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The effect of dietary pigments on the coloration of Japanese ornamental carp (koi, *Cyprinus carpio* L.)

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ABSTRACT

This study evaluated the effects of dietary supplementation with four pigment sources on the coloration of Japanese ornamental carp (*Showa* koi) (*Cyprinus carpio* L.). *Showa* koi (which are colored black with scattered red patches and white spots) initially weighing 18.04 ± 0.92 g were fed five dietary treatments in triplicate: a control diet with no added pigments, a diet with 1.5 g kg^{-1} Carophyll® red (synthetic, CR diet), a diet with 200 g kg^{-1} wet weight of a photosynthetic bacterium (*Rhodopseudanonas palustris*, PB diet), a diet with 200 g kg^{-1} wet weight of effective microorganisms (EM diet) and a diet with 75 g kg^{-1} dry weight feed-grade *Spirulina platensis* (SP diet). After a 99 day feeding trial, the fish's color was evaluated with a colorimeter to measure the chroma, lightness, redness and yellowness of different color zones. The carotenoid and xanthophyll concentration in the skin and the scales of the fish's red, black and white zones were tested. *S. platensis* significantly increased the growth and feeding efficiency of koi ($P<0.05$). *S. platensis* and Carophyll® red significantly improved the chroma of the black zone, the redness and the chroma of the red zone, and the lightness of the white zone ($P<0.05$). *S. platensis* and Carophyll® red increased the carotenoid content of the black and red scales and the xanthophyll content of the black and red skin and scales ($P<0.05$). The results indicate that *Showa* koi pigmentation can be modified by supplementing the diet with 1.5 g kg^{-1} Carophyll® red or 75.0 g kg^{-1} *S. platensis*. Dietary *R. palustris*, at levels up to 1.0 g dry matter kg^{-1} of diet, does not appear to affect the coloration of *Showa* koi. Furthermore, body coloration was generally correlated with the dose of dietary carotenoids and xanthophylls, and carotenoids had a deeper and greater influence than xanthophylls.

1. Introduction

Ornamental carp (koi) are characterized by a wide diversity of colors and color patterns (Gomelsky et al., 1996). More than 100 different types of coloration have been developed (Kuroki, 1981; Tamadachi, 1990) for these fish, which are valued as pets. Color is one of the most important quality criteria dictating the market value of koi. Because fish cannot synthesize carotenoids *de novo*, they rely on a dietary supply of these pigments to achieve their natural skin pigmentation (Gouveia et al., 2003; Paripatananont et al., 1999). Fish use carotenoids, one of the most important groups of natural pigments, for pigmentation of their skin and flesh. Carotenoids commonly occurring in freshwater food sources include β-carotene, lutein, taraxanthin, astaxanthin, tunaxanthin, α-, β-doradexanthins, and zeaxanthin (NRC (National Research Council), 1983, 1993).

Various synthetic pigments (β-carotene, canthaxanthin, zeaxanthin, and astaxanthin) and natural sources (yeast, bacteria, algae, higher plants, and crustacean meal) have been used as dietary supplements to enhance the pigmentation of fish and crustaceans (Kalinowski et al., 2005; Shahidi et al., 1998). Natural sources of carotenoids are usually composed of several carotenoids in various forms, and these sources vary in their digestibility, making their pigmentation efficiency complicated to interpret. In contrast, studies can clearly determine the pigmentation efficiency of synthetic carotenoids (which are always a single carotenoid). Given the high costs of synthetic pigments, however, efforts have been made to evaluate the potential of natural compounds. Some studies have shown that *Chlorella vulgaris* is as efficient as synthetic pigments in the pigmentation of rainbow trout *Oncorhynchus mykiss* (Gouveia et al., 1996b), gilthead seabream *Sparus aurata* (Gouveia et al., 2002), koi *Cyprinus carpio* and goldfish *Carassius auratus* (Gouveia et al., 2003). Pigments obtained from red yeast *Phaffia rhodozyma* (Bon et al., 1997), the marine bacteria *Agrobacterium aurantiacum* (Yokoyama and Miki, 1995), *Chlorococcum* sp. (Zhang et al., 1997), the green algae *Haematococcus*

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pluvialis (Harker et al., 1996; Yuan and Chen, 2000), *Chlorella zofingiensis* (Bar et al., 1995) and *C. vulgaris* (Gouveia et al., 1996a) were used as sources of dietary carotenoids.

In practice, we find out that some ornamental fish culturists used green algae *Spirulina platensis* to improve body color of fish such as goldfish (*Carassius auratus auratus*), ornamental Cichlid (*Cichlidae* sp.) and so on. On the other hand, we found that red color of *kohaku* koi had been significantly improved by photosynthetic bacteria (*Rhodopseudanonas palustris*) when we splashed this bacteria liquid into koi cultural ponds to improved the water quality of (Sun et al., 2010). Based on the practice and above researches, the present study evaluated the potential of these natural microorganisms, including *S. platensis*, single *R. palustris*, and effective microorganisms including *R. palustris*, as sources of dietary pigments for coloring the skin of *Showa* koi (*C. carpio* L.). These sources were compared to a positive control of synthetic astaxanthin (Carophyll® red) and a negative control, a diet with no pigment added.

