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Effects of *Hibiscus rosasiensis* as a natural carotenoid on growth performance, body composition, pigmentation and carotenoid in blood plasma of blue gourami, *Trichogaster trichopterus* at different stocking densities

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Abstract

This study examined the effects of China rose powder (*Hibiscus rosasiensis*) supplementation (1.5%) on the growth performance, body composition, and pigmentation of blue gourami at different stocking densities (0.6 and 0.9 fish L^{-1}). Four treatments were assigned in this study: China rose diet+0.6 fish L^{-1} (CG0.6), China rose diet+0.9 fish L^{-1} (CG0.9), control diet+0.6 fish L^{-1} (C0.6) and control diet+0.9 fish L^{-1} (C0.9). The results showed that after 70 days, there were no significant differences in growth and body composition between China rose treated groups and their corresponding control groups. Color parameters (L*Lightness, a*redness, b*yellowness) behind the operculum were measured on a weekly basis. Results indicated that in the most of weeks, there were no significant differences for (L*, a*, b*). However in the China rose treatments, the fish were brighter in the first weeks but turned darker in the last weeks. The fish in the China rose treatments had higher greenness and yellowness values at the end of the experiment. The amounts of skin total carotenoids, canthaxanthin and β-carotene in the China rose groups were significantly different compared with the corresponding control groups. Similar patterns were observed in muscle and caudal fin canthaxanthin and β-carotene contents. Significant differences were observed in the content of blood plasma canthaxanthin and β-carotene between C0.6 and CG0.6; whereas such differences were observed in astaxanthin, canthaxanthin and β-carotene between C0.9 and CG0.9 groups. The study shows that the China rose powder is a potent natural carotenoid source for blue gourami to enrich its color in low density.

Keywords: Carotenoid, China Rose, *Hibiscus rosasiensis*, Blue gourami, Stocking density.

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Introduction

Aquaculture is one of the fastest-growing economic sectors in the world. The market value of ornamental fish depends on their visual criterion. The worth of high-value colorful ornamental species is mostly attributed to their skin pigmentation. The beauty of the fish skin is a reflection of its surrounding condition. In general, color formation is affected by the physiology of the individual fish. Skin color of fish depends on the carotenoids present in its diet. Hence, microalga biomass in general and carotenoids in particular can be utilized as a feed additive to supply basic nutrients and pigments towards fish in aquaculture (Urban *et al*., 2013).

Blue gourami (*Trichogaster trichopterus*) is an ornamental fish that has great economic importance that fetches a good price because of its color and behavior. It is distributed throughout Central Africa, India and Southern Asia. It is a common species of fish in rice-field areas and in certain parts of the world such as Asia and Africa they are consumed as food (Morioka *et al.,* 2012).

The art of rearing and keeping fish in an aquarium has a long history. At the dawn of the 21st century fish keeping is reflected in ubiquitous aquaria that feature as an integral part of modern interior decoration (Katia, 2001). Stocking densities used in commercial aquaculture have been highlighted as an area of specific welfare concern. Fish farmers can keep on rearing fish at high densities, partly because operating at higher stocking densities can reduce production costs. However, stocking density has been demonstrated to affect various aspects of the wellbeing of farmed fish, although differences between species are distinct. High densities may impair the wellbeing of some fish species (e.g. salmon, Ewing and Ewing, 1995; seabream, Montero et al., 1999; trout, Ellis et al., 2002) while for instance Arctic charr grow more rapidly at high densities, (Jørgensen *et al*., 1993). Color changes in fish are often related to stress. Among the environmental factors that may influence the skin colors pigmentations of fish are background color, illumination intensity and light spectrum and also aquaculture-related stressors such as crowding.

Colored fishes often went through discoloration under intensive culture conditions. Fish cannot synthesize carotenoids and they are dependent on dietary sources. They should feed on food containing carotenoids to achieve coloration. The coloration of fish is thus enhanced by administering pigment enriched feed. However, there is a lack of studies on natural carotenoid containing dietary feed in combination with growing the fishes under different stocking densities towards the growth performance and skin coloration of fishes. Due to the lack of information on natural carotenoid and stocking density, this study attempted to ascertain the effect of stocking density and the addition of China rose (*Hibiscus rosasiensis*) powder as a natural carotenoid on the growth performance, body composition, skin

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color and carotenoid contents of blue gourami.

Materials and methods

Study site

The experiment was performed at the aquaculture laboratory of Inland Waters Aquatic Stocks Research Center (Gorgan, Iran).

Sampling preparation

Homogeneous fish of blue gourami used in this study were provided by Shahriyari Company (Gorgan, Iran). The fishes were subjected to 24 days acclimatization period in the laboratory. Each aquarium contained 35L of water with an average temperature of 26.5±0.02 °C, dissolved oxygen content of 7.15±0.02 mg L^{-1} and pH of 8.35±0.02. Each aquarium was aerated using a central pump and air stone. The fish were kept in this system until the fish reached an average weight of 0.80±0.02 g. Fish sex was not determined.

