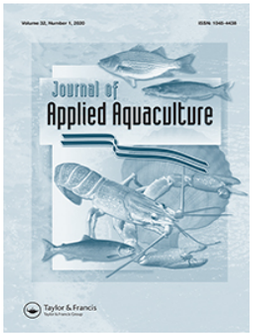

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Growth performance and color enhancement of goldfish, *Carassius auratus*, fed diets containing natural dyes extracted from annatto (*Bixa orellana*) seeds

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ABSTRACT

The present study was conducted to evaluate growth performance and color enhancement of goldfish, *Carassius auratus*, fed diets containing 0, 50, 100, 200, and 250 mg kg^{-1} diet of annatto dye (AD) for 60 days. The survival rate was significantly higher in fish fed 100, 200, and 250 mg AD kg^{-1} diet over than these fed control and 50 mg AD kg^{-1} diet ($p < 0.05$). AD significantly (p <0 .05) increased the pigmentation in the skin and caudal fin of goldfish in a concentration dependent manner (R^2 = 0.995, 0.997). The highest amount of total carotenoid deposition in fish skin and fins were given by diets containing 200–250 mg AD kg^{-1} diet. The highest redness (a*) of 43.21 and yellowness (b*) of 12.53 were obtained by 250 and 50 mg AD kg^{-1}, respectively. The present results show that AD can be successfully used as an alternative natural carotenoid source in goldfish diets at levels of 200–250 mg AD kg^{-1} diet.

KEYWORDS

Goldfish; Carassius auratus; carotenoid; annatto dye; pigmentation

Introduction

Ornamental fish farming is one of the commercially important sectors in the aquaculture industry all over the world. Beside the size of the fish, body shape and coloration (skin pigmentation) are vital characters, which directly affect the marketability of any ornamental fish. Carotenoids, which are lipids soluble pigments, are responsible for the skin color of fish. Level of carotenoid directly determines the commercial value of fish (Gouveia and Rema 2005; Liang et al. 2012; Paripatananont et al. 1999). It also influences fish growth, metabolism, and reproduction (Miki 1991). Fish are unable to perform de novo synthesis of carotenoids (Goodwin 1984) and therefore rely on the dietary supply of pigments to achieve their natural pigmentation.

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Goldfish, *Carassius auratus*, is one of the most popular freshwater ornamental fish, which has a high market value (Yanar et al. 2008). This fish must be pigmented to have an orange-red color to achieve good consumer acceptance (Gouveia and Rema 2005). Goldfish are generally reared in eutrophic ponds, with a high level of Chlorophyta and Cyanobacteria that are rich sources of carotenoids (Moreira et al. 2011). Goldfish cultured in such environments exhibit an excellent intensive coloring, nevertheless, if carotenoid sources are not included in the fish diets, and this coloration will not be achieved under intensive rearing conditions (Yanar et al. 2008). Therefore, skin pigmentation of goldfish in aquaria has been accomplished by supplementing their diets with synthetic or extracted carotenoids, such as zeaxanthin, lutein or astaxanthin (Matsuno, Matsutaka, and Nagata 1981; Ohkubo et al. 1999; Paripatananont et al. 1999). However, recent efforts have focused on natural compounds as alternative sources to synthetic carotenoids because of concerns on the use of synthetic additives as well as the high cost of pigmented feeds (Gupta et al. 2007). It has been reported that natural carotenoid sources, such as red yeast, *Xanthophyllomyces dendrorhous* (Xu et al. 2006), *Spirulina* (Kiriratnikom, Zaau, and Suwanpugdee 2005), *Chlorella vulgaris, Haematococcus pluvialis* and *Arthrospira maxima* (Gouveia et al. 2003), enhance the pigmentation of goldfish.

Annatto (*Bixa orellana*) is a tropical shrub originated from the American continent that bears an inedible, red fruit containing about 50 red seeds. The extract of annatto seeds are characterized by a high content of red pigments with a high absorption coefficient in the visible part of the solar spectrum. Among all natural colorants, annatto ranks second in economic importance worldwide and is widely used in industries such as textiles, varnishes, cosmetics, tattoos, and medicinal purposes (Scotter 2009; Yolmeh, Habibi Najafi, and Farhoosh 2014). The amont of total carotenoids in annatto seed was reported about 4.5–5.5% of seed weight (Giridhar, Venugopalan, and Parimalan 2014). The pericarps of the annatto seed contain a high concentration of carotenoids, and bixin makes up between 70 and 80% of the total mass of these carotenoids (Gomezortiz et al. 2010). In addition to bixin, a smaller quantity of many other carotenoids and related compounds are also present (Mccullagh and Ramos 2008). These include methyl bixin, norbixin, all-E- geranylgeraniol, geranylgeraniol esters phytofluene, neurosporene and phytoene (Figure 1). The annatto seed extract also has documented antioxidant and antimicrobial properties (Yolmeh, Habibi Najafi, and Farhoosh 2014). Previously we have purified bixin from AD and showed bixin based diet can enhance the skin color and pigmentation in goldfish (Dananjaya et al., 2017). Based on our results we continue the present study to investigate whether AD could have similar effects (enhance the skin color and pigmentation) on goldfish which may be useful to develop an inexpensive carotenoid source for fish diet formulations.

