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Effects of astaxanthin and *Dunaliella salina* on skin carotenoids, growth performance and immune response of *Astronotus ocellatus*

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Abstract In this study, *Astronotus ocellatus* were fed with food supplemented with *Dunaliella salina* (a natural β-carotene source) or astaxanthin (synthetic pigment), and their effects on fish skin carotenoids, growth indices and immune responses were evaluated. 135 *A. ocellatus*, weighing 25.6 ± 0.6 g, were randomly divided into three groups with three replicates (15 fish in each replicate). Group 1 and 2 were fed with a diet supplemented with 200 mg/kg astaxanthin or *D. salina*, respectively. Control group received the same diet without supplemented carotenoids. After 50 days, the growth indices were compared with the groups. Blood, mucus and skin samples were taken from each group. The immunological parameters of dietary *D. salina* and astaxanthin were studied in terms of serum and mucus lysozyme and bactericidal activity, as well as resistance against *Aeromonas hydrophila* infection. Carotenoid content of skin was assayed as well. Results showed that growth indices increased significantly in fish fed diet supplemented either with astaxanthin or *D. salina* ($P < 0.05$). Serum and mucus lysozyme and bactericidal activity increased in *D. salina* and astaxanthin groups ($P < 0.05$). Skin carotenoid content was statistically higher in astaxanthin group (6.48 ± 0.84 mg g^{-1}) and *D. salina* group (4.89 ± 0.83 mg g^{-1}) compared with the control group (3.05 ± 0.32 mg g^{-1}) ($P < 0.05$). Rate of mortality following the challenge with *A. hydrophila* was significantly lower in *D. salina* group (50 ± 10) and astaxanthin (56.7 ± 5.8) group compared with control group (76.7 ± 5.8) ($P < 0.05$). Conclusively, *D. salina* and astaxanthin as a food additive can affect positively the growth and immunological parameters as well as skin carotenoid of *A. ocellatus*.

Keywords *Astronotus ocellatus* · *Dunaliella salina* · Astaxanthin · Immunological parameters · Growth indices

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Introduction

As fish cannot synthesize their own pigments de novo, the pigments, which are synthesized by plants, algae and microorganisms, need to be incorporated in their diet. One of the greatest challenges in the ornamental fish industry is to replicate the accurate natural colour of the fish in the captive environment. In addition to affecting the skin colour of fish, these pigments also have other positive influence on strengthening the immune system and stimulating the growth indices of fish (Tanaka et al. 1976; Tacon 1981). There are four main pigment groups that give colour to the skin and tissues of animals and plants, namely melanines, purines, pteridiums and carotenoids. Carotenoids, which dissolve in fat, give the skin the yellow and red colours. They also give the orange and green colours to the egg, skin and flesh of many fish (Fuji 1969). Carotenoids, which are produced primarily by phytoplankton and plants, are divided into two groups as carotenes and xanthophylls. Although more than 600 carotenoids in nature have been defined, only a few of them are used in animal feeding, pharmaceuticals, cosmetics and food colouring (Bricaud et al. 1998, 1998). Carotenoids, particularly those that are vitamin A precursors, have received increasing attention due to their reported health benefits in mammals (Ong and Tee 1992; Krinski 1991). Microalgae, which are important in the production of larval fish because of their nutritive ingredients, can also be used as a natural pigment source in fish foods. The use of microalgal biomass has been recently investigated with regard to its potential as a colouring agent (Gourveia et al. 1997; Raymundo et al. 2005), but the use of synthetic pigment sources is more common because they are easy to obtain (Sales and Janssens 2003).

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a carotenoid from dietary origin that fish cannot synthesize. The wild fish derive astaxanthin through their prey organisms, while in aquaculture (aquariums and fish farms) astaxanthin is added to the feed in the form of nature identical synthetic beadlets (Bjerkeng et al. 1999). Although astaxanthin is an effective carotenoid, it is too expensive. The cost of astaxanthin supplementation represents about 10–20 % of the ingredient cost of fish feeds (Baker et al. 2002).

