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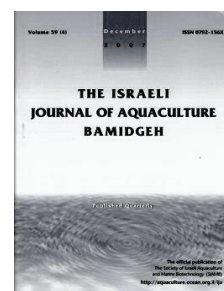
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## Efficacy of Alfalfa Saponins on Promoting Pigmentation by Astaxanthin in Blood Parrot Fish (*Vieja synspila* ♀ × *Amphilophus citrinellus* ♂)

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**Keywords:** blood parrot; alfalfa saponins; carotenoid; chroma value; physiological and biochemical indicators

### Abstract

This study was conducted to evaluate the effect of alfalfa saponins on pigmentation induced by astaxanthin in 540 blood parrot fish (*Vieja synspila* ♀ × *Amphilophus citrinellus* ♂). The fish were divided into six treatment groups: basal diet; a diet supplemented with 3‰ astaxanthin; three diets with 3‰ astaxanthin plus 3‰, 6‰ or 12‰ alfalfa saponins respectively; and a diet supplemented with 4‰ alfalfa saponins alone. The fish were maintained in three aquaria per treatment. At 20, 40, 60, and 80 days, 6 blood parrot fish per aquarium were sampled. At the end of the experiment, the carotenoid content of scales, skin, and caudal fin in groups supplied with 3‰ astaxanthin plus alfalfa saponins were significantly higher than the control. The redness values,  $a^*$ , of the body and caudal fins in these groups increased significantly. Conversely, the cholesterol and triglyceride content decreased significantly. Non-esterified fatty acid content and lysozyme activity were significantly higher in the three groups fed supplemented astaxanthin diets with the addition of alfalfa saponins, than the group fed a diet supplemented with 3‰ astaxanthin alone ( $P < 0.05$ ). The results show that the addition of alfalfa saponins to diets supplemented with astaxanthin, improves astaxanthin absorption and utilization.

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## **Introduction**

Coloration, body shape, fin shape, and body size are all important qualities that affect the market value of ornamental fish. Blood parrot fish are derived from the hybridization of male *Vieja synspila* (Hubbs, 1935) and female *Amphilophus citrinellus* (Günther, 1864). These fish are among the most important ornamental fish in China. Blood parrot fish have higher ornamental value than their parents because of their blood-red body color, rounded body, and heart-shaped mouth. Demand and high prices for blood parrot fish depends on red body pigmentation. Like other animals without the ability to perform de novo synthesis of carotenoids (Johnson and An 1991), blood parrot fish depend entirely on dietary supplements to achieve their blood-red body color. Suitable colorants could improve the body color of blood parrot fish.

Astaxanthin is a red carotenoid commonly found in marine and aquatic animals. It functions in pigmentation and benefits animal production and health by scavenging free radicals and quenching singlet oxygen (Miao et al. 2006). As an antioxidant, it is more effective than other carotenoids, such as  $\beta$ -carotene, lutein, canthaxanthin, and zeaxanthin (Lim et al. 2002). However, the chemical is unstable and easily oxidized (Yuan et al. 2008). Astaxanthin also has poor utilization and supplementation is very expensive. There is a need to identify feed compounds that promote effective coloring with utilization of astaxanthin.

Skin pigmentation in fish is affected by intrinsic factors such as species, age, weight, and growth stage such as courting. Body pigmentation is also affected by extrinsic factors such as light, temperature, and feeding rate (Wang and Shao 2008). Absorption and transport of astaxanthin in the blood determines the fish pigmentation (Barbosa et al. 1999). Coloring feed synergists, such as fat (Torrissen et al. 1990), vitamins (Liu et al. 2005) or sodium taurocholate (Yang et al. 2012), can improve the utilization rate of carotenoid. However, fat added to the feed can become rancid, vitamins are easily oxidized and sodium taurocholate is difficult to extract. To improve the utilization rate of astaxanthin, absorption and deposition rate of astaxanthin could be increased by adding lipid metabolism regulators to the diet. However, this may decrease the amount of oxidized carotenoid in the deposition process and requires supplementation with antioxidants. Further studies are needed to find ways to improve pigmentation efficiency and reduce coloring feed costs.