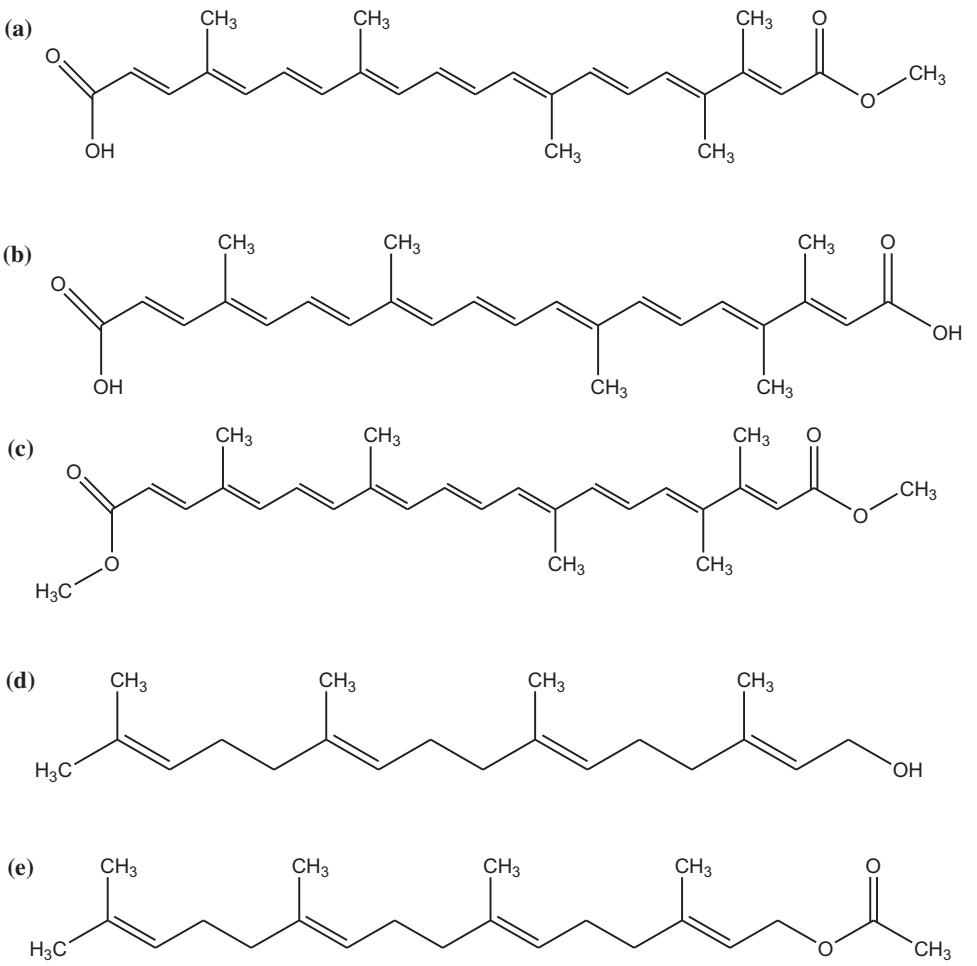

Figure 1. Chemical structures of the main carotenoid compounds in annatto dye. (a) bixin; (b) ethyl bixin; (c) nor-bixin (d); geranylgeraniol and (e); geranylgeraniol esters.

In the present study, we applied the ultrasound assisted extraction procedure to extract AD (from annatto seed) with a higher recovery rate. Four diets were formulated by varying levels of AD and tested with goldfish to evaluate its effect on the growth and enhancement of skin and fin coloration. Finally, the optimum AD concentration was determined for formulating feed to obtain effective color enhancement in goldfish.

Materials and methods

Experimental design

Three months old red variety of goldfish, *Carassius auratus*, were obtained from a local commercial breeder and kept under quarantine conditions for three weeks and then acclimatized to the experimental conditions in aquarium system

for two weeks before the commencement of the experiment. During this period, fish were fed by a control diet (CD).

A total of 315 fish (7 treatment X 3 replicates x 15 fish/tank) with an average body weight 7–11 g and similar in color were randomly distributed in 21 tanks (30 x 30 × 60 cm^3) at a stocking density of 15 fish/tank. Six experimental diets with different levels of AD (0, 50, 100, 150, 200, and 250 mg/kg) were prepared and labeled as CD, 50 AD, 100 AD, 150 AD, 200 AD, and 250 AD, respectively. A commercial color feed (CCF) containing astaxanthin was used as the seventh treatment to compare the coloration of fish in aquarium conditions. Fish were fed ad libitum with one of the tested diets in triplicates twice a day for 60 days. Feed consumption was recorded daily. Body weight (g) of fish were measured initially and then 10 days at the intervals. Total carotenoid content of the skin and the fin of fish (n = 3) from each tank were analyzed at 0, 20, 40, and 60 days. Uneaten feed and fish faces were siphoned out daily and water in the aquarium was changed at a rate of 60% volume per day. Water temperature and pH were determined daily with a glass electrode (Thermo Orion, Beverly, Massachusetts, USA). Total ammonia (un-ionized ammonia) (ng L^{-1}) was measured by an indophenol method and nitrite was measured (ug L^{-1}) weekly by an azo method (Boyd and Tucker, 1992). Tanks were aerated continually and natural photoperiod (12 h: 12 h light – dark cycle) was maintained in the aquarium during the experimental period.