2. Materials and methods

2.1. Experimental diets

A basal diet containing 300 g kg^{-1} crude protein and 50 g kg^{-1} crude fat without added pigments was used as a control diet. Using this basal mixture, four experimental diets were formulated by adding pigments in accordance with recommended doses: diet CR containing 1.5 g kg^{-1} Carophyll® red (DSM Nutritional Products Ltd.), diet PB containing 200 g kg^{-1} (wet weight) of the photosynthetic bacteria *R. palustris* (University of Science and Technology of Beijing, China), diet EM containing 200 g kg^{-1} (wet weight) of effective microorganisms, primarily photosynthetic bacteria (Shanghai Sanzhi Biotech. Co., Ltd., China), and diet SP containing 75 g kg^{-1} of the algae *S. platensis* (Beijing Sunpu Biochemical and Technology Co., Ltd., China). The ingredients and biochemical composition of the diets are shown in Table 1.

The pigments in the CR and SP diets were mixed with the basal diet completely and dry-pelleted using a steamless pelleting machine fitted with a 2.5-mm diameter screen. During the pelleting process, the temperature varied from 40 to 50 °C. The pigments in the PB and EM were evenly spray-coated onto the surface of the basal pellets and air-dried. To preserve the pigments, all diets were stored at 4 °C and were protected from light throughout the experiment.

2.2. Fish and rearing conditions

The homogeneous *Showa* koi carp were obtained from a commercial fish farm in Beijing, China. Before the beginning of the experiment, fish were fed with the control diet for 2 weeks to acclimatize them to the laboratory culturing system. At the beginning of the test, the fish, with an initial mean body weight of 18.04 ± 0.92 g, were randomly divided into fifteen groups of 20 fish each (5 treatments in triplicate). Each group of fish was stocked in a 0.21 m^3 indoor tank with a freshwater input of 0.5 l min^{-1}. The oxygen level was more than 7 mg l^{-1}. The fish were subjected to a natural photoperiod, and the average temperature of the water was 23 °C during the 99 day experimental period.

The fish were fed the appropriate experimental pellet corresponding to 3–5% of their body weight three times per day (8:00, 13:00, and 18:00). The amount fed was corrected every month after the fish were individually weighed. Mortality was recorded daily. During the experiment, fish in each tank were batch-weighed every 2 weeks to adjust the amount of feed and clean the tank. At initiation and at the end of feeding, after 1 day starvation, three fish were randomly selected from each tank, killed by means of a sharp blow to the head and batch weighed. At the end of the trial, three fish from each tank were sampled randomly for color analysis. Three other fish in each treatment were sacrificed by a blow to the head, and samples of the black, red and white skin and scales of each fish were excised, immediately frozen in liquid nitrogen, and kept at −50 °C until measurement of their carotenoid and xanthophyll contents.

Table 1
Ingredients and proximate composition of the experimental diets (g kg^{-1}).

Ingredients	Control diet	CR diet	PB diet	EM diet	SP diet
Fishmeal	150.0	150.0	150.0	150.0	150.0
Soybean meal	170.0	170.0	170.0	170.0	170.0
Soybean, full fat	80.0	80.0	80.0	80.0	20.0
Solvent-extracted cottonseed meal	110.0	110.0	110.0	110.0	80
Wheat shorts	250.0	250.0	250.0	250.0	240.0
Wheat flour	150.0	148.5	150.0	150.0	180.0
Attapulgite meal	40.0	40.0	40.0	40.0	40.0
Vitamin/minerals premix[a]	10.0	10.0	10.0	10.0	10.0
Soybean oil	20.0	20.0	20.0	20.0	15.0
$Ca(H_2PO_4)_2 \cdot H_2O$	20.0	20.0	20.0	20.0	20.0
Carophyll® red[b]		1.5			
Photosynthetic bacterium (wet weight)[c]			200.0		
Effective microorganism (wet weight)[d]				200.0	
Spirulina platensis[e]					75.0
Proximate composition (%)	**Control diet**	**CR diet**	**PB diet**	**EM diet**	**SP diet**
Dry matter	88.3	88.3	88.3	88.3	87.6
Crude protein	30.7	30.7	30.7	30.7	30.2
Crude fat	5.3	5.3	5.3	5.3	5.3
Ash	10.4	10.4	10.4	10.4	9.55
Total carotenoid (mg kg^{-1})	4.72	23.27	7.97	6.83	12.07
Xanthophyll (mg kg^{-1})	2.47	15.84	2.71	3.01	5.19

[a] Vitamin premix (mg kg^{-1}): thiamine-HCl, 8.0; riboflavin, 8.0; niacin mix, 100.0; pyridoxine-HCl, 20.0; cyanocobalamine, 0.1; pantothenate, 20.0; biotin, 1.0; inositol, 100.0; folic acid, 5.0; ascorbic acid, 250.0; Vitamin A, 20.0; Vitamin D, 8.0; Vitamin E, 150.0; Vitamin K, 10.0; BHT,10.0; α-cellulose, 1289.9. Mineral premix (mg kg^{-1}): $MgSO_4 \cdot 7H_2O$, 300.0; $FeSO_4 \cdot 7H_2O$, 180.0; $ZnSO_4 \cdot 7H_2O$, 120.0; $MnSO_4 \cdot 7H_2O$, 35.0; KI, 0.65; Na_2SeO_3, 0.5; $CoCl \cdot 6H_2O$ (1%), 7.0; $CuSO_4 \cdot 5H_2O$, 5.0; zeolite, 7351.85.
[b] Pigments: 10% canthaxanthin.
[c] Pigments: 1.5 g dry PB in per kg PB liquid.
[d] Pigments: 0.7 g dry PB in per kg EM liquid.
[e] Pigments: 1 g β-carotene and 2.6 g zeaxanthin per kg algae.