In some fish species such as ornamental fish and salmonids, skin and fillet colour constitutes an important quality parameter, and improving the fish colouration increases directly economical value of fish (Kop and Durmaz 2008). Since fish, like other animals, is unable to synthesize carotenoids de novo (Tacon 1981), skin colour is highly dependent on the carotenoids present in the diet.

Dunaliella salina is a type of halophile green microalgae especially found in hypersaline waters. It is well known for its antioxidant activity and large amount of carotenoids (Borowitzka and Borowitzka 1988). *Dunaliella* spp. are grown as a food source in aquaculture, and *D. salina* is the richest algal source of *β*-carotene and glycerol (Ben-Amotz and Avron 1983; Raja et al. 2007). *D. salina* can produce *β*-carotene up to 14 % of its dry weight under conditions of high salinity, light and temperature, even under nutrient limitation. *D. salina* as a natural source of *β*-carotene can improve skin and fillet pigmentation in fish.

In last decades, ornamental fish industry has developed and become more popular in Iran society. *Astronorus ocellatus* is a market-friendly and preferred ornamental fish in different countries especially in Iran. One of the most attractive features of this species is wide spectrum of body colour. Unfortunately, bacterial infection (especially motile *Aeromonas* infection) is very common health problem among this species in Iran, which leads the aquarists to use large amount of antibiotics and as a result, they are encountering bacterial resistance to antibiotics plus increase of production cost, so substances that either

induce skin pigmentation or stimulate immune response and resistance against bacterial infections of this species can be the best choice as food additive.

Price and efficiency are indicators to choose the sources of pigment that can be used in fish feed. Although carotenoids are known substances to enhance immune function and disease resistance in higher animal (Bendich and Shapiro 1986; Jyounchi et al. 1993), little information is available for fish and no data about *A. ocellatus*. Therefore, in this study, effects of dietary astaxanthin and *D. salina* on skin carotenoids, growth performance, immune responses and disease resistance of *A. ocellatus* were evaluated.

Materials and methods

Fish

Albino Oscar fish (*A. ocellatus*) (weighing 25.6 ± 0.6 g), having similar skin colour, were purchase from an ornamental fish hatchery in suburb Ahvaz, Iran. Fish were transferred in oxygenated plastic bags to the wet laboratory of Shahid Chamran University, Ahvaz, Iran. Fish were fed with commercial granulated food (Biomar company, France), without any other supplementation for 1 month. After that, fish with similar skin colour were selected for the experimental study.

To acclimate and ascertain the constant colour of fish, impure fish were omitted, after 1-month period, in initial study.

Dunaliella salina preparation

Dunaliella sallina coded as *Dunaliella* M1 an isolate from Uremia Lake, Iran, was cultivated within a 14-l photobioreactor using the modified Junson medium described by Hejazi et al. (2010). NaCl concentration of the medium was 1.5 M. The photobioreactor was illuminated by Osram lamps (SPOT R80 NATURA E27/ESW) with the average light intensity of 400 $\mu mol\ m^{-2}\ s^{-1}$. The temperature and pH were 26 ± 0.5 °C and 7.5 ± 0.3, respectively. After 7 days, stress condition was applied for over-production of β-carotene. During the stress stage, NaCl concentration of the medium and the average light were increased to 3 M and 2,000 $\mu mol\ m^{-2}\ s^{-1}$, respectively. The cells were harvested after 8 days when the colour of the algae in photobioreactor turned to orange(Hejazi et al. 2010). *D. sallina* was harvested, lyophilized and stored in refrigerator until use. Then 200 mg/kg of alga mixed in a small amount of water and sprayed on commercial granulated fish food, and in order to prevent wasting of the alga in the water during feeding time, the surface of feed granules was coated with 5 % gelatin (Merk, Germany) then spread on a tray and air-dried in room temperature.

Astaxanthin (CAROPHYLL® 135 pink, DSM, Basel, Switzerland) at concentration of 200 mg/kg food was dissolved in distilled water and sprayed on the thin layer of basal food then air-dried in room temperature.

