and pigmentation of Oscar fish (*Astronotus ocellatus*)

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Abstract: Skin coloration has a key role in ornamental fish trade. The amount of carotenoid pigments has a decisive influence on the color of muscle and skin of edible and ornamental fish. Also, there is a need to add carotenoids in the diet of cultured fish. Live foods especially *Artemia* play an important role in ornamental fish feeding regarding growth and skin color and thus marketing. In this study, the effects of different diets including concentrate, 67% concentrate and 33% frozen earthworm, 67% concentrate and 33% frozen *Artemia urmiana* as well as 67% concentrate and 33% frozen cow heart on total carotenoids and carotenoid pigments of Oscar fish skin was studied. A total of 360 Oscar fish with initial weight of approximately 1.6 g were cultured for 2 months in 4 feeding treatments, each in 3 replicates. The total carotenoids, astaxanthin, cantaxanthin and β-Carotene contents of the fish were analyzed. Results indicated that in all treatments asthaxanthin was the highest amount of carotenoid pigmentation of Oscar skin. Adding *Artemia urmiana* in the diet induced the higher total carotenoid and asthaxanthin levels in the skin of Oscar fish.

Keywords: *Astronotus* ocellatus, carotenoid pigments, *Artemia urmiana*, Earthworm, Cow heart

Introduction

The production and trade of aquarium fish as living jewels is a profitable sector in aquaculture and socio-economic development. Ornamental fish keeping is one of the most popular hobbies of millions people worldwide. European Union countries and the Unites States are the largest market of ornamental fish. The industry has been estimated to be worth about 15 US dollars (Yilmaz and Arslan, 2013; Koru and Turkmen, 2014).

Oscar fish (*Astronotus ocellatus*) from Cichlidae family is one of the most popular and beautiful freshwater ornamental fish in the world. It is highly interested for aquarists due to its unique color, fighting behavior, movement and display (Yilmaz and Arslan, 2013; Saghaei *et al*., 2015). Oscar fish are omnivorous but mostly carnivorous. They mainly feed on aquatic and terrestrial insects, small fish and invertebrates, fruits, benthic algae and plants (Yilmaz and Arslan, 2013). Nowadays, ornamental fish feeding is based on live foods such as *Artemia*, moina, fairy shrimps, daphnia (Munuswamy, 2005; Seidgar *et al*., 2015). Live feed is an important food source for cultured ornamental fish especially in larval stages. Formulated diets aggregate on the water surface or sink to the bottom, but the movement of live feed in the water column can stimulate larval feeding responses. The cultivation of Earthworms has become common as a source of income, protein and essential amino acids, due to easy production, different sizes in different lifespans and low production cost (Ahmadifard *et al.,* 2016). Cow heart is also used as a main food for carnivorous aquarium fish such as Oscar, but compare to other types of food provides rapid drop in water quality due to contamination (Heidary and Akbary, 2013). Artificial feed can be formulated for aquacultural use, to suit different life stages, enjoy a constant quality, available in wide types and varieties but they are expensive, require refrigerated and dry storage and may create water pollution and undesirable growth due to decreased in digestion and absorption (Sharifian, 2015; Holt, 1993).

Live foods as a nutrition rich diet can swim in water column and stimulate larval feeding response. Also, their size suit the mouth size of fish larvae but they can potentially introduce fish disease (Das et al., 2012; Sharifian, 2015)

Artemia has been an ideal food due to ease of handling, its adaptation to different environmental conditions, non-selective filter feeding habit, enabling bioencapsulation with algae, antibiotics, vitamins, fast

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growth at high densities, high nutritive value, short generation time, high fecundity (Koru and Turkmen, 2014). The quality of fish color is very important in terms of business and has a direct relation to the price, consumer acceptance and sale (Supamattaya *et al*., 2005). Carotenoids are responsible for skin color in aquarium fish and muscle pigmentation in food fish. A combination of genetic, environmental, nervous, hormonal and rearing factors affect the storage of pigments in cultured fish (Fujii, 2000). The use of carotenoids in aquatic animals has become widespread due to their role in growth, immune stimulation, color and anti-oxidant, anti-cancer effects (Alishahi *et al*., 2014; Gupta *et al*., 2007). As fish cannot synthesize carotenoids *de nova*, there is a need to add carotenoids in the diet of cultured ornamental fish. Synthetic carotenoids such as astaxanthin are used extensively in cultured ornamental fish, but due to their high price and deteriorating effects on the environment, there is a great interest for natural available cheap alternative carotenoids (such as micro algal pigments, yeast extracts, marigold, capsicum, animal and vegetable derivatives) in aquafeed to obtain bright coloration in fish (Alishahi *et al*., 2014; Gupta *et al*., 2007). In this study, the effect of feeding with *Artemia urmiana*, Earthworm, Cow heart and concentrate diets on pigmentation and skin color of Oscar fish (*Astronotus ocellatus*) has been compared.