2.3. Biochemical analysis

2.3.1. Feed analysis

Biochemical analysis of feed was conducted in triplicate according to the AOAC (1995). Briefly, the crude protein (N × 6.25) content was determined by the Kjeldahl method after acid digestion using an Auto Kjeldahl System (2100-Auto-analyzer, Foss, Hillerød, Denmark). The crude fat content was determined by the ether extraction method using a Soxtec System HT (Soxtec System HT6, Foss, Hillerød, Denmark). The moisture content was determined by oven-drying at 105 °C for 24 h. The ash content was determined by combustion at 550 °C for 12 h.

2.3.2. Total carotenoid and xanthophyll analysis

The total carotenoid and xanthophyll content from feed, skin and scale samples were measured according to the methods of the AOAC (1995) with some modifications. At first, the black, red or white scales at sample area were pulled out by forceps separately, and skin under the scale was picked from the meat. Individual feed(1 g, dry weight), skin or scale samples (1 g, wet weight) were placed into a brown 25 ml Erlenmeyer flask and mixed with 7.5 ml of n-hexane/acetone/ethanol/toluene (10:7:6:7 v/v), and the flask was firmly closed and shaken vigorously by hand for 1 min. A total of 1 ml 40% KOH methanol solution was then added. The flask was

Table 2
Growth performance and feed utilization of koi fed experimental diets for 99 days[1].

Diets	Control diet	CR diet	PB diet	EM diet	SP diet
WGR[2] (%)	2.11 ± 0.10^{a}	2.04 ± 0.08^{a}	1.93 ± 0.09^{a}	1.89 ± 0.04^{a}	2.71 ± 0.12^{b}
SGR[3] (%)	1.14 ± 0.03^{a}	1.12 ± 0.02^{a}	1.10 ± 0.06^{a}	1.10 ± 0.02^{a}	1.32 ± 0.03^{b}
FCR[4]	1.86 ± 0.08^{b}	1.93 ± 0.07^{bc}	2.02 ± 0.10^{cd}	2.08 ± 0.04^{d}	1.45 ± 0.07^{a}
K factor[5]	2.85 ± 0.08	2.84 ± 0.06	2.83 ± 0.13	2.87 ± 0.10	2.81 ± 0.41
VSI[6] (%)	5.63 ± 0.56	6.39 ± 0.52	5.73 ± 0.43	6.16 ± 0.76	5.98 ± 0.26
HSI[7] (%)	1.56 ± 0.23	1.79 ± 0.16	1.42 ± 0.36	1.68 ± 0.30	1.66 ± 0.25

[1] Values are expressed as the means ± S.E.M. Values in same row with different superscripts are significantly different ($P<0.05$).
[2] WGR (weight gain rate) = 100% × (Wf + Wd − Wi)/Wi, where Wf is the total final body weight (g), Wd is the total dead body weight (g), and Wi is the total initial body weight (g).
[3] SGR (Specific growth rate) = 100% × (ln Wf − ln Wi)/days.
[4] FCR (feed conversion ratio) = total dry feed offered (g)/total wet weight gain (g).
[5] K factor = 100 × (L/W^3), where L is fish length (cm) and W is fish weight (g).
[6] VSI (Viscerosomatic index) = 100% × visceral weight (g)/fish weight (g).
[7] HSI (Hepatosomatic index) = 100% × hepatopancreas weight (g)/fish weight (g).

heated in a 55.5 °C water bath for 20 min after being shaken for 1 min to mix the solution and sample. After cooling, 7.5 ml of n-hexane was added and the flask contents were stirred for 1 min, then a 10% sodium sulfate solution was added to bring the sample up to 25 ml and shaken vigorously by hand for 1 min. After 1 h in the dark, the saponified sample solution was separated chromatographically. The separation was performed using a tandem-installed ChromSpher 5 μm C_{18} (100 × 3 mm I.D.) column with a guard column of C_{18} material (Chromsep guard column SS) preceding the main column. A total of 10 ml saponifying sample solution was injected into the column, and then an elution solution of n-hexane/acetone (96/4, v/v) was slowly added into the column until all of the carotenoids were eluted. The total carotenoid content from the supernatant was measured using a spectrophotometer (Shimadzu, UV-120-02) at 448 nm against a hexane (+BHT) blank, using an $E_{1\%,1\ cm}$ of 2500.

$$\textit{Total carotenoid content}\left(\text{mg kg}^{-1}\right) = (\text{A} \times \text{K} \times \text{V})/(\text{E} \times \text{G})$$

$$\textit{Total carotenoid content}\left(\text{mg kg}^{-1}\right) = (\text{A} \times \text{K} \times \text{V})/(\text{E} \times \text{G})$$

Here, A is the absorbency, K is dilution, V is the amount of supernatant (ml), E is the absorbency index (2500) and G is the sample weight (g).