Rearing animals at different stocking densities and China rose 1.5%

Twelve (12) glass aquaria (60cm×30cm ×30cm) were used in this study. There are two different stocking densities studied in this experiment. This treatment was fed with 1.5% China rose powder which has been referred to as C0.6. The second group contained 30 fish per aquarium (0.9 fish L^{-1}), this group fed with 1.5% China rose powder which has been referred to as C0.9. For all of these treatments, a control group was used which was not fed with any China rose powder. These control groups have been referred to as CG0.6 (control C0.6) and CG0.9 (control C0.9). For each treatment, three replicates were used. The fish were fed two times a day and the aquarium water was exchanged once a day (30-50%). This experiment was carried out for ten weeks. For 70 days (Teimouri *et al.*, 2013) of the experiment, the fishes were hand-fed at 2.5-3.5% of their body mass per day.

Proximate analysis of the body composition of blue gourami at the end of experiment

The body composition of the fish was analyzed. Crude protein, crude fat, ash and energy were measured at the end of the experiment (AOAC) (1995).

Analysis of growth factors of blue gourami at the end of experiment

Growth factors comprising Specific Growth Rate (SGR), Feed Conversion Ratio (FCR), Condition Factor (CF), Weight Gain (WG), Survival Rate (SR) and Hepatosomatic Index (HSI) were computed at the end of experiment.

- SGR $= \frac{\ln \text{final weight} - \ln \text{initial weight}}{\text{day}} \times 100$ (Hung *et al.*, 1989)
- FCR $= \frac{\text{Total dry food fed}}{\text{Total weight gain}}$ (Hung *et al.*, 1989)
- CF $= \frac{\text{Weight (gram)}}{\text{Length}^3 \text{ (cm)}} \times 100$ (Hung *et al.*, 1989)
- WG= $(Mean\ final\ weight - Mean\ initial\ weight)$ (Hung *et al.*, 1989)

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- SR= $\frac{\text{Initial fish number} - \text{Number of dead fish}}{\text{initial fish number}} \times 100$ (Hung *et al.*, 1989)
- HSI = $\frac{\text{Liver weight}}{\text{Fish weight}} \times 100$ (Sun *et al.*, 2012)

Color analysis of blue gourami at the during of experiment

The color analysis of the fish was measured weekly using a portable Konica Minolta color reader (CR-10, Osaka, Japan,). Possible color change was observed at the b zone. The color parameters were determined based on the tri stimulus values, L* for lightness ranging from 0 (black) to 100 (white), a* values for red/green and b* values for yellow/blue.

Total carotenoid analysis

The carotenoid analysis for fish fed with each of the formulated diets was carried out by Pars Biopharmacy Co. Iran. The method proposed by Torrissen and Naevdal (1984) was used to extract the carotenoid content of the skin, muscle and caudal fin. A total of 500 mg samples were grounded from the skin, muscle and caudal fin in cold acetone solvent system separately with a homogenizer. In order to avoid any carotenoid loss, the extraction was done repeatedly in each solvent system. The total extracts were combined, centrifuged at 3000 rpm for 5 minutes and then the optical density was measured using a spectrophotometer (Model: WPA) at 474 nm (modified from Fuji Chemical Industry, 2010).

Total Carotenoids= $\frac{\text{Absorbance} \times \text{Volume}}{210 / \text{Weight}} \times 100$ (modified from Fuji Chemical Industry, 2010)

V = Volume of acetone (4 ml)

210 =Absorbance of 1mg ml^{-1} carotenoid solution in acetone

W = Sample weight (mg)

Astaxanthin and canthaxanthin and β-carotene analysis in skin, muscle and caudal fin of blue gourami (T. trichopterus) at the end of experiment

The concentration of astaxanthin, canthaxanthin and β-carotene was measured by comparing it with the standards for astaxanthin, canthaxanthin and β-carotene. The standard solution for astaxanthin, canthaxanthin and β-carotene were prepared in the concentration range of 2.5-540 µg L^{-1} (Sigma Aldrich and Fluka). The production and extraction of the samples was conducted at an ambient temperature of 22ºC and under dim light. A total of 2.5 ml of carotenoid extract sample from each treatment was subjected to astaxanthin, canthaxanthin and β-carotene analysis on High Performance Liquid Chromatography (HPLC). Then, two solvent mixture containing 2 ml of hexane and 0.5 ml of water were added to 2.5 ml volume of the upper layer. It was then vortexed again for 30 seconds followed by 5 minutes centrifugation at 3000 RPM. Then, the hexane layer was dried in a clean tube under an inert gas atmosphere of nitrogen. Then 250 µl of methanol was added to the residue and 70 µl of this solution was injected into the HPLC (Younglin HPLC system

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equipped with a pump (SP930D), UV detector (730D), reodyne injector, and Autochro 2000 integrator software). The HPLC separation condition includes: 1.4 ml min^{-1}; wave length: 474 nm; temperature: 25ºC; Column: C18, Inertsil ODS- 3V250×4.6mm. The Mobile phase A included methanol - water (97:3) and mobile phase B consisted of methanol, THF (Tetrahydrofuran) and water (37:60:3) (Suhnel *et al*., 2009).