Materials and Methods

This study was carried out in 2018 by National *Artemia* Research Center in collaboration with the Azarmahi ornamental fish farm, East Azarbaijan, Iran. 360 Oscar fish with average initial weight ranging from 1.59 ± 0.10 to 1.70 ± 0.11 g were reared for 2 months under identical and controlled conditions. Fish were randomly divided in 4 feeding treatments each in 3 replicates and in twelve glass aquariums (120×60×45 cm). The height of 40 cm were watered (Each aquarium contains 30 fish). The treatments were as follows: treatment 1 (Concentrate diet) as control and treatment 2 (67% concentrate and 33% frozen earthworm), treatment 3 (67% concentrate and 33% frozen *Artemia urmiana*) and treatment 4 (67% concentrate and 33% frozen cow heart). Concentrate food (21 Beyza Cooperative Production Company) contains a maximum moisture content of 11%, crude protein of 46-54%, a crude fat of 11-15%. The frozen earthworm was purchased from Vermicomposting Nademi and stored in the -20 ºC refrigerator. The frozen *A. urmiana* biomass was obtained from National *Artemia* Research Center. Cow heart was purchased from the market and after fat separation was stored at -20 ºC until consumption. In all treatments, physico-chemical factors such as tempe-rature, pH, and dissolved oxygen were provided under optimum conditions. In order to prevent the accumulation of ammonia and other toxic compounds, uneaten food at the bottom of the aquariums were siphoned out daily before feeding. Appropriate oxygen levels were provided by aeration during the experiment period. The average temperature of the water was 28 ± 1 ºC and photoperiod of 12 h light: 12 h dark were maintained during rearing period.

Daily feeding was carried out in three meals at 8 a.m, 13 and 18 by 3% body weight until satiation. All fish fed with concentrate diet at meals of 8 a.m. and 18 and with concentrate diet, frozen earth worm, frozen *Artemia urmiana* and frozen cow heart as supplemen-tary diet at 13. The feed was planned to be used by fish for 5 minutes. To determine the total carotenoid and carotenoid pigmentation of the skin of Oscar fish, at the end of rearing period, 3 fish were randomly sampled from each treatment and after anesthesia with clove powder (150 mg/L), their skins were separated and transferred to laboratory in adjacent to the ice.

Survival and growth of fish

Growth indices (initial and final weight, initial and final length) were measured at beginning and end of the experiment. Daily mortality was recorded to determine the survival percentage.

Total Carotenoid Analysis

The carotenoid content of fish skin was extracted according to the method of Torrissen and Naevdal, 1984. Three fish were randomly sampled from each diet treatment at the end of culture and used for carotenoid analyses, which carried out in triplicate. The samples of 200-300 mg skin were collected from both sides between the abdominal and dorsal regions of the fish. These samples were transferred into 10-ml pre weighed glass tubes. The samples were then grounded in acetone containing 1.5 g of anhydrous sodium sulphate with a homogenizer; the extractions were reached up to 10 ml with acetone. The samples were stored for 3 days at 4 °C and then extracted three or four times until no more colors would be obtained. The solution was centrifuged at 5000 rpm for 5 min and then optical absorption was calculated and recorded with a WPA spectrophotometer in a wave length of 450 nm.

Measuring carotenoid pigments

The Oscar fish skin carotenoid pigments were measured according to Torrissen and Naevdal, 1984 adapted as follows:

Materials and solutions: Methanol, Tetra hydro furan (THF), Acetone, Hexane, Distilled water.

Method of preparation of standards: 1000 ml/ml of each standard including Astaxanthin, cantaxanthin and β-carotene was prepared in acetone and then the working standards were prepared by mixing these three standards in the range of 2.5-540 µg/ml.