Alfalfa saponins are active substances with unique biological properties. Pentacyclic triterpenoid extracted from alfalfa has improved animal production (Peng et al. 2011). Alfalfa saponins have medicinal value (Sun et al. 2013) as they scavenge free radicals (Ilsley et al. 2005), promote lipid metabolism (Cookson and Fedoroff 1968), and function as antioxidants (Yalinkilic and Enginar 2008). However, there are no reports of their use as additives to promote absorption and pigmentation of astaxanthin in blood parrot fish or other aquatic animals. This study investigated the effect of alfalfa saponins as a coloring synergist to enhance pigmentation in blood parrot fish.

## **Materials and Methods**

We obtained 540 blood parrot fish (average weight  $34.6 \pm 0.79$  g, total length  $6.96 \pm 0.46$  cm) from an ornamental fish farm at the Pearl River Fisheries Research Institute. The fish had uniform body color of more than 95% faded surface area. The fish were acclimatized to the experimental conditions for two weeks before the experiment. During this period, they were fed a basal diet (Guangzhou Tai Feng Co., Ltd, Guangzhou, China) containing 8.6% fat. The astaxanthin (BASF, Germany) used in the study was 10% pure. The alfalfa saponins (Baoen Bio, Hebei, China) were 20% pure.

540 blood parrot fish were divided into six treatment groups. Each group consisted of three aquaria containing 30 fish per aquarium. The following 6 diet treatments were tested: (A) basal diet; (B) a diet supplemented with 3‰ astaxanthin; three diets with 3‰ astaxanthin plus alfalfa saponins (C) 3‰; (D) 6‰; (E) 12‰; and (F) a diet supplemented with 4‰ alfalfa saponins alone.

Compressed air and an air stone with a cotton filter were used to maintain soluble oxygen and water quality throughout the experiment. Water temperature was constantly maintained at  $27 \pm 1^\circ\text{C}$ . The water was exchanged at 1/3 volume, and feces was siphoned out three times a week. During the experimental period the fish were fed twice a day (9:00

and 16:00) with a diet equal to 3% body weight per feed.

### Methods

Carotenoids were extracted from scales, skin, and caudal fins based on the study by Boonyaratpalin et al. (2001), with some modifications.

Six fish were randomly sampled from each aquarium and were used for carotenoid analysis at days 20, 40, 60 and 80. The analyses were performed in triplicate. The fish were oven dried at 35°C for 1.5 hours. Caudal fin (0.5 g), scales around lateral line, and skin without scales, were taken. Scales and skin were weighed and placed into 1.5mL centrifuge tubes with 0.01g anhydrous sodium sulfate. Then 1mL acetone was added to each tube. The tissues were cut into pieces. Each sample was mixed thoroughly before each analysis.

Each sample was transferred into a 15 mL centrifuge tube and held overnight. The combined extracts were centrifuged at 4000 rpm for 5 min. The clear supernatant was made up to a cuvette volume (3.5mL). The UV/visible absorption spectrum was recorded against a blank of acetone. Carotenoid concentration was determined as astaxanthin equivalents, from the absorbance at  $\lambda_{max}$  ( $\approx 480$  nm). The carotenoid content was calculated using the following formula:

$$S = (A \times K \times V) / (E \times G)$$

where S is the carotenoid content (mg/kg); A is the absorbance; K is a constant ( $10^4$ ); V is the volume of extracting solution (mL); E is the extinction coefficient (2500); and G is the weight of the sample (g).

Recommendations of the International Commission on Illumination (CIE 1976) denote the color parameters: L\* for lightness, a\* for red/green chromaticity, and b\* for yellow/blue chromaticity. Body color of the fish was measured by a CR-400 (KONICA MINOLTA, Japan) color meter. Every 20 days during the experimental period, 18 blood parrot fish per group were randomly selected. Body color between the abdominal and dorsal regions and caudal fin of the fish was measured.

The following physiological and biochemical parameters were tested using a Nanjing Jiancheng biochemical kit: cholesterol, triglycerides in blood, non-esterified fatty acids and protein in liver and liver lysozyme activity.