Carotenoid analysis from the blood plasma of blue gourami at the end of experiment

Blood samples were taken from the caudal vessel of the fish using heparinized syringes. The samples were then centrifuged for 15 minutes at 4000 rpm. The plasma were collected and stored at −20°C before proceeding with the measurements of astaxanthin, canthaxanthin and β-carotene in experiment.

Statistical analysis

The analysis of growth factors and carotenoids (color, total carotenoids, astaxanthin, canthaxanthin and β-carotene) were subjected to T-test independent analysis. The level of significance was considered to be for $p<0.05$. Data were obtainable as mean ± SEM. Standard error values were reported. Levene's test was used to check for the equality of variance.

Results

Analysis of growth factors blue gourami fed China rose powder under stocking densities at the end of experiment

Table 1 shows growth factor of fishes reared at densities of C0.6 and C0.9 fish per aquarium and their comparison with CG0.6 and CG0.9.

Results showed no significant differences in the growth factors such as SGR, FCR, CF, WG, SR and HSI between CG0.6 and CG0.9 compared with C0.6 and C0.9, respectively ($p>0.05$) (Table 1).

Table 1: Comparison of growth performances of blue gourami fed 1.5% China rose powder under different stocking densities compared with their control.

Treatment	SGR (%/day^{-1})	FCR	CF (%)	WG (gr)	SR (%)	HSI (%)
CG0.6	2.34 ± 0.14	1.14 ± 0 .10	1.28 ± 0.02	3.00 ± 0.04	100	2.37 ± 0.19
C0.6	2.51 ± 0.17	1.07 ± 0.10	1.28 ± 0.02	3.48 ± 0.05	100	1.80 ± 0.17
CG0.9	2.25 ± 0.10	1.16 ± 0 .05	1.33 ± 0.03	3.17 ± 0.05	100	1.95 ± 0.21
C0.9	2.44 ± 0.07	1.06 ± 0.05	1.32 ± 0.02	3.72 ± 0.05	100	2.17 ± 0.32

Each value is a mean±S.E of three replicates
CG0.6 = Control blue gourami reared under stocking density of 20 fish/aquarium,
C0.6 = Blue gourami fed 1.5% China rose under stocking density of 20 fish/aquarium,
CG0.9 = Control blue gourami reared under stocking density of 30 fish/aquarium,
C0.9 = Blue gourami fed 1.5% China rose under stocking densityof 30 fish/aquarium.

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Proximate analysis of body composition of blue gourami fed China rose powder under stocking densities at the end of experiment

Table 2 shows proximate analysis of body composition in fishes reared at densities of 20 and 30 fish per aquarium (C0.3, C0. 6, CG0.6 and C0.9, CG0.9). Based on the results shown in Table 2, no significant differences (p>0.05) have been observed in the composition of crude protein, crude fat, ash, energy contents between C0.6 and C0.9 compared with CG0.6 and CG0.9, respectively (Table 2).

Table 2: Comparison of proximate analysis of blue gourami body composition fed 1.5% China rose powder under different stocking densities compared with their control.

Proximate analysis	CG0.6	C0.6	CG0.9	C0.9
Crude protein (%)	64.02 ± 0.52	65.31 ± 0.34	63.99 ± 0.27	64.94 ± 0.52
Crude fat (%)	23.47 ± 0.31	22.61 ± 0.67	23.33 ± 0.39	23.85 ± 0.25
Ash (%)	11.66 ± 0.17	11.85 ± 0.17	12.40 ± 0.14	11.73 ± 0.26
Energy (Cal g^{-1})	4621.24 ± 46.07	4488.94 ± 93.44	4589.87 ± 47.25	4642.38 ± 32.68

Each value is a mean ± S.E of three replicates.
CG0.6 = Control blue gourami reared under stocking density of 20 fish/aquarium,
C0.6 = Blue gourami fed 1.5% China rose under stocking density of 20 fish/aquarium,
CG0.9 = Control blue gourami reared under stocking density of 30 fish/aquarium,
C0.9 = Blue gourami fed 1.5% China rose under stocking densityof 30 fish/aquarium.

Color analysis of blue gourami fed China rose powder under stocking densities at the during of experiment

For lightness color at density of 20 fish per aquarium (0.6 fish L^{-1}), significant differences were observed between of C0.6 and CG0.6 on the 10^{th} week (p<0.05) as shown in Table 3. There were no significant differences in the lightness color in week 1 to week 9 except in week 10 between C0.6 and CG0.6 (Table 3). At density of 30 fish per aquarium (0.9 fish L^{-1}), no significant difference was observed between the lightness colors of C0.9 and CG0.9 during the experiment (p>0.05) as shown in Table 3.

Table 3: Comparison of weekly lightness values at the behind of operculum in blue gourami fed 1.5% China rose powder under different stocking densities compared with their control during the experiment.