Sample preparation method: All tubes were washed with acetone before use. All sample preparation steps were performed in a very low light environment. 50 mg of the sample was homogenized in 3 ml cold acetone and then was vortexed for 30 seconds and centrifuged for 5 minutes at 1500 rpm. 0.5 ml of distilled water and 2 ml of hexane were added to 2.5 ml of the supernatant, again vortexed for 30 seconds and centrifuged at 1500 rpm for 5 minutes. The supernatant (hexane) was transferred to a clean tube and dried under Nitrogen. 250 µl of methanol was added and after vortexification, 70 µL of i, was injected into a Younglin HPLC apparature equipped with a SP930D pump, a UV detector (730D), a reodyne injector, and an Autochro 2000 integrator. HPLC conditions include the moving phase: A methanol/Water: (3/97) and mobile phase B: methanol/THF/water: (3, 60 and 37) and gradient program were as Table S1.

The data were analyzed by SPSS 18 software. Analysis of variance at p= 0.05 was used to analyze data. Means were separated by Duncan Multiple Range Test.

Results

Biometric (Mean ± SE, n = 9) analysis of weight and length and survival rate of Oscar fish are sumerized in Table 1. Also, total carotenoid analysis results at the end of experiment (day 60) by spectrophotometric method at 450 nm wavelength are given in Table 2. Figure 1 shows the fish tested in different feeding treatments.

Tab. 1: Survival and growth (Mean ± SE) of Oscar fish fed different diets.

	Treatment			
Factors	**Con**	**Con+FEW**	**Con+FA.u**	**Con+FCH**
Initial weight (g)	1.59± 0.10^{a}	1.69± 0.11^{a}	1.66± 0.11^{a}	1.70± 0.11^{a}
Final weight(g)	4.60± 0.29^{a}	7.74± 0.47^{b}	7.56± 0.22^{a}	9.13± 0.48^{b}
Initial length (mm)	28.52± 1.24^{a}	28.94± 1.28^{a}	28.98± 1.28^{a}	28.80± 1.26^{a}
Final length (mm)	64.53± 1.49^{a}	73.19± 1.64^{b}	73.54± 1.40^{a}	78.78± 1.40^{b}
Survival rate (%)	94.47± 1.65^{a}	70.53± 2.67^{b}	93.43± 1.65^{c}	90.43± 1.33^{d}

- dissimilar letters in each column represent a significant difference in treatments (p <0.05) .
- Con= Concentrate, FEW= Frozen Earth Worm, FA.u= Frozen *Artemia urmiana*, FCH= Frozen Cow Heart

Tab. 2: Carotenoid pigments values (Mean ± SE, n=3) in Oscar skin of different treatments.

	Treatment			
Factors	**Con**	**Con+FEW**	**Con+FA.u**	**Con+FCH**
Total Carotenoid (mg /g)	6.31±0.30^{a}	5.17±0.09^{b}	9.17±0.10^{c}	7.42±0.10^{d}
Astaxanthin (ng/g)	413.68±1.54^{a}	148.05±1.24^{b}	503.63±1.88^{c}	70.48±0.99^{d}
Canthaxanthin (ng/g)	56.41±0.11^{a}	2.18±0.11^{b}	29.02±0.10^{c}	3.14±0.90^{d}
β-Carotene (ng/g)	209.68±1.17^{a}	16.34±0.11^{b}	73.40±0.43^{c}	3.45±0.11^{d}

- dissimilar letters in each column represent a significant difference in treatments (p<0.05).
- Con= Concentrate, FEW= Frozen Earth Worm, FA.u= Frozen *Artemia urmiana*, FCH= Frozen Cow Heart

The results of measuring carotenoid pigments in different treatments at the end of experiment (day 60) are shown in Table 2.

In spite of the fact that the highest final weight and length were observed from diet containing 67% concentrate and 33% frozen earth worm, the lowest survival rate were related to the same diet.