After the carotenoids were eluted, the xanthophyll remained in the column. A xanthophyll elution solution of n-hexane/acetone/methanol (80:10:10, v/v) was slowly added into the column until all of the xanthophylls were eluted. The total xanthophyll content from the supernatant was measured by spectrophotometer (Shimadzu, Kyoto, Japan, UV-120-02) at 474 nm. Each sample was analyzed in triplicate.

$$\textit{Xanthophyll content}\left(\text{mg kg}^{1}\right) = (\text{A}_{474} \times 1000 \times \text{f})/(263 \times \text{b} \times \text{d})$$

Here A_{474} is absorbency, f (instrument error) = 0.561/observed A_{474}, b is the comparison tube length (cm), d (diluents index) = (the sample weight(g) × saponification (ml))/(top phase value (ml) × the last diluents (ml)).

2.3.3. Color analysis

At the end of the trial, three fish from each treatment were randomly selected to evaluate their skin color. Because koi are ornamental fish, investigators need a simple, rapid and accurate way to analyze color on living animals. In this study, skin color was assessed with reflectance spectroscopy with transformation into color parameters based on the tristimulus values, L*, a*, b* and dE, representing lightness, redness, yellowness and chromatic aberration, respectively (Skrede, 1987), using a portable Hanpu Chroma Meter HP-200 (Hanpu, Shanghai, China) calibrated with a white standard (the original adjusted value of the white standard was $L^* = 97.40 \pm 0.01$; $a^* = -0.10 \pm 0.01$; $b^* = 1.92 \pm 0.01$). The measurements were performed on the largest zone of black, red and white from each fish. L* and dE were measured in the black color zones; L* and b* were measured in white color zones; L*, a* and dE were measured in red color zones.

2.4. Statistics

All data were subjected to a one-way analysis of variance

2.4. Statistics

All data were subjected to a one-way analysis of variance (ANOVA) using the Statistica 8.0 software environment to test the effects of the experimental diets. Duncan's multiple range test and critical ranges were used to test differences among the individual means. The differences were regarded as significant when $P<0.05$. All of the results are expressed as the means ± S.E.M. The slopes of the color parameters and scale and skin pigment responses to the diets were compared after fitting a linear regression model using Statistica 8.0. Correlations were regarded as significant when the correlation coefficient $R>0.5$.

3. Results

3.1. Effects of experimental diet on the growth performance

The changes in growth during the experiment are shown in Table 2. The weight gain and feed conversion ratio were significantly affected by dietary treatment. The fish in the SP diet group had a significantly higher rate of weight gain (WGR) and specific growth rate (SGR) than the other fish groups ($P<0.05$). The food conversion

Table 3
The concentration of total carotenoids and xanthophylls in scale of koi (mg kg^{-1}).

Items		Control diet	CR diet	PB diet	EM diet	SP diet
Total carotenoids	White scale	4.49 ± 0.07	6.40 ± 1.07	5.57 ± 0.30	6.77 ± 0.8	6.13 ± 1.11
	Black scale	3.66 ± 0.37^{a}	9.72 ± 1.99^{b}	4.47 ± 0.02^{ab}	3.24 ± 1.62^{a}	4.45 ± 0.14^{ab}
	Red scale	6.75 ± 0.66^{ab}	10.69 ± 1.28^{b}	4.14 ± 1.90^{a}	4.87 ± 1.14^{a}	7.85 ± 1.40^{ab}
Xanthophylls	White scale	0.56 ± 0.15	0.62 ± 0.12	0.55 ± 0.10	0.77 ± 0.20	0.41 ± 0.18
	Black scale	0.27 ± 0.07^{a}	1.16 ± 0.44^{b}	0.87 ± 0.16^{ab}	0.61 ± 0.08^{ab}	0.89 ± 0.25^{ab}
	Red scale	1.24 ± 0.21^{a}	1.72 ± 0.31^{a}	1.11 ± 0.35^{a}	1.56 ± 0.53^{a}	3.45 ± 0.70^{b}

Values are expressed as the means ± S.E.M. Values in same row with different superscripts are significantly different ($P<0.05$).

Table 4
The correlation of fish scale pigments with dietary pigments.

Items	Correlation	Regression models
White scale carotenoid by feed carotenoid	$R=0.48$; $P<0.5$	
Black scale carotenoid by feed carotenoid	$R=0.96$; $P<0.01$	$y=1.34+0.34x$; $R^2=0.93$
Red scale carotenoid by feed carotenoid	$R=0.85$; $P<0.1$	$y=3.57+0.30x$; $R^2=0.72$
White scale xanthophyll by feed xanthophyll	$R=0.04$; $P>0.5$	
Black scale xanthophyll by feed xanthophyll	$R=0.73$; $P<0.5$	$y=0.51+0.04x$; $R^2=0.54$
Red scale xanthophyll by feed xanthophyll	$R=0.13$; $P>0.5$	

Table 6
The relationship between fish skin pigments and feed pigments.