Week	CG0.6	C0.6	CG0.9	C0.9
Week 1	75.68 ± 2.16	76.09 ± 0.25	76.60 ± 1.37	77.39 ± 0.84
Week 2	72.06 ± 1.23	69.94 ± 0.79	71.59 ± 0.20	68.98 ± 1.97
Week 3	67.96 ± 1.06	68.54 ± 0.25	70.72 ± 1.11	70.02 ± 0.35
Week 4	69.90 ± 1.22	70.17 ± 1.13	68.35 ± 1.24	72.1 ± 0.85
Week 5	71.14 ± 0.88	69.70 ±1.48	70.76 ± 0.81	72.04 ± 1.22
Week 6	67.28 ± 2.05	70.46 ± 0.95	69.64 ± 1.34	70.54 ±1.78
Week 7	69.18 ± 1.80	68.06 ± 1.03	70.18 ± 0.86	68.98 ± 0.90
Week 8	69.85 ± 2.19	69.72 ± 0.57	70.73 ± 0.74	70.54 ± 0.93
Week 9	67.47 ± 0.82	69.30 ± 1.26	69.38 ± 0.92	71.58 ± 0.46
Week 10	73.84 ± 0.68 [a]	70.15 ± 0.52 [b]	71.30 ± 0.37	70.76 ± 0.98

Each value is a mean ± S.E of three replicates.
CG0.6 = Control blue gourami reared under stocking density of 20 fish/aquarium,
C0.6 = Blue gourami fed 1.5% China rose under stocking density of 20 fish/aquarium,
CG0.9 = Control blue gourami reared under stocking density of 30 fish/aquarium,
C0.9 = Blue gourami fed 1.5% China rose under stocking densityof 30 fish/aquarium.
Lowercase letters show significant difference between CG0.6 and C0.6 (p<0.05).

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At the density of 20 fish per aquarium (0.6 fish L^{-1}), no significant difference were observed between the greenness colors of C0.6 and CG0.6 during the experiment except in week 4 (p<0.05) as shown in Table 4. C0.6 produced a relatively higher amount of greenness color compared to CG0.6 in the 4th week. C0.6 and CG0.6 had negative values which indicates green color.

At the density of 30 fish per aquarium (0.9 fish L^{-1}), significant differences were observed between the greenness color of C0.9 and CG0.9 in weeks 4 and 8 (p<0.05) as shown in Table 4. There were no significant differences between C0.9 and CG0.9 from week 1 to week 10 except in weeks 4 and 8 in the greenness color (Table 4). CG0.9 produced a relatively higher amount of greenness color compared to C0.9 in weeks 4 and 8. Negative value indicates greenness color. Both of C0.9 and CG0.9 had negative values in week 4 and week 8 (Table 4).

Table 4: Comparison of weekly redness/greenness values at the behind of operculum in the blue gourami fed 1.5% China rose powder under different stocking densities compared with their control during the experiment.

Week	CG0.6	C0.6	CG0.9	C0.9
Week 1	-2.88 ± 1.02	-4.16 ± 0.23	-3.98 ± 0.10	-3.21 ± 0.36
Week 2	-0.22 ± 1.67	-0.62 ± 1.13	-1.39 ± 0.60	0.01 ± 0.94
Week 3	-2.77 ± 0.57	-3.30 ± 1.67	-3.57 ± 0.67	-2.62 ± 0.55
Week 4	-6.37 ± 0.32 [a]	-2.03 ±1.12 [b]	-7.04 ± 0.74 [A]	-5.13 ± 0.87 [B]
Week 5	1.17 ±1.02	-2.68 ± 0.83	-0.35 ± 0.63	0.20 ± 1.11
Week 6	-0.52 ± 0.76	-2.81 ± 2.33	-0.34 ± 1.13	-0.29 ±1.33
Week 7	-2.53 ± 0.57	-3.94 ± 0.91	-6.35 ± 0.43	-5.24 ± 0.37
Week 8	-3.70 ± 0.54	-2.51 ± 1.03	-6.08 ± 0.24 [A]	-4.58 ± 0.38 [B]
Week 9	-7.08 ± 0.33	-6.35 ± 0.88	-7.18 ± 0.14	-6.06 ± 0.90
Week 10	-5.47 ± 0.40	-6.23 ± 0.57	-5.81 ± 0.25	-6.24± 0.59

Each value is a mean ± S.E of three replicates.
CG0.6 = Control blue gourami reared under stocking density of 20 fish/aquarium,
C0.6 = Blue gourami fed 1.5% China rose under stocking density of 20 fish/aquarium,
CG0.9 = Control blue gourami reared under stocking density of 30 fish/aquarium,
C0.9 = Blue gourami fed 1.5% China rose under stocking densityof 30 fish/aquarium.
Note: Positive values indicate redness color and negative values indicate greenness.
Lowercase letters show significant difference between CG0.6 and C0.6 (p<0.05).
Uppercase letters show significant difference between CG0.9 and C0.9 (p<0.05).