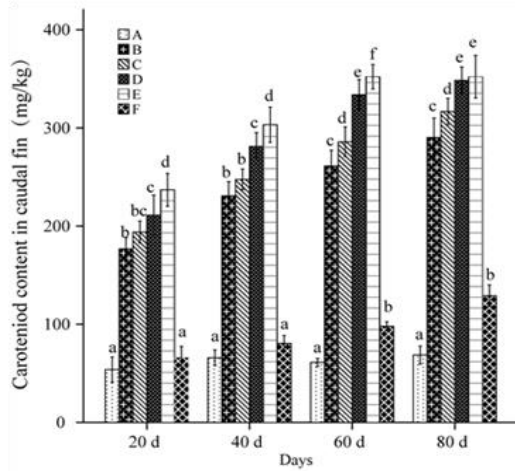
### Data Analysis

Statistical analysis was performed by SPSS Statistics 21.0 and MATLAB R2011a. A general linear model procedure was applied to perform one-way analysis of variance (ANOVA). Results are presented as means $\pm$ SD (n=3). Statistical significance was indicated when  $P < 0.05$ .

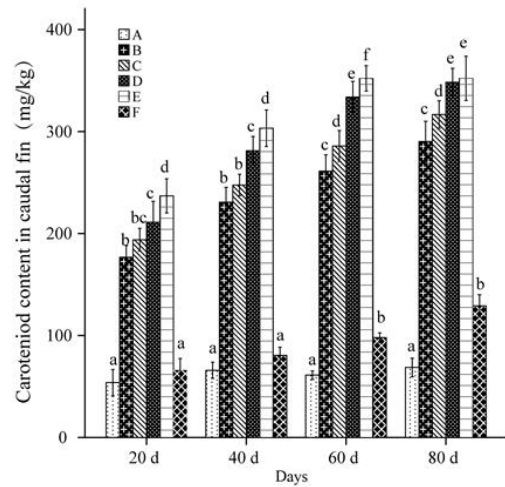
### Results

*The carotenoid content in scales, skin, and caudal fins.* The carotenoid content in the scales, skin, and caudal fins of the fish fed diets containing both alfalfa saponins and astaxanthin was measured every 20 days. The carotenoid content of the fish fed a diet containing both alfalfa saponins and astaxanthin increased with the increase of alfalfa saponins. However, the carotenoid content of the scales in group E was lower than group F at the 80th day. The carotenoid content of the skin (Fig. 1) and caudal fins (Fig. 2) in group D were 48.45 mg/kg and 211.18 mg/kg, respectively. These levels were higher than the 39.80 mg/kg in skin and 176.81 mg/kg in caudal fins found in group B at day 20. The carotenoid content in scales was 188.72 mg/kg (Fig. 3) in group D, and this value was significantly higher than group B with value of 158.14 mg/kg at day 40. The scales, skin, and caudal fins of the fish in group C had a higher total carotenoid content than those in group B at 60 days, and the average values in group C were 223.75, 76.87 and 285.87 mg/kg, respectively. There were significant differences in carotenoid content in the skin after 40 days between group F and group A. The average values for skin were 25.68 and 15.72 mg/kg in group F and group A, respectively. After 60 days, there was a higher carotenoid deposition level found in caudal fins (97.95 mg/kg) and scales (73.07 mg/kg) in group F compared to the control group.

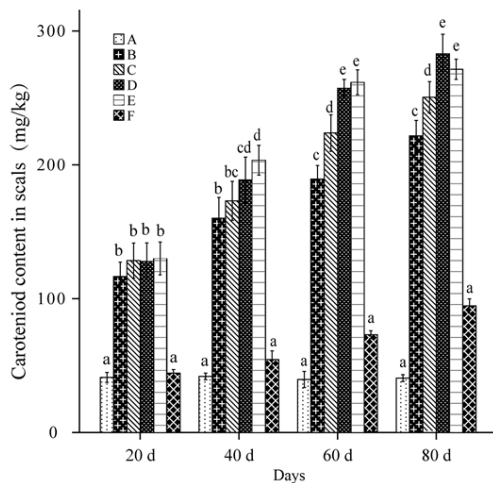
## Efficacy of alfalfa saponins on promoting pigmentation