Items	Correlation	Regression models
White skin carotenoid by feed carotenoid	$R=-0.92$; $P<0.05$	$y=8.55-0.27x$; $R^2=0.85$
Black skin carotenoid by feed carotenoid	$R=-0.30$; $P>0.5$	
Red skin carotenoid by feed carotenoid	$R=-0.32$; $P>0.5$	
White skin xanthophyll by feed xanthophyll	$R=0.31$; $P>0.5$	
Black skin xanthophyll by feed xanthophyll	$R=0.37$; $P>0.5$	
Red skin xanthophyll by feed xanthophyll	$R=0.87$; $P<0.1$	$y=0.70+0.12x$; $R^2=0.75$

ratio (FCR) of the fish fed the SP diet was significantly lower than that of the other fish groups ($P<0.05$). The FCR of the control fish was significantly lower than that of fish fed the PB diet or the EM diet ($P<0.05$). The FCR of the fish fed the CR diet was significantly lower than that of the fish fed the EM diet ($P<0.05$).

The K factor, VSI and HSI of the fish were unaffected by the experimental diets ($P>0.05$). No mortality was associated with experimental treatments.

3.2. Effects of the experimental diets on total carotenoid and xanthophyll concentrations of koi skin and scales

The amounts of total carotenoid and xanthophyll found in koi skin are shown in Table 3. No significant differences were found in the total carotenoid content of white scales among all of the experiment diet groups ($P>0.05$). The fish on the CR diet had a significantly higher total carotenoid content in their black scales compared with the control and EM diets and a higher content in their red scales compared with the PB and EM diets ($P<0.05$).

The xanthophyll concentration of the white scales did not differ among any of the diets ($P>0.05$). All pigments increased the xanthophyll content in black scales, and there were significant differences between the CR and the control diets ($P<0.05$). The fish fed the SP between the CR and the control diets ($P<0.05$). The fish fed the SP diet had markedly higher xanthophyll content in their red scales than the fish fed other diets ($P<0.05$).

The relationship between dietary pigments and scale pigments is shown in Table 4. The level of black scale pigments correlated well with dietary pigment doses, with correlation coefficients (R values) of 0.96 and 0.73 for total carotenoids and xanthophylls, respectively. The level of total carotenoids in red scales was correlated with the total dietary carotenoid dose ($R=0.85$). A regression analysis of the scale pigment level revealed that black and red scale deposition was linearly related to the total carotenoid levels in the feed, with R^2 values of 0.93 and 0.72 for black and red scales, respectively. An increase in the total feed carotenoids resulted in significantly elevated levels of carotenoids in the black scales ($P<0.01$).

Table 5 shows the total carotenoid and xanthophyll content in koi skin. The CR and SP diet groups had significantly lower total carotenoid content in their white skin than the fish in the PB, EM and control diet groups ($P<0.05$). Fish fed the EM diet had markedly higher total carotenoid content in their black skin than the other diet groups did ($P<0.05$). Fish fed the PB diet had significantly higher total carotenoid content in their red skin than that of fish fed the CR or control diets ($P<0.05$). There were no significant differences in the xanthophyll content of the white skin among all diets ($P>0.05$). Fish fed the SP diet had significantly higher xanthophylls in their black skin than the fish fed the PB or EM diets ($P<0.05$). Fish fed the CR and SP diets had markedly higher xanthophyll content derived from red skin than the fish fed the PB or EM diets ($P<0.05$).

The relationship between feed pigments and skin pigments is shown in Table 6. The total carotenoid level in white skin was significantly correlated with dietary carotenoid dose ($R=0.92$). A regression analysis showed that carotenoid levels in the white skin of fish were linearly related to the feed carotenoid level ($R^2=0.85$). An increase in the total amount of carotenoids in the feed resulted in a significant decline of carotenoids in the white skin ($P<0.05$). Red skin-derived xanthophyll levels were correlated with the dietary dose of xanthophylls ($R=0.87$). A regression analysis showed that the xanthophyll levels in red skin were linearly related to the xanthophyll level in the feed ($R^2=0.75$).

3.3. Effects of experimental diet on the body color

The intensity of color, red and yellow tonalities, and chromatic aberration for the chromatic varieties of *Showa* koi are shown in Table 7. All of the pigments increased the lightness (L^*) of the black zones, and there was a significant difference between the SP and the control diet groups ($P<0.05$). The black and white chromatic aberrations (dE of the black zones) of the CR and PB diet groups were significantly higher than those of the EM, SP and control diet groups ($P<0.05$).

The CR diet groups showed a significantly lighter red zone than that of groups fed the control, PB or SP diets ($P<0.05$). The group fed the control diet showed weak red tonality (a^*) and dE of the red

Table 5
The concentration of carotenoids and xanthophylls in skin of koi (mg kg^{-1}).

Items		Control diet	CR diet	PB diet	EM diet	SP diet
Total carotenoids	White skin	7.54 ± 1.07^{b}	2.78 ± 0.23^{a}	6.35 ± 0.25^{b}	7.44 ± 0.41^{b}	3.89 ± 0.28^{a}
	Black skin	2.15 ± 0.20^{a}	2.20 ± 0.33^{a}	1.93 ± 0.44^{a}	9.81 ± 2.59^{b}	2.38 ± 0.87^{a}
	Red skin	2.44 ± 0.39^{a}	2.61 ± 0.30^{a}	4.25 ± 0.54^{b}	3.58 ± 0.44^{ab}	3.42 ± 0.35^{ab}
Xanthophylls	White skin	0.48 ± 0.06	0.67 ± 0.15	0.46 ± 0.15	0.68 ± 0.12	0.85 ± 0.21
	Black skin	0.54 ± 0.15^{ab}	0.65 ± 0.02^{ab}	0.27 ± 0.05^{a}	0.44 ± 0.10^{a}	0.89 ± 0.03^{b}
	Red skin	1.05 ± 0.27^{ab}	2.42 ± 0.77^{b}	0.72 ± 0.07^{a}	0.76 ± 0.14^{a}	1.92 ± 0.56^{b}

Values are expressed as the means ± S.E.M. Values in same row with different superscripts are significantly different ($P<0.05$).