At density of 20 fish per aquarium (0.6 fish L^{-1}), significant differences were observed between the yellowness color of C0.6 and CG0.6 in weeks 9 and 10 (p>0.05) as shown in Table 5. There were no significant differences between C0.6 and CG0.6 from week 1 to week 8 in the yellowness color (Table 5). C0.6 produced a relatively higher amount of yellowness color compared with C0.6 in the 9th week, while CG0.6 produced a relatively higher amount of yellowness color compared to C0.6 in the 10th week because of their positive values.

At the stocking density of 30 fish per aquarium (0.9 fish L^{-1}), no significant differences were observed between the yellowness of C0.3 and C0.9 compared with CG0.3 and CG0.9, respectively, during the treatment (p>0.05) as shown in Table 5. Results indicated that this

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zone is also dominated by yellowness because all the data collected showed positive values.

Table 5: Comparison of weekly yellowness/blueness values behind the operculum of blue gourami fed 1.5% China rose powder under different stocking densities compared with their controls during treatment.

Week	CG0.6	C0.6	CG0.9	C0.9
Week 1	10.41 ± 0.58	9.29 ± 0.59	9.95 ± 0.43	9.96 ± 0.50
Week 2	12.48 ± 0.80	10.63 ± 1.71	10.99 ± 0.62	12.66 ± 0.38
Week 3	9.93 ± 0.51	10.40 ± 0.76	10.64 ± 0.65	13.06 ± 0.81
Week 4	10.35 ± 0.75	11.20 ± 0.31	11.16 ± 1.03	13.58 ± 1.15
Week 5	13.76 ± 0.40	12.62 ± 0.22	13.91 ± 0.37	14.35 ± 0.47
Week 6	12.56 ± 0.63	13.08 ± 1.05	13.04 ± 0.32	13.78 ± 1.35
Week 7	13.08 ± 1.02	12.78 ± 0.91	9.98 ± 1.12	12.45 ± 1.16
Week 8	12.55 ± 1.23	13.88 ± 0.87	11.33 ± 1.19	13.19 ± 0.55
Week 9	10.23 ± 0.70 [b]	12.31± 1.05[a]	7.80 ± 0.83	11.17 ± 1.79
Week 10	12.55 ± 0.31 [a]	11.15 ± 0.27 [b]	11.93 ± 0.24	11.56 ± 0.50

Each value is a mean ± S.E of three replicates
CG0.6 = Control blue gourami reared under stocking density of 20 fish/aquarium,
C0.6 = Blue gourami fed 1.5% China rose under stocking density of 20 fish/aquarium,
CG0.9 = Control blue gourami reared under stocking density of 30 fish/aquarium,
C0.9 = Blue gourami fed 1.5% China rose under stocking densityof 30 fish/aquarium.
Note: Positive values indicate yellowness and negative values indicate blueness.
Lowercase letters show significant difference between CG0.6 and C0.6 (p<0.05).

Total carotenoid, astaxanthin, canthaxanthin and β-carotene analysis in skin of blue gourami fed China rose powder under stocking densities at the end of experiment.

Table 6 shows total carotenoids, astaxanthin, canthaxanthin and β-carotene contents in blue gourami skin fed 1.5% China rose powder at different stocking densities of 20 and 30 fish per aquarium (0.6 and 0.9 fish L^{-1}), and their comparison with their control (CG0.6 and CG 0.9 fish L^{-1}).

Results showed that at both stocking densities of 20 and 30 fish per aquarium (0.6 and 0.9 fish L^{-1}), total carotenoids in treated fish significantly increased compared with untreated fishes (p<0.05). Total carotenoid contents of C0.6 and C0.9 treated fishes increased to 101 and 36%, respectively compared with their control (Table 6).

Results indicated that there were no (p>0.05) significant differences for astaxanthin contents of C0.6 and C0.9 treated fishes compared with CG0.6 and CG0.9, respectively (Table 6).

Canthaxanthin contents significantly increased in C0.6 and C0.9 treated fishes compared with their control (p<0.05) (Table 6). Canthaxanthin contents of C0.6 treated fishes increased three times compared with their control. Canthaxanthin contents of C0.9 treated fishes showed 25 times increase compared with CG0.9.

Results also showed that β-carotene contents significantly increased (p<0.05) (Table 6) 3.50 and 5 times in C0.6 and C0.9 treated fishes compared with their control CG0.6 and CG0.9, respectively.

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Table 6: Carotenoids analysis in skin of blue gourami fed 1.5% China rose powder under different stocking densities compared with their control.