Annatto dye extraction

The annatto dye was extracted according to the ultrasound-assisted extraction method according to Yolmeh, Habibi Najafi, and Farhoosh (2014) with some modifications. First, annatto seeds (50 g) were soaked in n-hexane (Sigma, USA) for 6 hours in order to remove oils and defatted seeds were used for dye extraction. Samples were placed in capped glass tubes and mixed with chloroform (400 mL, Sigma, USA) and then immersed in a water bath at 50°C. The ultrasound-assisted extraction process was performed using ultrasonic device (20 kHz, 550 W, Misonix, Germany). The working frequency, power and time were fixed at 20 kHz, 200 W, and 12 min, respectively. After ultrasonic extraction, the extracts were filtered through Whatman filter paper (No.1) and then vacuum-dried to make dye powder. The obtained powder was weighed and the mass ratio (powder to seeds) was taken into account as the extraction yield. Consequently, the extraction yield was calculated according to the following formula; Extraction yield % = (W1 -W2)/W1 X100, where W1 = weight of powder and W2 = weight of annatto seeds.

Diet preparation

The control diet was formulated using fish meal, soybean meal, wheat flour, wheat gluten, meat meal, coconut oil, fish oil and vitamin-mineral mixture as

main ingredients, and AD was added in different amounts (0, 50, 100, 150, 200 and 250 mg/kg) to each diet to prepare other five experimental diets. Dietary feed ingredients were ground using a laboratory grinder and then blended into homogenous dough matter by adding water and pellets that were made by pressing through a die of 3 mm diameter in a grinding machine. Then, prepared pellets were dried and stored in plastic containers in a freezer at −20 °C until taken for the feeding trial. The formulation and proximate composition are given in Table 1. Proximate compositions of formulated diets were determined according to analytical procedures of Association of Official Analytical Chemists (AOAC) (2005). Dry matter was calculated after drying in an oven at 105 °C to a constant weight; ash content was determined by incineration in a muffle furnace at 550 °C for 12 h; crude protein (N- 6.25) by the Kjeldahl method after acid digestion; amount of crude fiber was analyzed after digestion with H_2SO_4 and NaOH. Crude lipid content was measured by petroleum ether extraction in a Soxlet apparatus for 40 min in a Tecator HT6 equipment. Triplicate samples of each ingredient or diet were used for each analysis.

Analysis of carotenoid content

The carotenoid content was extracted according to the method of Torrissen and Naevdal (1984), with some modifications. Sample (skin or fin) of 200–1000 mg were taken from both sides of the abdominal and dorsal regions of the fish, being

Table 1. Feed ingredients and nutrient composition (% dry weight) of the control diet.

Ingredients	% dry weight
Fish meal	45
Wheat flour	30
Soy bean meal	7.5
Wheat gluten	5.5
Meat meal	2.5
Coconut oil	3.5
Fish oil	3.5
Vitamin-Mineral premix	2.5
Proximate composition	
Crude protein	38 ± 1.6
Crude lipid	12 ± 0.8
Dry matter	85 ± 2.4
Crude fiber	1.4 ± 0.5
Ash	10 ± 0.6

Each value is mean of three samples.

[a]Vitamin-Mineral premix consisted of (mg/kg) Vitamin A 500,000 IU, Vitamin D3 100,000 IU, Vitamin E 3,000 IU, Vitamin B1 3,000 mg, Vitamin B2 100 mg, Vitamin B6 200 mg Vitamin B12 1 mg, Vitamin C 2,000 mg, Vitamin K3 200 mg, Pantothenic acid 500 mg, Nicotinic acid 1,000 mg, Inositol 1,000 mg, Choline chloride 10,000 mg, Folic acid 100 mg, Biotin 2 mg, Dl-methionine 1,000 mg, Zinc sulfate 5,000 mg, Ferrous sulfate 4,000 mg, Copper sulfate 1,000 mg, Cobalt sulfate 250 mg, Magnesium oxide 1,000 mg, L-Lysine 1,000 mg, DC Methionine 1,000 mg. Fish meal with 66% crude protein level (TripleNine Group A/S, Denmark), and Soy bean meal with 46% crude protein content (The Delong Co.Inc, USA) was used.

careful to remove adhering adipose tissue. The grounded sample was transferred to 10 mL pre-weighed glass tubes containing 10 mL acetone (98%, Merck, Germany) and 1.5 g of anhydrous sodium sulfate. It was homogenized using a Bio homogenizer (Biospec and Samro, USA). The samples were stored for one day at 4°C, and then extracted with acetone, two or three times until no more color could be obtained. The solutions were centrifuged at 3500 rpm for 10 min, and then absorptions were measured at 476 nm by a spectrophotometer (Hach DR/4000U Spectrophotometer, USA). A similar method was adapted for the analysis of total carotenoid in the feed samples, but anhydrous sodium sulphate was not used and absorption was measured at 450 nm (Ramamoorthy et al. 2010).