Table 7
Color parameters (L*, a*, b* and dE) for koi fed experimental diets.

Items	Control diet	CR diet	PB diet	EM diet	SP diet
L* of black zone	35.47 ± 6.50^{a}	44.77 ± 5.82^{ab}	48.35 ± 4.85^{ab}	52.13 ± 2.17^{ab}	55.80 ± 5.72^{b}
dE of black zone	19.40 ± 1.90^{a}	49.15 ± 2.85^{b}	24.13 ± 2.88^{a}	16.25 ± 0.45^{a}	50.45 ± 4.75^{b}
L* of red zone	54.75 ± 3.75^{a}	71.25 ± 10.55^{b}	54.20 ± 2.80^{a}	60.90 ± 2.61^{ab}	54.17 ± 2.46^{a}
a* of red zone	14.80 ± 2.30^{a}	34.15 ± 0.05^{b}	16.80 ± 0.76^{a}	26.33 ± 1.18^{ab}	38.40 ± 8.46^{b}
dE of red zone	20.75 ± 1.35^{a}	45.77 ± 5.72^{b}	18.90 ± 4.20^{a}	25.77 ± 3.03^{a}	39.23 ± 2.69^{b}
L* of white zone	74.55 ± 2.05^{a}	83.80 ± 2.00^{b}	71.70 ± 3.10^{a}	85.45 ± 1.25^{b}	85.63 ± 2.82^{b}
b* of white zone	4.05 ± 1.05^{a}	7.30 ± 0.84^{ab}	11.40 ± 1.80^{ab}	7.10 ± 4.29^{ab}	13.37 ± 0.70^{b}

Values are expressed as the means ± S.E.M. Values in same row with different superscripts are significantly different ($P<0.05$).

zone (red and white chromatic aberration), which differed significantly from values found for groups fed the CR or SP diets ($P<0.05$).

In the white zones, the CR, EM and SP diet groups had significantly stronger L* than that of the PB or the control diet groups ($P<0.05$). All of the pigments increased the yellow hue (b*) of the white zones, and there were significant differences between the SP and the control diet groups ($P<0.05$).

The relationship between dietary pigments and body color parameters is shown in Table 8. A regression analysis revealed that the dE of the black zones and the, L^* and dE of the red zones were linearly related to the feed carotenoid levels, with R^2 values of 0.662, 0.671 and 0.802, respectively. The L^* and dE of the red zones were linearly related to the level of xanthophylls in the feed, with R^2 values of 0.792 and 0.726, respectively. An increase in the total carotenoid levels in the feed resulted in a significant increase in the dE of the red zones ($P<0.05$); increasing the xanthophyll levels in the feed level resulted in a significant increase in the L^* of the red zones ($P<0.05$).

4. Discussion

In this study, the growth and feed utilization parameters were not markedly improved by any pigments except *S. platensis* ($P<0.05$). *S. platensis* has been identified as a potential protein source for animal feed owing to its high protein content and the presence of essential amino acids, vitamins and minerals. In addition, this type of microalgae has been reported to have no cell wall, which results in improved digestion and absorption (Becker and Venkataraman, 1984). Improvement in the growth of fish by the dietary inclusion of *Spirulina* has been reported earlier in a number of studies (Mustafa et al., 1994; Nakazoe et al., 1986). Some studies have revealed that *S. platensis* could be used as the sole source of protein in common carp and catla (*Catla catla*) diets (Nandeesha et al., 1998, 2001). In this trial, *S. platensis* replaced 75% full-fat soybean and 27% solvent-extracted cottonseed meal to keep the diet isonitrogenous. If *S. platensis* replaced other plant proteins, it could improve the growth of the fish in this trial and replace fishmeal. These results are also in agreement with detailed investigations on the utilization of microalgae as a feed for fish in Israel, which found that fish grew better on algae-enriched diets than on any conventional fish feed (Sandbank and Hepher, 1978).

Table 8
The relationship of color parameters and feed pigments.

Items	Correlation	Regression models
L* of black zone by feed carotenoid	$R=0.13$; $P>0.5$	
dE of black zone by feed carotenoid	$R=0.81$; $P<0.1$	$y=11.78+1.83x$; $R^2=0.662$
L* of red zone by feed carotenoid	$R=0.82$; $P<0.1$	$y=50.06+0.82x$; $R^2=0.671$
a* of red zone by feed carotenoid	$R=0.68$; $P<0.5$	$y=15.51+0.97x$; $R^2=0.471$
dE of red zone by feed carotenoid	$R=0.90$; $P<0.05$	$y=14.32+1.44x$; $R^2=0.802$
L* of white zone by feed carotenoid	$R=0.46$; $P<0.5$	
b* of white zone by feed carotenoid	$R=0.14$; $P>0.5$	
L* of black zone by feed xanthophyll	$R=0.04$; $P>0.5$	
dE of black zone by feed xanthophyll	$R=0.72$; $P<0.5$	$y=19.65+2.09x$; $R^2=0.514$
L* of red zone by feed xanthophyll	$R=0.89$; $P<0.05$	$y=52.31+1.16x$; $R^2=0.792$
a* of red zone by feed xanthophyll	$R=0.59$; $P<0.5$	$y=19.83+1.07x$; $R^2=0.347$
dE of red zone by feed xanthophyll	$R=0.85$; $P<0.1$	$y=19.73+1.77x$; $R^2=0.726$
L* of white zone by feed xanthophyll	$R=0.42$; $P<0.5$	
b* of white zone by feed xanthophyll	$R=0.02$; $P>0.5$	