Sampling procedure

A total of 135 apparently healthy Oscar fish, which adapted to wet laboratory situation randomly distributed in three equal groups, in triplicate, following a completely randomized design (CRD) Group 1 and 2, were fed with a diet supplemented with 200 mg/kg

astaxanthin (CAROPHYLL® pink, DSM, Basel, Switzerland) and *D. sallina*, respectively. Control group received the same food without supplementation. Fish sex was not taken into consideration because sex affects Oscar fish colour just after sexual maturation, and experimental fish were immature. Feeding trial was conducted for 50 days. The fish of each replicate weighed in day 0 and day 50, at the end of experiment. Fish were anaesthetized with 100 ppm MS_{222}, and blood samples were taken randomly from six fish of each replicate of experimental groups. Sera samples were separated via centrifugation at 3,000g for 10 min and stored at −20 °C until used. Skin sample (around 0.5 g) of lateral flank near the dorsal fin was taken from each fish after taking the blood and euthanasia of fish with 1,000 ppm MS222 (Choubert and Storebakken, 1989).

Mucus sample preparation

After anaesthesia with 100 ppm MS222, the fish were placed with the ventral side of the body facing downward and cutaneous mucus from the dorsal side of the fish was collected by a cell scraper and transferred to a tube. Samples were kept on ice during transportation to the laboratory and kept frozen to avoid bacterial growth and degradation at −80 °C until used. The mucus was centrifuged (3,000 rpm for 10 min), and supernatant was used to analyse the lysozyme activity and antibacterial activity of mucus.

Lysozyme and bactericidal activity

Serum and mucus lysozyme activities were measured as described by Ellis (1990). Briefly, 10 µl of serum/mucus was mixed with 200 µl of a lyophilized *Micrococcus lisodeichticus* (Sigma) suspension at 0.2 mg ml^{-1} in 0.05 M sodium phosphate buffer (pH 6.2). The mixture was incubated at 27 °C, and its optical density (OD) was detected after 1 and 6 min at 530 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech, Netherland). One unit of lysozyme activity was defined as the amount of enzyme that produced a decrease in absorbance of 0.001 min ml^{-1} serum.

The method used for serum bactericidal activity was followed a modified version of that adopted by Kajita et al. (1990). Briefly, the serum samples were diluted three times with 0.1 % gelatin-Veronal buffer, and mucus samples were used without dilution. *Aeromonas hydrophila* (live washed cells) were suspended in the same buffer, and bacteria were mixed at a ratio of 1:1 and incubated for 60 min at 25 °C and continuously agitated. The number of viable bacteria was then calculated by counting the resultant colonies from the incubated mixture on tryptic soy agar after incubation for 24 h in duplicate.

Growth performance indices

The growth indices including weight gain percentage (WGP), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (FER) were calculated according to the following equations:

$$\text{WGP } (\%) = 100 \times [\text{Average final weight (g)} - \text{Average initial weight (g)} / \text{Average initial weight (g)}].$$

$$\text{SGR } (\%/\text{day}) = 100[\text{In final body weight (g)}$$
$$- \text{ ln initial body weight (g) } / \text{ experimental period (day)}].$$

$$\text{FCR } = \text{Feed intake (g)}/\text{weight gain (g)}.$$

$$\text{PER } = \text{Wet weight gain (g)}/\text{protein intake (g)}.$$

Total carotenoids

Skin total carotenoids of five fish in each replicate were determined at the end of the experiment by using spectrophotometer (Choubert and Storebakken 1989). After that, 10 mg of dry skin sample was homogenized with the addition of 5 ml acetone (98 %, Merck Germany) and centrifuged for 10 min at 3,500 rpm.[1] Supernatant of the samples was read at 475 nm wavelength on the spectrophotometer (JENWAY 6305).

Challenge study with *A. hydrophila*

At the end of experiment (day 50) of feeding, 0.1 ml of pathogenic isolates of *A. hydrophila* in sterile PBS (2×10^7 CFU ml^{-1}) were intraperitoneally injected to ten fish per replicate. Mortality was recorded for 10 days. Tissues were taken from the dead fish for bacteriological culture to confirm *A. hydrophila* as the cause of death (Schaperclaus et al. 1992).