Analysis of skin color

Skin color analysis was performed by reflectance spectroscopy. The color parameters of L* (lightness), a* (greenness & redness), and b* (blueness & yellowness) were obtained according to the CEB lab criteria using a portable 'Minolta colorimeter' (CR-300, AE.PT.01) calibrated towards a white standard. The color of the dorsal region of the fish skin from each tank was measured at the beginning of the experiment and also the end of the experimental period of 60 days.

Analysis of biological indices

Biological indices (specific growth rate, weight gain and feed conversion ratio) were obtained according to Gouveia et al. (2003) and Kalinowski et al. (2005).

Specific growth rate (% weight gain/day) = [(Final weight-initial weight)/number of days] *100.

% SGR

Weight gain (WG; g) = Final weight-initial weight

Feed intake = feed intake (g) per 100 g fish for the period

Feed conversion ratio (FCR) = Feed intake/average daily weight gain

Statistical analysis

All the data were expressed as the mean ± SD. The results were subjected to one-way analysis of variance (ANOVA) using Minitab 16 software (Minitab, Inc., USA). Turkey's studentized range test for mean separation procedure was applied to compare differences between the means at 5% significance level. The functional relationship between diets including annatto dye and carotenoid content of the skin and caudal fin of the fish was analyzed by using second order polynomial regression fit in

R (3.2.1 for windows, 2007). The function $\hat{Y} = b_0 \pm b_1x \pm b_2x^2 \pm \ldots \pm b_kx^k$ was used to fit our data, where $\hat{Y}$ is the predicted carotenoid content (mg/kg), x is the annatto dye level (mg/kg) in diet and b_0, b_1, and b_2 are the constants determined by the regression. Subjective goodness of fit was assessed by plotting the data and the fitted curve.

Results

Annatto dye extraction and diet preparation

The extraction yield of annatto dye was 4.18%. The feed ingredients and proximate compositions of the experimental diet are given in Table 1. The carotenoid content of the control diet and the experimental diet with different amounts of annatto dye are shown in Table 2.

Biological indices of experimented gold fish

The water temperature was maintained at 27.5 ± 2°C using an aquarium heater. Other physico-chemical parameters throughout the experimental period were pH 7.8 ± 0.9, total ammonia, 90 ± 15 ng/L and nitrite, 34.56 ± 5.7 µg/L. All diets were readily accepted by fish, which proved that there was a relationship between palatability and feed intake of AD supplemented diets. All fish showed a good health condition and grew normally. Thereby, zero mortality was observed in all groups except the control group during the experimental period. However, the survival rate in each treatment at the end of the experimental time was significant ($p < 0.05$) compared to control and 50 mg/kg AD group (Table 2).

The biological indices revealed that the initial body weight of the fish was ranged from 6.77 to 7.77 g and fish body weight increased by 2.2–2.6 folds at the end of the experimental period of 60 days (Table 2). The inclusion of AD in fish diets did not affect the final weight, weight gain and specific growth rate of fish ($p > .05$). Furthermore, the highest and the lowest mean final body weights of 18.85 g and 16.26 g were observed in fish fed with 250 mg/kg AD in diet and the control diet, respectively. The highest TWG and SGR values were 11.65 g and 1.60% g/day, respectively and the lowest TWG and SGR values were 8.91 g and 1.11% g/day in that order. The FCR of the fish was adversely affected by the higher level of dietary AD in diets. The lowest significant FCR value of 1.42 was observed in fish fed with 250 AD, whereas, the highest FCR value of 1.72 was exhibited by the control group ($p < 0.05$).

Table 2. Growth parameter and total carotenoid level in the skin and caudal fin of goldfish fed formulated diets for 60 days of the experimental period.