In colorimetry and color theory, hue is one of the main properties of a color, defined technically (in the CIECAM02 model), as "the degree to which a stimulus can be described as similar to or different from stimuli that are described as red, green, blue, and yellow". Chroma is the colorfulness relative to the brightness of another color that appears white under similar viewing conditions. Lightness is a property of a color, or a dimension of a color space, that reflects the subjective brightness perception of a color to humans along a lightness–darkness axis. With three attributes—colorfulness (or chroma or saturation), lightness (or brightness), and hue—any color can be described (http://en.wikipedia.org/wiki/Colorfulness). The black, red and white zones of koi were tested separately in this trial. Lightness (L*) and chroma (dE) were the main indexes for the black zones. Higher dE of the black zones correlated with better black zones. Meanwhile, a higher L* of the black zone indicates that the black color is brighter. The fish fed the SP diet had the highest L* and dE of the black zones, followed by the fish fed the CR diet, which had significantly higher dE. A higher level of red hue (a*) indicates that the red zones are more similar to red. Higher a*, L* and dE indicated significant improvement in the coloration of the fish fed the CR and SP diets. Less yellowness (b*) means that white zones is more pure. A higher yellow hue in the white zones of the fish fed the experimental diets indicates that these zones could become more variegated with increased amounts of dietary pigments. The reason might be that *Showa* koi is colored black with scattered red and white patches, and the grounding color of the white patches is black. The white zones would not be snow-white when the black grounding became darker and brighter. Also for this reason, those fish fed SP and CR diets had a high yellowness morphologically when the diets reduced carotenoids content in the white skin. Overall, the CR and SP diets yielded better coloring for the *Showa* koi in this trial.

Carotenoids can be broadly classified into two classes, carotenes ("which are purely hydrocarbons and contain no oxygen") and xanthophylls ("which contain hydroxyl groups or pairs of hydrogen atoms that are substituted by oxygen atoms", http://en.wikipedia.org/wiki/Xanthophylls). The total carotenoid and xanthophyll concentrations in the diets were measured to analyze the relationship between dietary pigment content and fish body color. The feed pigment doses targeted in this study were selected to be in range with doses commonly employed in the market. Thus, the carotenoid and xanthophyll content in the experimental diets were not equal

because of the different pigment contents of the sources and the different amounts of the sources added to the diets. The control diet contained some carotenoids and xanthophylls because it included plant proteins and solvent-extracted cottonseed meal.

As showed in Table 4, the level of dietary pigments is correlated with the level of black scale pigments for carotenoids ($P<0.01$) and xanthophyll ($P<0.5$). Feeding fish with carotenoids significantly ($R^2=0.93$) increases the level of carotenoid pigments in their black scales. Therefore, dietary supplementation with carotenoids primarily affects the level of carotenoids in black scales. In addition, the level of dietary carotenoid pigments correlates with the level of white-skin carotenoid pigments because the P value is less than 0.05. As more carotenoid was fed, the level of white-skin carotenoid pigment decreased ($R^2=0.85$). Dietary supplementation with xanthophylls correlates with the level of red-skin xanthophyll pigments ($P<0.1$), but their linear relationship is not significant because the R^2 value is only 0.75. All of these results suggested that the applied dietary carotenoids were correlated with the carotenoids levels of the colored scales of koi, directly affecting body coloration. The results based on Tables 4 and 6 also suggested that scales might play more important role than skin on showing the body color. Dietary supplementation with carotenoids is positively and linearly dependent on the dE of the red zone (Table 8, $R^2=0.802$, $P<0.05$) whereas dietary supplementation with xanthophlls can increase the L^* of red zones ($R^2=0.792$, $P<0.05$). The color-improving functions of xanthophylls are