Statistical analysis

Statistical analysis consisted of one-way ANOVA, using the probability level of 0.05 for rejection of the null hypothesis. After ANOVA, significant differences between means were determined by Dancan's multiple range test. All statistical analysis was performed using SPSS version 16.0.

Results

Growth performance

Significant differences were seen between astaxanthin- and *D. salina*-treated groups and control in all evaluated growth indices ($P = 0.015$). These data indicate a deep influence of carotenoids on growth performance. Although PER and FCR did not vary markedly between astaxanthin and *D. salina* groups ($P = 0.0735$), WGP and SGR showed significant difference between these two groups ($P < 0.01$) (Fig. 1).

Lysozyme and bactericidal activity

The serum and mucus lysozyme activity was significantly increased with astaxanthin and *D. salina* groups when compared with the control group (Fig. 2). Serum and mucus

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[1] Rounds per minute.
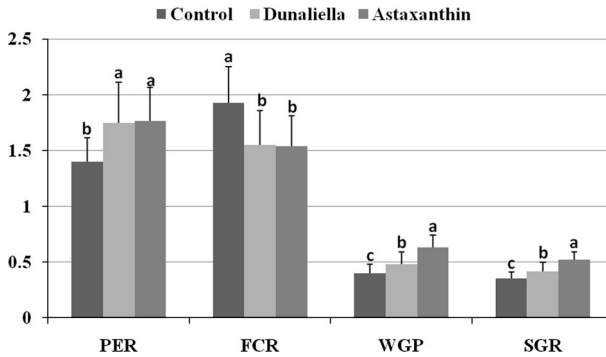
Fig. 1 Effect of astaxanthin and *Dunaliella salina* on growth performance of *Astronorus ocellatus*: Protein efficacy rate (PER), food conversion ratio (FCR), weight gain percentage (WGP) and specific growth rate (SGR) of *A. ocellatus*. $N = 45$ and values are mean ± SD
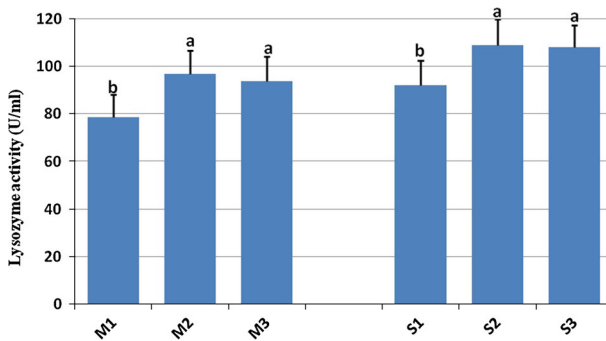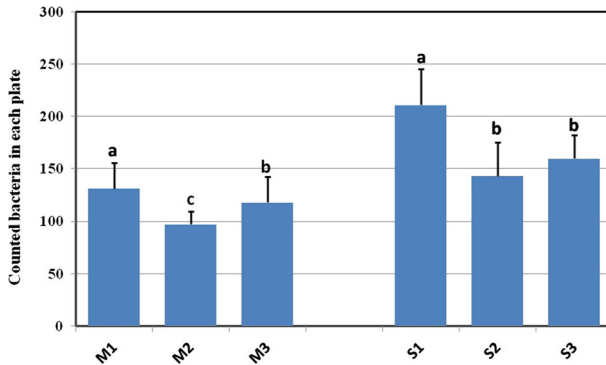

Fig. 2 Effect astaxanthin and *Dunaliella sallina* on Lysozyme activity of serum and mucus of *Astronorus ocellatus*: M1 and S1: mucus and serum samples of fish fed with free supplementation food. M2 and S2: mucus and serum samples of fish fed with 200 mg/kg *D. salina* supplemented food. M3 and S3: mucus and serum samples of fish fed with 200 mg/kg astaxanthin supplemented food. Different letters show significant difference ($P < 0.05$) between groups. $N = 15$ and values are mean ± SD

Lysozyme activity between astaxanthin and *Dunaliella* did not show significant difference ($P = 0.115$).