	(Annatto dye (AD) supplementation level mg/kg)						
Rearing parameter	Control	50AD	100AD	150AD	200AD	250AD	CCF
Initial body weight (g)	7.34 ± 0.86	7.77 ± 0.70	7.01 ± 0.41	6.77 ± 0.49	7.01 ± 0.40	7.21 ± 0.45	7.18 ± 0.58
Final body weight (g)	16.26^{a} ± 1.22	17.44^{a} ± 0.51	16.75^{a} ± 0.45	16.89^{a} ± 0.66	18.17^{a} ± 1.03	18.85^{a} ± 1.37	17.77^{a} ± 1.19
Total weight Gain (g)	8.91^{a} ± 1.56	9.66^{a} ± 1.21	9.75^{a} ± 0.07	10.11^{a} ± 1.05	11.16^{a} ± 0.73	11.65^{a} ± 1.74	10.58^{a} ± 1.77
Specific growth rate (SGR) (%g/day)	1.33^{a} ± 0.24	1.114^{a} ± 0.19	1.45^{a} ± 0.20	1.45^{a} ± 0.06	1.53^{a} ± 0.17	1.601^{a} ± 0.22	1.51^{a} ± 0.07
Feed conversion ratio (FCR)	1.72^{a} ± 0.06	1.70^{a} ± 0.07	1.64^{a} ± 0.11	1.56^{a} ± 0.07	1.43^{b} ± 0.06	1.42^{b} ± 0.10	1.58^{a} ± 0.07
Initial total carotenoids in skin (mg/kg)	16.02 ± 1.20	16.02 ± 1.20	16.02 ± 1.20	16.02 ± 1.20	16.02 ± 1.20	16.02 ± 1.20	16.02 ± 1.20
Initial carotenoids in fin (mg/kg)	26.68 ± 1.60	26.68 ± 1.60	26.68 ± 1.60	26.68 ± 1.60	26.68 ± 1.60	26.68 ± 1.60	26.68 ± 1.60
Final total carotenoids in skin (mg/kg)	17.23^{a} ± 1.38	36.77^{b} ± 5.06	46.44^{cb} ± 4.41	59.14^{d} ± 3.69	66.28^{eg} ± 1.97	76.95^{fg} ± 4.29	73.34^{g} ± 2.15
Final total carotenoids in fin (mg/kg)	27.23^{a} ± 2.98	50.84^{b} ± 3.44	65.68^{c} ± 3.62	77.64^{d} ± 2.76	88.51^{eg} ± 1.04	95.11^{fg} ± 4.23	87.12^{g} ± 3.17
Fish survival (%)	87.25^{a} ± 00	87.25^{a} ± 00	100^{b} ± 00	100^{b} ± 00	100^{b} ± 00	100^{b} ± 00	100^{b} ± 00

Values are a means ± S.D. Means within the same row with different superscript letters differ significantly ($P < 0.05$)

Analysis of the effect of annatto dye on pigmentation of goldfish skin and caudal fin

The results of pigmentation in goldfish skin and caudal fin were significant ($p < .05$). Initial carotenoid value of 16.02 mg/kg in fish skin for all the treatments was increased in an ascending rate of accumulation in the first 20 days. However, the rate of carotenoid accumulation was observed to be at a descending rate at day 60. The total carotenoid level was increased with time in all treatments (Figure 2A). Thereby, highest carotenoid amount of 76.95 mg/kg was given by fish fed with the diet of 250 mg AD/kg diet at the 60 days of sampling (Table 2) ($p < .05$). The range of total carotenoid levels at each sampling time (i.e. 20d, 40d, 60d) were 16.45–53.10; 17.08–17.65; 17.24 – 76.95 mg/kg, respectively. Furthermore, the highest significant total carotenoid amount in the skin at each sampling period was observed in fish fed with 250 mg AD/kg, followed by a commercial diet and 200 mg AD/kg diet.

A similar pattern of total carotenoid level in the caudal fin was observed during the experimental period. The initial carotenoid level was 1.6 times higher than that of the skin (26.68 mg/kg). The highest rate of carotenoid accumulation during the first 20 days was observed and became constant at day 60 (Figure 2A). Mean total carotenoid amount range of fish fin during 20d, 40d, and 60d were 26.66 – 75.55; 28.47–87.65 and 27.23–95.11 mg/kg, respectively. The diets of 250, 0.0 (commercial diet) and 200 mg AD/kg diets gave the highest significant mean total carotenoid amounts in the caudal fin ($p < .05$) in descending order at each sampling period. The lowest carotenoid amount in both skin and fin at each sampling period was observed in fish fed with the control diet.

The relationship between the levels of AD and pigmentation in skin and fin was significantly ($P < .05$) correlated (Figure 3 A & B). A regression analysis results on skin and fin carotenoid amount with the level of dietary AD at 60 days

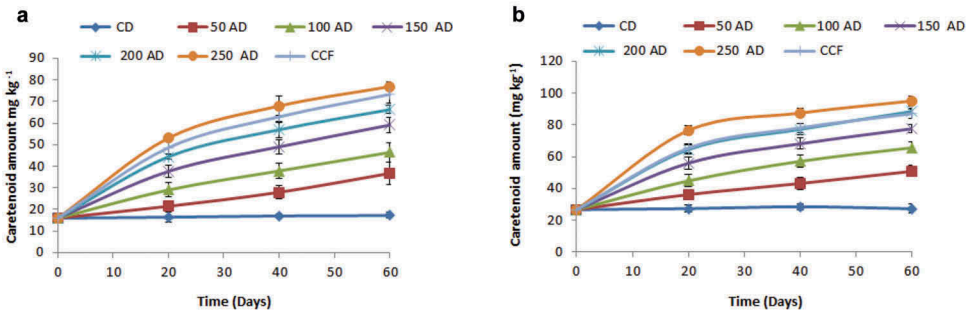

Figure 2. Effect of AD supplemented diets on total carotenoid amount in goldfish during the experimental period. a) skin and b) caudal fin. Value is a means ± S.D (n = 3).