Treatments (Skin)	Total Carotenoids ($\mu g g^{-1}$)	Astaxanthin ($ng g^{-1}$)	Canthaxanthin ($ng g^{-1}$)	β-Carotene ($ng g^{-1}$)
CG0.6	4.87 ± 0.36^{b}	5.93 ± 0.14^{a}	24.78 ± 2.53^{b}	20.80 ± 0.02^{b}
C0.6	9.81 ± 0.29^{a}	6.11 ± 0.40^{a}	80.87 ± 6.02^{a}	72.01 ± 0.04^{a}
CG0.9	5.37 ± 0.37^{B}	5.23 ± 0.31^{A}	6.50 ± 1.71^{B}	20.40 ± 0.02^{B}
C0.9	7.29 ± 0.16^{A}	6.61 ± 0.57^{A}	164.88 ± 23.50^{A}	98.20 ± 0.06^{A}

Each value is a mean ± S.E of three replicates.
CG0.6 = Control blue gourami reared under stocking density of 20 fish/aquarium,
C0.6 = Blue gourami fed 1.5% China rose under stocking density of 20 fish/aquarium,
CG0.9 = Control blue gourami reared under stocking density of 30 fish/aquarium,
C0.9 = Blue gourami fed 1.5% China rose under stocking density of 30 fish/aquarium.
Lowercase letters show significant difference between CG0.6 and C0.6 ($p<0.05$).
Uppercase letters show significant difference between CG0.9 and C0.9 ($p<0.05$).

Carotenoid, astaxanthin, canthaxanthin and β-carotene analysis in muscle of blue gourami fed China rose powder under stocking densities at the end of experiment

Table 7 shows total carotenoids, astaxanthin, canthaxanthin and β-carotene contents in blue gourami muscle which supplemented with 1.5% China Rose powder at different stocking densities of 20 and 30 fish per aquarium (0.6 and 0.9) and comparison with their control.

There were also no significant differences in regard to total carotenoids and astaxanthin contents at treated fishes (0.6 and 0.9 fish L^{-1}), compared to their control ($p>0.05$) (Table 7).

Results revealed that canthaxanthin contents significantly increased ($p<0.05$) at C0.6 and C0.9 treated fishes compared to their control. Canthaxanthin contents of C0.6 and C0.9 increased 3 and 19 times in treated fishes compared to control and CG0.6 and CG0.9 respectively (Table 7). β-carotene contents of C0.6 and C0.9 treated fishes compared to control significantly increased 17 and 7%, compared to CG0.6 and CG0.9, respectively ($p<0.05$) (Table 7).

Table 7: Carotenoids analysis in muscle of blue gourami fed 1.5% China rose powder under different stocking densities compared with their control.

Treatments (Muscle)	Total Carotenoid ($\mu g g^{-1}$)	Astaxanthin ($ng g^{-1}$)	Canthaxanthin ($ng g^{-1}$)	β-Carotene (ngg-1)
C0.6	1.19 ± 0.05^{a}	1.26 ± 0.28^{a}	1.78 ± 0.12^{b}	0.50 ± 0.01^{b}
C0.6	1.22 ± 0.01^{a}	2.21 ± 0.22^{a}	4.60 ± 0.84^{a}	80.03 ± 0.02^{a}
CG0.9	1.55 ± 0.01^{A}	1.48 ± 0.35^{A}	1.64 ± 0.17^{B}	0.70 ± 0.01^{B}
C0.9	1.93 ± 0.25^{A}	2.18 ± 0.37^{A}	30.52 ± 3.92^{A}	50.05 ± 0.01^{A}

Each value is a mean ± S.E of three replicates.
CG0.6 = Control blue gourami reared under stocking density of 20 fish/aquarium,
C0.6 = Blue gourami fed 1.5% China rose under stocking density of 20 fish/aquarium,
CG0.9 = Control blue gourami reared under stocking density of 30 fish/aquarium,
C0.9 = Blue gourami fed 1.5% China rose under stocking density of 30 fish/aquarium.
Lowercase letters show significant difference between CG0.6 and C0.6 ($p<0.05$).
Uppercase letters show significant difference between CG0.9 and C0.9 ($p<0.05$).

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Total carotenoid, astaxanthin, canthaxanthin and β-carotene analysis caudal fin of blue gourami fed China rose powder under stocking densities at the end of experiment

Table 8 shows the total content of carotenoids, astaxanthin, canthaxanthin and β-carotene in the caudal fin of blue gourami fed with dietary supplements of 1.5% of China rose powder at stocking densities of 20 and 30 fish per aquarium (0.6 and 0.9).

β-carotene values for C0.3 increased significantly in comparison with CG0.3 (p<0.05), as shown in Table 8.

Results showed that no significant differences were observed in the contents of carotenoids and astaxanthin in the caudal fin of blue gourami between C0.6 and C0.9 compared with CG0.6 and CG0.9, respectively (p>0.05), as shown in Table 8. Conversely, canthaxanthin values for C0.6 and C0.9 increased significantly in comparison with CG0.6 and CG0.9 (p<0.05), respectively as observed in Table 8. Canthaxanthin values for C0.6 and C0.9 increased at the rate of 197 and 51% in comparison with CG0.6 and CG0.9, respectively (Table 8). β-carotene contents increased significantly with increasing stocking densities (p<0.05) (Table 8).

Table 8: Carotenoids analysis of caudal fin of blue gourami fed 1.5% China rose powder under different stocking densities compared with their control.