The number of the counted bacterial colonies was lower with *D. salina* group followed by astaxanthin group when compared with the control group ($P < 0.05$) as shown in Fig. 3. Serum bactericidal activity did not show markedly difference between astaxanthin and *D. salina* group ($P = 0.0675$), but Mucus bactericidal activity in *D. salina* group was significantly higher than astaxanthin group ($P < 0.01$).

Skin carotenoid content

Supplementation of the food with *D. salina* and astaxanthin significantly affected the skin carotenoid rate of A. *ocellatus* (Fig. 4) ($P = 0.023$). Skin carotenoids were higher with astaxanthin group followed by *D. salina* group when compared with the control group.

Fig. 3 Effect of astaxanthin and *Dunaliella sallina* on bactericidal activity of serum and mucus of *Astronorus ocellatus*: Legends are the same as on Fig. 2
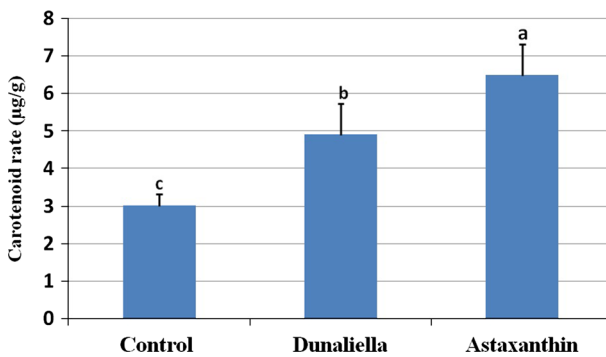
Fig. 4 Skin carotenoid content of *A. ocellatus* fed with basal diet (control) and 200 mg/kg *D. salina* or astaxanthin supplemented food for 7 weeks. Legends are the same as on Fig. 2

Disease resistance

The mortality was observed up to 10 days. The clinical signs observed in fish were swollen abdomen, reddish vent, exophthalmia and loss of equilibrium. Percentage cumulative mortality of *A. ocellatus* after challenging with *A. hydrophila* in different experimental groups showed that the highest mortality was recorded in control group which was significantly ($P < 0.01$) higher than *D. salina*- and astaxanthin-treated groups. Lowest mortality was observed in astaxanthin group (Fig. 5).

Discussion

Although no adverse effects of astaxanthin have been reported and that is permitted to be used in many countries, there is a great tendency for finding alternative similar materials, because astaxanthin derivates are expensive and add about an extra 10–15 % to the cost of feed. Microalgae (especially *Dunaliella*) are of the most favourite of these alternative materials both because of its nutritive quality and being a good source of carotenoid

Fig. 5 Cumulative mortality (%) of *A. ocellatus* following an experimental *Aeromonas hydrophila* infection, *different letters* show significant difference ($P < 0.01$) between groups. Values are mean ± SD
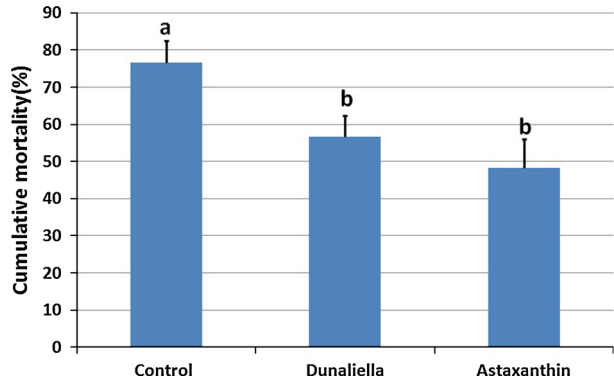
(Becker 1994). The two dietary supplements (astaxanthin and *D. salina*) affected growth indices in *A. ocellatus*. SGR, PER, FCR and WGP were promoted in fish fed with astaxanthin and *D. salina* supplemented diet compared with control. Nevejena et al. reported an increase in growth following administration of *D. salina* as a natural source of *β*-carotene in *Argopecten purpuratus* that is in agreement with our results. But Amar et al. (2004) and Choubert et al. (2006) reported no change in growth indices following administration of supplemented food with not only natural but synthetic carotenoid sources in rainbow trout.