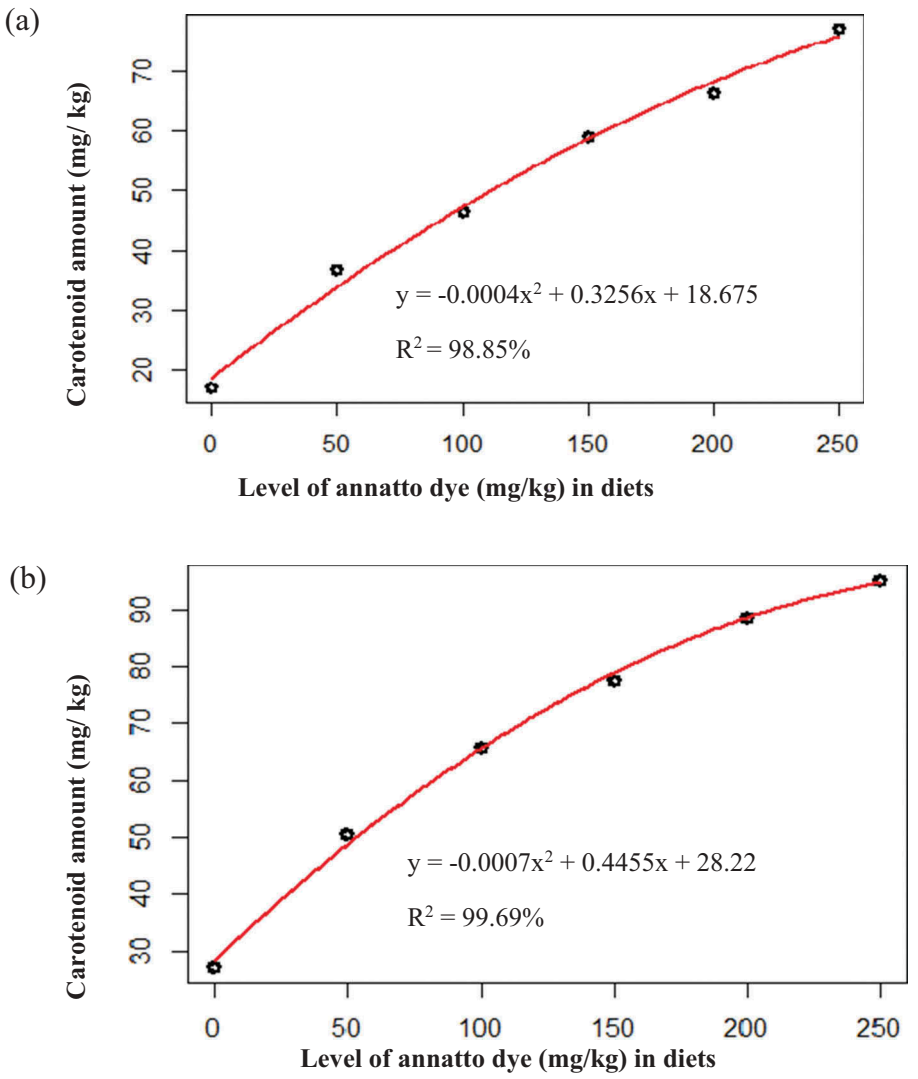

Figure 3. The relationship between total carotenoid content of goldfish and AD supplemented diet at the end of the experiment period (60 days). a) skin and b) caudal fin.

have nonlinear relation. The coefficient of correlation (R^2) values for skin and fin were 0.995, and 0.997, respectively.

Analysis of external skin color of goldfish

An effect of dietary AD on the body color was investigated using CIElab coordinating criteria. Average values for L* (lightness), a* (redness), b*(intensity of yellowness) and H° (hue) were shown in Table 3. According to the results only H°, a* and L* values of the fish skin were significant ($p < .05$) while b* values were not significant among treatments ($p > .05$). Feeding of the control diet

Table 3. Summary of color values of fish skin.

	Color Parameter			
Treatment	L*	a*	b*	H°
Control	67.59^{a} ±1.73	10.33^{d} ±1.18	11.70^{a}±0.71	48.73^{a} ±3.44
50AD	59.37^{b}±2.07	15.17^{a} ±1.76	12.53^{a} ±1.48	39.76^{b} ±6.32
100AD	50.69^{c} ±1.43	21.33^{b} ±0.74	12.01^{a}±0.67	29.33^{c} ±0.54
150AD	45.04^{d} ±1.13	27.82^{c} ±1.68	12.23^{a}±1.20	23.94^{c} ± 3.40
200AD	37.50^{e} ±1.59	35.57^{e} ±1.38	11.89^{a}±0.85	18.46^{d} ±0.79
250AD	29.19^{f} ±1.86	43.21^{h} ±1.37	11.64^{a}±0.66	15.05^{d} ±0.45
CCF	65.43^{ag} ±1.65	35.97^{e} ±2.02	11.69^{a}±0.58	18.05^{d} ±1.09

L*: lightness, a*: redness, b*: yellowness, H°: hue, CCF: commercial color feed, AD: annatto dye, Value are a means ± S.D. Means with different superscript letters within the same Colom differ significantly ($P < 0.05$)

increased L*, H° compared to the other diets. However, the highest a* and b* values of 12.53, 43.21, were observed in fish fed with diets included 50 and 250 mg AD/kg diet respectively.