Treatments (Caudal fin)	Total Carotenoids (μg g^{-1})	Astaxanthin (ng g^{-1})	Canthaxanthin (ng g^{-1})	β-Carotene (ng g^{-1})
CG0.6	8.96 ± 0.76 ^{a}	10.66 ± 0.79 ^{a}	18.88 ± 3.86^{b}	30.44 ± 0.05 ^{b}
C0.6	9.46 ±1.83 ^{a}	12.91 ± 2.48 ^{a}	55.98 ± 1.03^{a}	65.55 ± 0.12 ^{a}
CG0.9	7.48 ± 0.68 ^{A}	13.55 ± 0.81 ^{A}	65.61 ± 9.13 ^{B}	23.01 ± 0.09 ^{B}
C0.9	9.74 ± 1.95 ^{A}	10.39 ± 1.80 ^{A}	98.89 ± 5.54 ^{A}	72.50. ± 0.02 ^{A}

Each value is a mean ± S.E of three replicates.
CG0.6 = Control blue gourami reared under stocking density of 20 fish/aquarium,
C0.6 = Blue gourami fed 1.5% China rose under stocking density of 20 fish/aquarium,
CG0.9 = Control blue gourami reared under stocking density of 30 fish/aquarium,
C0.9 = Blue gourami fed 1.5% China rose under stocking density of 30 fish/aquarium.
Lowercase letters show significant difference between CG0.6 and C0.6 (p<0.05).
Uppercase letters show significant difference between CG0.9 and C0.9 (p<0.05).

Astaxanthin, canthaxanthin and β-carotene analysis in blood plasma of blue gourami fed China rose powder under stocking densities at the end of experiment

In this section, samples of blood plasma were not efficient for detecting total carotenoids. Table 9 shows astaxanthin, canthaxanthin and β-carotene contents in the blood plasma of blue gourami fed with 1.5 % of China rose powder, at different stocking densities of 20 and 30 fish per aquarium (0.6 and 0.9).

A comparison between group C0.6 and CG0.6 showed no significant differences in the amount of astaxanthin between these two groups (p>0.05) (Table 9).On the contrary, there were significant differences in the canthaxanthin and β-carotene contents in the blood plasma of fish between groups C0.6 and CG0.6.

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Significant differences were observed between astaxanthin, canthaxanthin and β-carotene contents in the blood plasma of fish in groups C0.9 and CG0.9 (p<0.05) (Table 9).

Table 9: Carotenoids analysis in blood plasma of the fish fed with China rose powder.

Treatments (blood plasma)	Astaxanthin (ng/g)	Canthaxanthin (ng/g)	β-Carotene (µg/g)
CG0.6	27.07 ± 3.52 [a]	72.88 ± 4.34 [b]	0.27 ± 0.04 [b]
C0.6	27.38 ± 1.37 [a]	642.35 ± 54.66 [a]	3.11 ± 0.02 [a]
CG0.9	16.35 ± 4.30 [B]	21.83 ± 1.99 [B]	0.65 ± 0.09 [B]
C0.9	34.36 ± 3.77 [A]	1252.74 ± 132.55 [A]	3.81± 0.68 [A]

Each value is a mean ± S.E of three replicates.
CG0.6 = Control blue gourami reared under stocking density of 20 fish/aquarium,
C0.6 = Blue gourami fed 1.5% China rose under stocking density of 20 fish/aquarium,
CG0.9 = Control blue gourami reared under stocking density of 30 fish/aquarium,
C0.9 = Blue gourami fed 1.5% China rose under stocking density of 30 fish/aquarium.
Lowercase letters show significant difference between CG0.6 and C0.6 (p<0.05).
Uppercase letters show significant difference between CG0.9 and C0.9 (p<0.05).

Discussion

The research on stocking density in aquaculture has always been a priority to most aquaculturists (Di Marco *et al.*, 2008). This study showed that there was no significant difference in fish survival when exposed to different stocking densities. Thus, it can be inferred that blue gourami are hardy and can endure poor conditions such as high stocking densities and will maintain their reproduction ability even at very high stock densities.

In this study, results showed that dietary supplement of China rose have not shown any significant effect on the growth of blue gourami. The findings in this study suggested that high stocking density might not affect the growth factors of blue gourami. Previous studies reported that stocking densities have not shown any significant effect on the growth of juvenile rainbow trout (North *et al.*, 2006); Cobia (Webb *et al.*, 2007); olive flounder (Kang *et al.*, 2011); adult burbot (Wocher *et al.*, 2011); and trout (Cagiltay *et al.*, 2015). Additionally, this research revealed that China rose treatments and different stocking densities have not shown any significant effects on fish survival rates. This may prove that blue gourami is a resistant fish which is able to tolerate hard conditions such as high stocking densities.

However, no significant differences were observed in the protein and fat contents in fish fed China rose powder with their control group for stocking densities of 20 and 30 (fish per aquarium). This result is consistent with the reported effect of high stocking density on body composition of larvae of Florida pompano, *Argyrosomus regius* (Roo *et al*., 2010). These results suggest that high stocking density has little effect on protein metabolism.