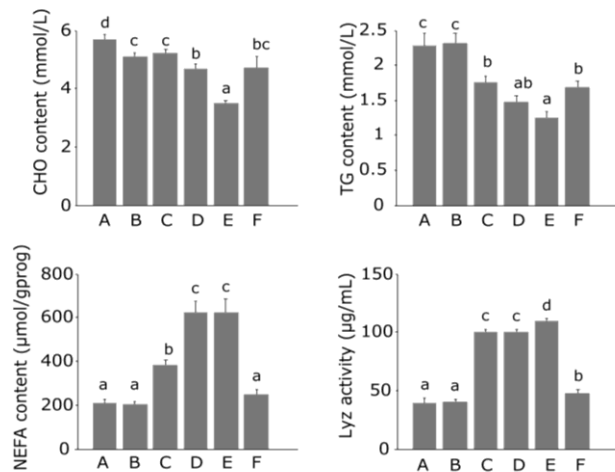
**Fig.1** Carotenoid content of scales of blood parrot fish at 20, 40, 60 and 80 days. A represents a basal diet; B represents a diet with 3‰ alfalfa saponins; C represents a diet with 3‰ alfalfa saponins plus 3‰ astaxanthin; D represents a diet with 3‰ alfalfa saponins plus 6‰ astaxanthin; E represents a diet with 3‰ alfalfa saponins plus 12‰ astaxanthin; and F represents a diet with 4‰ alfalfa saponins alone.



**Fig.2.** Carotenoid content of skin of blood parrot fish at 20, 40, 60, and 80 days.



**Fig.3** Carotenoid content of caudal fin of blood parrot fish at 20, 40, 60 and 80 days. A represents a basal diet; B represents a diet with 3‰ astaxanthin; C, D, and E represent three diets with 3‰ astaxanthin plus 3‰, 6‰, or 12‰ alfalfa saponins, respectively; and F represents a diet with 4‰ alfalfa saponins alone.



**Fig. 4** Effects of diets on physiological and biochemical indicators in blood parrot fish after 80 days. A represents a basal diet; B represents a diet with 3‰ astaxanthin; C, D, and E represent three diets with 3‰ astaxanthin plus 3‰, 6‰, or 12‰ alfalfa saponins, respectively; and F represents a diet with 4‰ alfalfa saponins alone.

## Efficacy of alfalfa saponins on promoting pigmentation

*Effect of diet on scales, skin, and caudal fin color.* The chroma values of the body and caudal fins of the fish were measured every 20 days (Tables 1 & 2). The body lightness  $L^*$  of the fish fed diets containing both alfalfa saponins and astaxanthin was significantly lower than group B after 60 days. The body lightness  $L^*$  in group F was significantly lower than the control group (A) at 80 days. Groups D and E had higher redness values than group B at 60 days. No significant difference was found between group F and group A. The yellowness  $b^*$  value was significantly lower in group F than in control group A after 60 days. Only the value of  $b^*$  in group D was lower than group B after 80 days.  $L^*$  value in caudal fins in groups A and F was significantly higher than in the other groups.  $L^*$  value was lower in caudal fins in groups D and E than group B at 60 days.  $L^*$  value in caudal fins was lower in group F showed than the control group A at 80 days. At 20 days, the redness  $a^*$  in group E was significantly higher than group B. Additionally, group F had significantly higher  $a^*$  value than group A at 40 days. We observed significantly higher  $a^*$  values in groups D and E than group B after 60 days. There were no significant differences in caudal fin yellowness between diets supplemented with alfalfa saponins plus astaxanthin and astaxanthin alone.

*Effect of diets on physiological and biochemical indicators.* After 80 days cholesterol, triglycerides, non-esterified fatty acids, and lysozyme activity were all examined in fish fed experimental diets (Fig 5). The cholesterol levels were 4.67 and 3.49 mmol/L in group D and E, respectively, and were significantly lower than the group B value of 5.08 mmol/L.