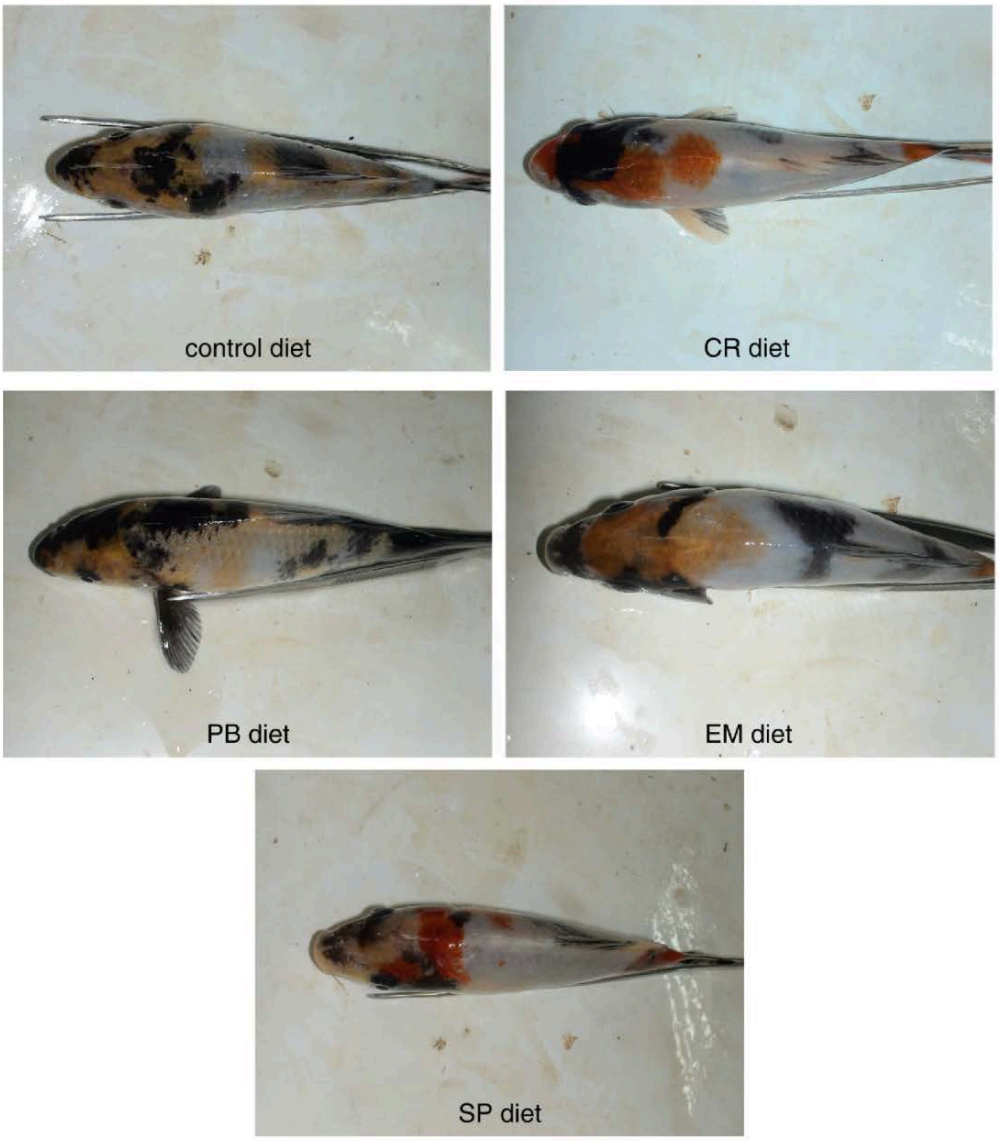

Fig. 1. The differences in coloration from different treatments.

thought to mainly improve red coloration. The results here suggest that the carotenoid and xanthophyll content in fish feeds were highly correlated with fish body coloration, and the carotenoids had a deeper and greater influence compared to the xanthophylls.

Gouveia et al. (2003) showed that *Showa* koi fed 80 mg total colorings kg^{-1} from *Arthrospira maxima* (*Spriulina*) had similar body color and total carotenoid content with fish fed the same content colorings from synthetic astaxanthin. This trial got similar even better results compared with Gouveia's. *Showa* koi fed SP diet containing less total pigment content (diet total carotenoids + xanthophylls, 17.26 mg kg^{-1}) had similar body color with fish fed CR diet containing more total pigment (39.11 mg kg^{-1}). Koi are a type of red carp that can convert zeaxanthin, canthaxanthin and lutein into astaxanthin and store it in the body (Simpson, 1981). *Spriulina* contains mainly zeaxanthin and β-carotene (Liao et al., 1993; Soejima et al., 1980). One reason that these compounds can produce an improved body coloration might be that zeaxanthin in *S. platensis* is able to be used by koi effectively. These results suggested that *S. platensis* could be a useful, even competitive pigment for inclusion in the diets of koi carp to improve skin pigmentation.

Li and Wang (1997) indicated that the main pigments in the cells of photosynthetic bacteria are bacteriochlomphyll a, bacteriophaeophytin and 3 carotenoids with absorption maxima at 485 nm and 516 nm, 481 nm and 510 nm, 486 nm and 521 nm. In the present study, the total pigment content in the PB and EM diets was 3.49 and 2.65 mg kg^{-1}, respectively. Both of these values are higher than the control diet values. Because body coloration generally correlated with the dietary pigment dose and koi can use astaxanthin, zeaxanthin, canthaxanthin and lutein effectively, the poorer coloration of the fish consuming the PB and EM diets may be due to the low total pigment content and low utilization rate of koi for chlorophyll.

The present study suggested that *Showa* koi pigmentation could be modified by supplementing the diet with 1.5 g kg^{-1} Carophyll® red or 75.0 g kg^{-1} *S. platensis*. Dietary *R. palustris* at levels up to 1.0 g dry matter kg^{-1} of diet does not appear to affect the coloration of *Showa* koi (Fig. 1). Furthermore, body coloration generally correlated with the dose of dietary carotenoids and xanthophylls. Future work will attempt to optimize the concentration of *S. platensis* in the diet and the period of pigment supplementation, and determine the optimal size of the fish to begin supplementation.

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