The results showed that in the skin of oscar fish, the highest levels of total carotenoids (9.17 ± 0.10 mg/g) and Astaxanthin (503.63 ± 1.88 ng/g) were obtained from diet containing 67% concentrate and 33% frozen *Artemia urmiana.* While, the highest levels of Canthaxanthin (56.41 ± 0.11ng/g) and β-Carotene (209.68 ± 1.17 ng/g) were observed in concentrate diet. There was a significant difference among different diets in this study ($p<0.05$). Although, fish fed with 67% concentrate and 33% cow heart showed higher final weight and final total length, Adding *Artemia urmiana* in the diet can improve total carotenoid and astaxanthin levels in the skin of Oscar fish. In all treatments astaxanthin was achieved the highest level of carotenoid pigmentation of Oscar skin.

Fig. 1: Apparent skin color comparisons in different dietary treatments (treatments from left to right: *Artemia*+Concentrate, Earthworm+Concentrate, Cowheart+Concentrate, Concentrate).

Discussion

Oscar fish are omnivorous but mainly carnivorous. Its food items included aquatic insects, crustaceans, invertebrates, small fish and vertebrates, fruits, algae, water plants; everything falls into water and prepared fish food (Yilmaz and Arslan, 2013; Consoli *et al*., 1992; Soares *et al*., 1986). Yilmaz and Arslan (2013) mentioned that live foods could increase growth rate but also may cause endoparasites. Also, cow heart should not be fed for long periods due to its contribution to fatty liver disease. Feeding with diet containing cow heart resulted in the highest total length and weight and the lowest survival rate and astaxanthin content in this study.

Carotenoid pigments have a positive role in metabolism of fishes (Segner *et al*., 1989). Although endocrine nervous systems control the color of fish, food sources with carotenoids are necessary to obtain good quality body coloration. Also, the effect of carotenoid sources on the skin color of fishes is species specific. Fish enjoy different ways for chromatin metabolisms, so transfer of carotenoids in tissues of fish are different (Chatzifotis *et al*., 2004). Mandal *et al*. (2010) showed that live organisms were utilized more efficiently than the artificial diet and provided more brightly color in fantail guppy (*Poecilla reticulate*). Also, Seidgar *et al*. (2015) mentioned that live food supplemented feed enhanced carotenoid pigments in goldfish (*Carassius auratus*) comparing with concentrate diet and significant difference were observed using supplemented feed with frozen fairy shrimp, frozen *Artemia* and alone concentrate diets. In this study, diets containing *Artemia urmiana* and cow heart created the highest and lowest astaxanthin levels in Oscar skin, respectively. Torrisen *et al*. (1989) mentioned that accumulation and absorption of astaxanthin in fish is more than other carotenoids that is in agreement to our findings on carotenoid pigments of Oscar fish skin with different diets. Shapoori *et al*. (2012) revealed that Oscar fish are sensitive to natural and synthetic edible carotenoids. Astaxanthin from different sources was used for coloration of the skin in different fish species including red sea bream, cichlid, Australian snapper, Albino Oscar, gilhead sea bream, *Heros severus*, *Poecilla reticulata* (Kop and Durmaz, 2008; Booth *et al*., 2004; Gomes *et al*., 2002; Shapoori *et al*., 2012; Alishahi *et al*., 2014; Mirzaee *et al*., 2012). As synthetic pigment sources are expensive and add about 10-15% to the cost of feed, using natural carotenoids with high nutritive quality that are environmentally friendly is preferred (Mirzaee *et al*., 2012; Ghiasvand and Shapouri, 2006).

In this study, astaxanthin was the most carotenoid pigment in all treatments compared to cantaxanthin and β-Carotene with the highest (503.63 ± 1.88 ng/g-treatment 3) and the lowest (70.48 ± 0.99 ng/g-tratment4) levels in Oscar skin. Therefore, it can be concluded that diets containing *Artemia* could improve total carotenoid and astaxanthin levels of Oscar fish skin, whereas, diets containing earth worm and cow heart create the lowest total carotenoid and astaxanthin levels, respectively. The highest canta-xanthin and β-Carotene levels were obtained in concentrate diet, whereas the lowest levels of these pigments were from diets containing earth worm and cow heart, respectively. Although the highest final weight and final length were obtained from diet containing cow heart, long term consumption of cow heart should be controlled due to probably fatty liver problem. This study showed that totally, *Artemia* as a food source for Oscar fish will produce the highest levels of pigmentation in the skin. On the other hand, *Artemi*a has the lowest side effects on fish. Therefore, it is suggested to use *Artemia* as a part of diet to increase the interest and selling of ornamental fish producing farms.

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