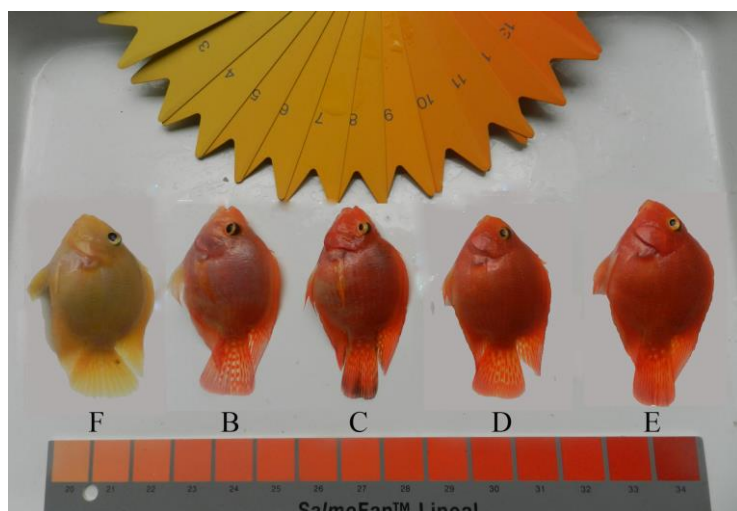


Fig 5. From left to right: (F) a diet with 4‰ alfalfa saponins; (B) a diet with 3‰ astaxanthin; and 3 diets with 3‰ astaxanthin plus (C) 3‰; (D) 6‰; (E) 12‰, alfalfa saponins, respectively.

The triglyceride content decreased with increasing alfalfa saponins in the diets with astaxanthin. Triglyceride content in group C, D, and E was lower than group B, which had values of 1.76, 1.48, 1.25 and 2.31 mmol/L, respectively. The non-esterified fatty acid content in groups C, D, E, and B, was 382.82, 761.94, 895 and 204.09  $\mu\text{mol/g}$ , respectively. In groups C, D and E there was a significant difference in non-esterified fatty acids compared to group B. The highest value of the lysozyme activity (109.52  $\mu\text{g/mL}$ ) was found in fish fed a diet supplemented with alfalfa saponins plus astaxanthin. The group fed a diet of astaxanthin alone averaged 40.16  $\mu\text{g/mL}$ . Data indicated that there was a significant effect on lysozyme activity. Cholesterol, triglycerides, non-esterified fatty acids, and lysozyme activity were examined after the fish were fed experimental diets for 80 days (Fig 4). Cholesterol values were 4.67 and 3.49 mmol/L in groups D and E, respectively. This value was significantly lower than that of group B (5.08 mmol/L). Triglyceride content decreased with increasing alfalfa saponins in the diets with astaxanthin. Triglyceride content in groups C, D, and E was lower than in group B and the values were 1.76, 1.48, 1.25 and 2.31 mmol/L, respectively. Non-esterified fatty acid content in groups C, D, E, and B was 382.82, 761.94, 895 and 204.09  $\mu\text{mol/g}$ , respectively. Groups C, D, and E were significantly different from group B. The highest value of lysozyme activity in fish fed a diet supplemented with alfalfa saponins plus astaxanthin reached 109.52  $\mu\text{g/mL}$ . The value of lysozyme in the diet with astaxanthin alone was 40.16  $\mu\text{g/mL}$ . There was a significant effect on lysozyme activity.

**Table 1.** Effect of the diets supplemented with alfalfa saponins plus astaxanthin on the lightness (L\*), redness (a\*) and yellowness (b\*) of the body surface after 20, 40, 60 and 80 days.