The L* and a* and H° of fish skin were significantly affected by ($p < .05$) higher levels of dietary AD while b* values were not significant ($p > .05$) with both treatment and carotenoid amount in the skin. Furthermore, as shown in Figure 4 (A) negative linear correlation was indicated by L*, b* and H°, whereas, a positive correlation for a* with a carotenoid amount in fish skin

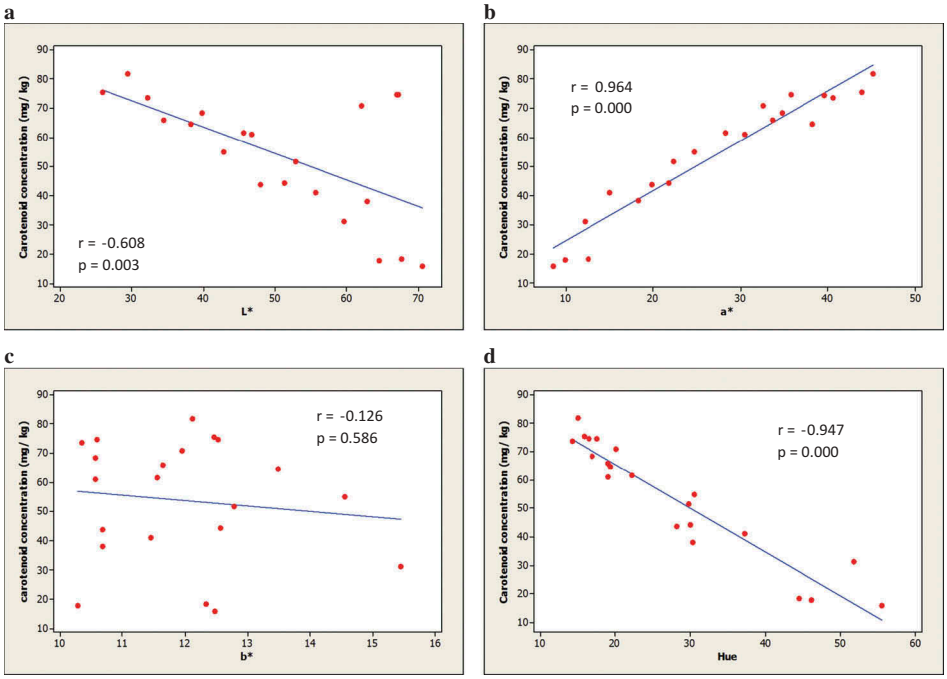

Figure 4. Comparisons between the carotenoid concentration in skin color parameters. A) lightness (L*); B) redness (a*); C) yellowness (b*) and D) hue (H°) in goldfish fed on a different AD supplemented diet for 60 days.

was obtained. The correlation coefficient (R^2) for L*, b*, H° and a* were −0.608, −0.126, −0.947 and 0.964, respectively.

Discussion

In the present study, the effect of AD formulated diets on growth, skin and fin pigmentation in goldfish were investigated. The primary source of coloration of fish skin is reported as carotenoid (Villar-Martinez et al. 2013). According to the results of the present study, it was found that carotenoid levels in goldfish skin and the caudal fin were increased by increasing the incorporating level of AD in the fish diet (50–250 mg/kg diet). Dietary carotenoid plays a significant role in the regulation of skin and muscle color in fish (Ezhil, Jeyanthi, and Narayanan 2008). Carotenoids have a positive role in the intermediary metabolism that could enhance nutrient utilization and ultimately results in improved growth of fish (Villar-Martinez et al. 2013). However, the inclusion of AD (50–250 mg/kg diet) in fish diets was not significantly affected final body weight, weight gain and specific growth rate of goldfish in the present study. In similar study, Zhengyu et al. (2006) reported that astaxanthin in goldfish diets had no effect on the weight gain and survival rate. In contrast, a similar level of annatto seed meal in the formulated diets has shown significant growth rate (SGR) of rainbow trout (Safari and Atash 2015). Furthermore, their findings indicated a positive correlation between the levels of annatto seed meal and the final body weight and SGR, while a negative correlation between FCR and blood carotenoid. Similarly, the negative correlation with FCR was observed when the AD level was increased in the diet of goldfish. The lowest FCR was observed in fish fed with diets containing 250 mg/kg diet. The lower FCR may be due to the pre-biotic effect of annatto seed extraction and ability to improve the fish gut bacterial colonization that ultimately increases the feed utilization (Safari and Atash 2015).