Body color is an important criterion in aquaculture which will determine the commercial value of fish particularly in those species that are sold fresh or live or as ornamental fish (Biswas, 2013). Results showed that, brighter fish were normally observed in the first weeks of China rose treatment, while the last

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weeks of treatment resulted in darker color. In line with this, (Van der Salm *et al.,* 2004) reported that increasing stocking density may promote crowding-stress in the aquarium, which may possibly affect the regulation of pigmentation and ultimately result in dark coloration of fish skin. Increasing trend of redness has been observed with increasing fish densities. With increasing fish densities, greenness decreased. At the end of China rose treatments, greenness values increased compared to the first week of treatment. In this study, higher yellowness values have been observed at the end of the treatment. Increasing fish densities resulted in increasing yellowness. In contrast Cagiltay *et al.* (2015) reported that different stocking densities did not result in significant differences in L* (lightness), a* (redness) and b* (yellowness) colors of rainbow trout (*Oncorhynchus mykiss*). Some studies reported that stocking density significantly decreased color parameters with increasing stocking density such as in dark barbel catfish (Zeng *et al*., 2010) and in cultured Arctic charr (*Salvelinus alpinus*) (Metusalach *et al.,* 1997). Previous studies have also revealed factors which affect fish pigmentation. For instance, Forsberg and Guttormsen (2006) showed that fish size and dietary factors affect fish pigmentation in Atlantic salmon. Other factors such as species genetic origin (Gjedrem, 2000) and stress level prior to sacrificing to collect samples may affect pigmentation (Robb *et al.,* 2000). The origin of these contradictions may come from different experiments media and farming management.

In the present study, usually, blue gourami fishes were subjected to carotenoid measurement at first maturation stage. Therefore, it is possible that the sexual maturation process may cause the carotenoid contents to decline in the muscle tissue. Besides, the saturation of pigment in blue gourami may decrease muscle carotenoid contents which is in line with findings of the study by Mukherjee *et al.* (2009) who stated that turmeric powder as a carotenoid resulted in the highest pigment concentration in caudal fin of guppy fish. James *et al.* (2006) reported that total carotenoid contents in fins, skin and muscle of red sword tail fish increased as *Spirulina* level increased. They concluded that maximum coloration was in the fins, followed by the skin and muscle in all treatments.

Results of this study showed that the amount of canthaxanthin varied significantly in China rose treatments under different stocking densities compared to their control. The results revealed that under high stocking density, China rose treatment resulted in more variation of canthaxanthin compared with astaxanthin contents. Thus, results revealed that high stocking density affects the synthesis of astaxanthin, while, canthaxanthin contents increased in fish fed natural carotenoids under high stocking densities.

The variation in value of carotenoids, astaxanthin, canthaxanthin and β-carotene shows that the adaptive

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responses of blue gourami to stocking density also depends on other factors including type of food, fish species, genome and environmental conditions (Rey Vazquez and Guerrero, 2007). The differences may also be affected by absorption, deposits or metabolism of carotenoids, astaxanthin and canthaxanthin and β-carotene pigments by blue gourami.

Furthermore, astaxanthin, canthaxanthin and β-carotene were detected in blood serum of blue gourami fed China rose under different densities. This deduction goes contrary to studies by Hsu *et al.* (1972), who reported that goldfish fed with diets rich in both lutein and β-carotene efficiently resulted in higher production levels of astaxanthin. This is attributed to the conversion and deposition of the dietary supplements to astaxanthin. The deposition rate of carotenoids in organs and tissues vary among species such as Atlantic salmon, brown trout (*Salmo trutta*) and rainbow trout (*O. mykiss*) (Storebakken *et al.,* 1987; Schiedt , 1998). Nonetheless, there is a dearth of literature on the amount of astaxanthin, canthaxanthin and β-carotene in blue gourami. The variations shows that the adaptive responses of the blood to stocking density is dependent on several factors such as fish species, size, type of food, genome and environmental conditions (Rey Vazquez and Guerrero, 2007). Therefore, the differences between the relative deposition of carotenoids, astaxanthin, and canthaxanthin and β-carotene in blue gourami may be attributed to differences in absorption, deposition or metabolism of carotenoids astaxanthin and canthaxanthin and β-carotene pigments.

This study has shown that the amount of astaxanthin and canthaxanthin can be affected by stocking density. High stocking density is less preferable due to the disadvantage of having the possible increment in canthaxanthin value and decrement in astaxanthin value. In humans, carotenoids are utilized as systemic photo protection. Higher canthaxanthin has dangerous toxic effects that can result in ocular lesion caused by the formation of macular crystal formation observed in fish, monkeys, other farmed animals and also sporadic human cases (Brizio *et al.,* 2013). High stocking density is less preferable due to the disadvantage of having the possible increment in canthaxanthin value. Therefore, it is highly recommended to use low stocking density.

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