Dietary treatment	L*				A*				B*			
	20d	40d	60d	80d	20d	40d	60d	80d	20d	40d	60d	80d
A	52.67±0.97 <sup>a</sup>	52.46±0.66 <sup>b</sup>	52.21±0.78 <sup>bc</sup>	54.48±1.10 <sup>d</sup>	0.82±0.41 <sup>a</sup>	2.76±0.69 <sup>a</sup>	1.93±1.18 <sup>a</sup>	0.55±1.27 <sup>a</sup>	25.42±1.36 <sup>a</sup>	21.62±1.03 <sup>ab</sup>	15.37±0.98 <sup>b</sup>	15.72±0.99 <sup>a</sup>
B	52.06±0.86 <sup>a</sup>	50.08±0.81 <sup>a</sup>	54.34±3.06 <sup>c</sup>	47.06±1.40 <sup>c</sup>	8.73±0.80 <sup>bc</sup>	17.28±0.81 <sup>b</sup>	20.25±1.24 <sup>b</sup>	22.50±0.95 <sup>b</sup>	23.43±2.14 <sup>a</sup>	26.59±1.56 <sup>b</sup>	29.38±1.74 <sup>c</sup>	27.66±2.32 <sup>b</sup>
C	53.30±0.94 <sup>a</sup>	49.62±0.82 <sup>a</sup>	49.40±0.84 <sup>b</sup>	43.01±1.19 <sup>b</sup>	8.56±0.68 <sup>b</sup>	15.16±1.17 <sup>b</sup>	22.44±0.80 <sup>bc</sup>	24.51±0.88 <sup>bc</sup>	24.25±1.08 <sup>a</sup>	24.87±0.93 <sup>b</sup>	28.54±1.52 <sup>c</sup>	23.73±1.76 <sup>b</sup>
D	52.66±1.31 <sup>a</sup>	50.52±0.96 <sup>c</sup>	44.71±0.65 <sup>a</sup>	39.49±0.49 <sup>a</sup>	7.73±0.91 <sup>b</sup>	15.43±0.73 <sup>b</sup>	23.45±0.81 <sup>c</sup>	25.62±0.86 <sup>cd</sup>	24.13±1.60 <sup>a</sup>	24.74±1.42 <sup>b</sup>	26.37±1.75 <sup>c</sup>	16.81±0.97 <sup>a</sup>
E	51.80±0.77 <sup>a</sup>	50.38±0.98 <sup>ab</sup>	42.24±0.96 <sup>a</sup>	38.93±0.69 <sup>a</sup>	10.48±0.91 <sup>c</sup>	15.85±0.73 <sup>b</sup>	23.97±1.04 <sup>c</sup>	27.16±0.43 <sup>d</sup>	23.18±1.20 <sup>a</sup>	24.25±1.03 <sup>b</sup>	27.96±1.05 <sup>c</sup>	26.34±1.27 <sup>b</sup>
F	52.01±1.23 <sup>a</sup>	49.03±0.71 <sup>a</sup>	49.06±0.72 <sup>c</sup>	49.18±1.06 <sup>c</sup>	-0.34±0.96 <sup>a</sup>	2.52±0.15 <sup>a</sup>	1.73±0.83 <sup>a</sup>	1.76±0.90 <sup>a</sup>	24.81±1.65 <sup>a</sup>	18.86±1.22 <sup>a</sup>	13.18±1.76 <sup>a</sup>	15.07±1.33 <sup>a</sup>

Values are the means ± SD. The means with different superscript letters are significantly different (t<0.05)

A: basal diet without astaxanthin; B: basal diet with 3‰ astaxanthin; C: basal diet with 3‰ astaxanthin plus 3‰ alfalfa saponins; D: basal diet with 3‰ astaxanthin plus 6‰ alfalfa saponins; E: basal diet with 3‰ astaxanthin plus 12‰ alfalfa saponins; F: basal diet with 4‰ alfalfa saponins.

**Table 2.** Effect of the diets supplemented with alfalfa saponins plus astaxanthin on the lightness (L\*), redness (a\*) and yellowness (b\*) of the caudal fin surface after 20, 40, 60 and 80 days.