Alishahi et al. (2014) reported similar growth stimulation effects of *D. salina* in *Heros severus*.

In fish, the effectiveness of carotenoid source in terms of deposition and physiological function is species-specific. In addition, all fish species do not possess the same pathways for the metabolism of carotenoids; therefore, there is no universal transformation of carotenoids in fish tissues (Kop and Durmaz 2008).

Natural *β*-carotene sources have been reported to act as a potent immunostimulants in fish (Raymundo et al. 2005; Supamattaya et al. 2005). In this study, some non-specific immune responses (i.e. serum and mucus Lysozyme activity, serum and mucus bactericidal activity and resistance against bacterial infection) were significantly increased following administration of astaxanthin and *D. salina* supplemented food. The lysozyme activity is an important indicator of the immune defence in both invertebrates and vertebrates (Ellis 1990). The lysozyme causes hydrolysis of the *N*-acetylmuramic acid and *N*-acetylglucosamine that are constituents of the peptidoglycan layer of bacterial cell wall and activation of the complement system and phagocytes by acting as an opsonin (Magnado'ttir 2006). Lysozyme is secreted by leucocytes and is an indicator of leucocyte activity, increasing concomitantly with phagocytic activity (Keshav et al. 1991). In this study, food supplemented with 200 mg/kg astaxanthin/*D. salina* significantly increased the serum and mucus lysozyme activity and stimulated the immune response in *A. ocellatus*. The increased lysozyme activity has been reported after supplementing the fish feed, with astaxanthin and *D. salina* in *H. severus* (Alishahi et al. 2014) rainbow trout (Amar et al.2004), *Aloe vera* extract (Alishahi et al. 2010) and injection of vaccines (Alishahi and Buchmann 2006).

β-carotene is a vitamin A precursor and a quencher of singlet oxygen; thus, it could affect the immune system through both the antioxidant and retinoid pathways (Krinski, 1991). *D. salina* also contains large amounts of 9-cis *β*-carotene, which has been shown to be more potent than all-trans *β*-carotene in enhancing cell–cell interaction through gap

junctions (Zhang et al. 1992). Thus, the augmentation of lysozyme activity by these carotenoid sources could be via its stimulation of phagocytic cells as is the case with most immunostimulants (Sakai 1999). Astaxanthin is also a precursor of vitamin A in fish (Matsuno 1991) and possesses free radical scavenging ability.

Our results also showed that supplemented diet with astaxanthin and *D. salina* significantly increased the serum and mucus bactericidal activity against *A. Hydrophila* ($P < 0.05$). Alishahi et al. (2014) reported increased lysozyme activity following oral administration of 100 and 200 mg/kg *D. salina* in *H. severus*. Divyagnaneswari et al. (2007) in tilapia and Kajita et al. (1990) in rainbow trout reported an increase in serum bactericidal activity after administration of herbal extracts as immunostimulants.

It is important to estimate bacterial resistance of treated fish to determine the efficiency of an immunostimulant. In the present study, the lowest mortality after challenge was registered in *D. salina* group followed by astaxanthin group, which was significantly lower than the control group. The higher survival rate of *D. salina* group may be because of presence of other beneficial substances in algae structure that are not available in pure astaxanthin source.

The study showed that supplementation of astaxanthin and *D. salina* had a positive influence on the survival of *A. ocellatus* by resisting the *A. hydrophila* infection. Based on the results of the present study, it can be concluded that growth indices are affected by dietary supplementation of *D. salina* and astaxanthin. It appears that *D. salina* and astaxanthin are effective in enhancing some of the non-specific immune responses in *A. ocellatus*. Moreover, mostly lower humoral responses observed with natural sources in some parts of this study compared with synthetic sources may be due to the isomers and esterified forms of carotenoids found in the former which may have a lower bioavailability. Nevertheless, natural carotenoids from *D. salina* are promising and advantageous sources of dietary supplements for fish, not only for pigmentation purposes but also for the health benefits they provide.

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