Most importantly, the effectiveness of carotenoids as a source of pigmentation is species dependent (Ha et al. 1993). Goldfish have the ability to use different feed materials effectively as sources of enhancing pigmentation. Especially they can utilize lutein and zeaxanthin from alfalfa (Yanar et al. 2008), spirulina (Kiriratnikom, Zaau, and Suwanpugdee 2005), *A. maxima* (Gouveia et al. 2003), red yeast, *X.dendrorhous* (Xu et al. 2006), and Marigold (Villar-Martinez et al. 2013). In the present study, dietary AD played the most important role in increasing pigmentation in skin and fin of goldfish with its high concentration. According to the findings of Safari and Atash (2015), bixin and nor-bixin in annatto seed meal have been identified as the main carotenoid sources responsible for the pigmentation. Furthermore, bixin is categorized as oil soluble, whereas nor-bixin as water soluble carotenoid. According to Giridhar, Venugopalan, and Parimalan (2014), total carotenoids availability in annatto seed meal is about 4.5–5.5% of seed weight. Moreover, his finding revealed that annatto seed as an effective natural carotenoid source for

pigmentation of rainbow trout. Carotenoids metabolism and pigmentation of ornamental fish depends on the source of feed, availability and other parameters such as carotenoids extraction methods, body size and weight, life cycle, environment, proximate composition of the feed ingredients and especially the lipid content that should be carefully considered when presenting the results of fish coloration and growth (Safari and Atash 2015).

The pigmentation rate of goldfish varied during different time periods depending upon whether they ingest the diets with astaxanthin or not. In this regard, goldfish can absorb un-esterified axtaxanthin and transform into an esterified type for deposition (Xu et al. 2006). Similarly, the inclusion of 10% of *S. platensis* as a natural source that contains zeaxanthin resulted in a high carotenoid deposition in tissues (Mahdi, Amirkolaie, and Yeganeh 2013). These results support for evidence that physical and chemical properties of the fish diet play a crucial role in fish body metabolism and growth. Similarly, the outcomes of some previous experiments showed that artificial diets result in different skin coloration in goldfish (Yeşilayer et al. 2012). Different concentrations of the AD used in the feed resulted in considerably higher differences in color of skin and caudal fin. Interestingly Xu et al. (2006) showed that incorporation of different dosage of astaxanthin (60–80 mg/kg) by supplementing with red yeast on goldfish diets significantly increased the carotenoid concentration. Villar-Martinez et al. (2013) stated that tiger barbs (*Barbus tetrazona*) fed diets containing carotenoid from shrimp meal, marigold petal meal, and annatto seed extract had successfully increased the skin color. The correlation between body coloration and dose of dietary carotenoids is supported by similar findings by Sun et al. (2012) using dietary carotenoids of xanthophyll in Japanese ornamental carp.

The concentration of carotenoids in fish skin and the caudal fin has shown an increasing pattern with a decreasing rate of deposition. The regression analysis showed 76.95 and 95.11 mg/kg as the highest levels of carotenoids in goldfish skin and caudal fin, respectively at 250 mg AD/kg diet. A similar pattern of increasing carotenoid levels in the skin was observed by Yanar et al. (2008), in which goldfish diets contained dietary alfalfa for 60 days. Even though saturation level for goldfish has not previously been published for AD supplemented diets, Yanar et al. (2008) reported the saturation level of carotenoid in gold fish skin was 102 mg/kg when it was fed with alfalfa 26–31% in the diet. Moreover, Rezende et al. (2012) have shown four levels (125, 250, 375 and 500 mg of bixin/kg of diet) of red carotenoid extracted from annatto plant (*B. orellana*) included in diets granted the greatest intensity of red – yellowish color in pearl Gouramy (*Trichogaster leeri*) skin. These findings support the assumption that the additional carotenoid accumulation may be possible with feeding the fish beyond the current AD level of 250 mg/kg diet.

When evaluating a goldfish, body conformation and fin placement are the most important criteria. In addition, color variation and brightness are the

essences of goldfish. The scale type strongly influences the intensity and stability of color. Each scale consists of different integumentary cells called cromatophores. The orange/red color is produced by the combination effect of red pigmented cells, (erythrophores), yellow pigmented cells (xanthophores), and white cells (leucophores) in the scales and skin (Kottler et al. 2014). Diet and environment may influence the color saturation, but seldom changes the hue (Gouveia and Rema 2005). Linear correlations are shown in redness (a*) lightness (L*) and yellowness (b*) and hue (Ho) in the current study is supported by the findings of Mahdi, Amirkolaie, and Yeganeh (2013) and Wathne et al. (1998).

The higher survival rate of goldfish was observed in AD containing treatments during the experiment, compared to that of the control one. Reasons for this observation may be due to the identified influence of carotenoids on fish immunity (Miki 1991) and also the antibacterial and antifungal effect of annatto seed extraction (Akshatha and Parvatam 2012). Furthermore, carotenoid intake affects both humoral and cell mediated immunity in Atlantic salmon, ultimately producing higher survival rates (Paripatananont et al. 1999). Nevertheless, a previous finding (Yeşilayer et al. 2012) showed that the higher survival rates of goldfish fed diets containing gammarus and canthaxanthin compared to diets supplemented with astaxanthin and oleoresin.

According to the obtained results, it can be concluded that the goldfish can utilize annatto dye effectively. The inclusion of 200–250 mgAD/kg diet in goldfish diets showed the most effective level in pigmentation and higher survival rate compared to the control diet. Therefore, annatto dye can be suggested as an effective alternative to natural carotenoid source for goldfish color enhancement.

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