Dietary treatment	L*				a*				b*			
	20d	40d	60d	80d	20d	40d	60d	80d	20d	40d	60d	80d
A	68.51±0.33 <sup>a</sup>	69.06±0.53 <sup>b</sup>	70.56±1.00 <sup>d</sup>	70.72±1.41 <sup>d</sup>	13.19±2.00 <sup>a</sup>	10.54±0.86 <sup>a</sup>	8.86±0.92 <sup>a</sup>	9.19±0.75 <sup>a</sup>	47.70±2.06 <sup>ab</sup>	45.46±1.48 <sup>ab</sup>	43.69±1.21 <sup>a</sup>	43.29±1.54 <sup>a</sup>
B	68.61±0.77 <sup>a</sup>	64.34±1.21 <sup>a</sup>	61.06±1.21 <sup>c</sup>	57.32±1.42 <sup>b</sup>	24.89±1.39 <sup>b</sup>	28.20±1.29 <sup>c</sup>	32.38±1.33 <sup>c</sup>	37.12±1.85 <sup>c</sup>	44.14±1.71 <sup>a</sup>	45.12±1.73 <sup>ab</sup>	45.87±1.87 <sup>a</sup>	44.54±1.56 <sup>a</sup>
C	68.02±0.49 <sup>a</sup>	64.50±0.54 <sup>a</sup>	60.06±1.24 <sup>bc</sup>	54.25±1.03 <sup>b</sup>	23.90±1.42 <sup>b</sup>	29.93±0.93 <sup>cd</sup>	32.92±1.25 <sup>cd</sup>	35.61±1.69 <sup>c</sup>	42.92±1.74 <sup>a</sup>	50.64±1.11 <sup>c</sup>	43.22±1.74 <sup>a</sup>	41.32±2.04 <sup>a</sup>
D	68.34±0.49 <sup>a</sup>	64.56±0.85 <sup>a</sup>	57.91±0.86 <sup>b</sup>	56.07±1.13 <sup>b</sup>	22.51±1.48 <sup>b</sup>	29.36±1.02 <sup>c</sup>	36.33±0.82 <sup>de</sup>	42.60±1.48 <sup>d</sup>	42.78±2.05 <sup>a</sup>	46.12±1.83 <sup>ab</sup>	46.69±1.52 <sup>a</sup>	43.93±1.65 <sup>a</sup>
E	67.20±0.54 <sup>a</sup>	63.67±0.49 <sup>a</sup>	49.35±0.86 <sup>a</sup>	48.96±0.93 <sup>a</sup>	30.59±1.26 <sup>c</sup>	32.93±1.01 <sup>d</sup>	38.55±1.25 <sup>e</sup>	44.62±0.83 <sup>d</sup>	49.13±1.35 <sup>b</sup>	49.46±1.41 <sup>bc</sup>	44.84±1.10 <sup>a</sup>	52.48±1.28 <sup>a</sup>
F	67.32±0.69 <sup>a</sup>	68.51±0.75 <sup>b</sup>	68.77±0.92 <sup>c</sup>	65.79±1.25 <sup>c</sup>	14.43±1.52 <sup>a</sup>	14.35±1.58 <sup>b</sup>	13.17±1.58 <sup>b</sup>	14.54±1.63 <sup>b</sup>	49.25±1.54 <sup>b</sup>	44.48±1.66 <sup>a</sup>	46.59±1.37 <sup>a</sup>	45.23±2.35 <sup>a</sup>

Values are the means ± SD. The means not bearing the same superscript letters are significantly different (t<0.05).

A: basal diet without astaxanthin; B: basal diet with 3‰ astaxanthin; C: basal diet with 3‰ astaxanthin plus 3‰ alfalfa saponins; D: basal diet with 3‰ astaxanthin plus 6‰ alfalfa saponins; E: basal diet with 3‰ astaxanthin plus 12‰ alfalfa saponins; F: basal diet with 4‰ alfalfa saponins.

### **Discussion**

Many factors including fat, protein, and vitamins can affect carotenoid absorption in fish (Leng and Li 2006). The highest increase in flesh pigmentation occurred when Atlantic salmon were fed high-fat feeds (Young et al. 2006). Optical pigment density of the fins and skin of red *Carassius auratus* was significantly higher than in other groups when the crude protein content was 36.20% (Huang et al. 2008). Vitamin E and carotenoid have similar absorption processes and can be absorbed together. Vitamin E also improves the ability of  $\beta$ -carotene to be transported into plasma (Wang et al. 1995). Fat is subject to becoming rancid, and protein increases feed costs. Vitamins are easily oxidized. In the present experiment the carotenoid content in scales, skin, and caudal fins was lowest in group C which had the lowest content of alfalfa saponins. Values were significantly higher than the control group, indicating that alfalfa saponins are an effective natural additive to diets for ornamental fish.

In caudal fins and skin, there was a significant difference in the carotenoid content between groups C and B at 20 days. Carotenoid was initially deposited in caudal fins and skin, and then in the scales. In fish fed bait containing 65 mg/g canthaxanthin for 24 weeks, carotenoids were primarily deposited in the muscles before accumulating in the skin, (Metusalach et al. 1996). Carotenoid content in the skin was significantly different between groups F and A at 40 days. These differences appeared later when the diet was supplemented with alfalfa saponins and astaxanthin. This suggests that the carotenoid combined differently with lipoprotein carriers and resulted in different deposition sites and amounts. At the end of the experiment pigmentation levels in the caudal fins and scales did not accumulate. This suggests that the tissues were already saturated.

Our results demonstrate that the lightness ( $L^*$ ) value decreased and the redness ( $a^*$ ) value increased on both body and caudal fins in the groups fed diets containing both alfalfa saponins and astaxanthin. But the yellowness ( $b^*$ ) value in the body decreased when alfalfa saponins were added. There was no impact on the yellowness value in the caudal fin. The  $a^*$  value was consistent with the carotenoid content. The  $a^*$  values of Atlantic salmon (*Salmo salar*) muscle were related to the amount of astaxanthin in skin and muscle (Yagiz et al. 2010). As supplementation time increased, the skin redness value increased. This could be attributed to the increase of esterified astaxanthin deposition in skin (Pu et al. 2010; Sevdan Yilmaz et al. 2013). This suggests that alfalfa saponins can increase the amount of astaxanthin in the caudal fin and body, and this may reflect the rate of absorption and utilization of astaxanthin in the body, scales, skin, and caudal fin in blood parrot fish. This is in agreement with Wathne (1998) who tested alternate frequency feedings with and without supplemental astaxanthin concluding that maximum pigment deposition is obtained by frequent feeding of diets containing additional astaxanthin. The addition of both alfalfa saponins and astaxanthin led to significantly lower lightness values than in group B (no supplemented alfalfa saponins), but the yellowness of the body was higher. However, there was no significant difference compared with group B. The  $a^*$  values were significantly higher than group B. Our observation that  $L^*$  negatively correlated with  $a^*$  and  $b^*$  on the surface of the fish is consistent with reports that the  $L^*$  value of muscle of fish fed with astaxanthin decreased, while  $a^*$  and  $b^*$  gradually increased during a 42 day experiment (Choubert et al. 2009). Carotenoid (which the redness value  $a^*$  represented) was a type of carotenoid in erythrophores, but the carotenoid content was all the carotenoid in the tissue. The interaction of skin carotenoids and light-reflective guanine crystals may control the color and brightness of the fish (Brown et al. 2013).

When compared with the diet supplemented with astaxanthin alone, cholesterol and triglyceride content decreased but the non-esterified fatty acid content and lysozyme activity increased in fish fed diets supplemented with astaxanthin and alfalfa saponins. The saponins had a beneficial effect on the immune system, stimulating immune cells and increasing antibody production and proliferation of immune cells (Lacaille-Dubois et al. 1999). Triglyceride and cholesterol content was reduced and immune performance was improved in weaned pigs after being fed alfalfa saponins (Wang et al. 2011). Cholesterol metabolism was enhanced in the liver of hyperlipidemic rats fed 240 mg/kg



alfalfa saponins (Wang et al. 2012). In addition to enhancing pigmentation, saponins may enhance fish immune performance and metabolism.

Non-esterified fatty acids (NEFA) are generated from hydrolysis of triglycerides in adipose tissue (fat mobilization). The majority of fatty acids are supplied to the liver to form lipoprotein (Meng and Yin 1999). Fatty acids are absorbed by the small intestine in free form and are combined with lipoprotein for transport after entering the blood. Carotenoids in foods are hydrolyzed and digested after extraction in the digestive tract (Barbosa et al. 1999). Therefore, the increase of NEFA may increase lipoprotein levels and allow more astaxanthin to be transported to tissues. In this study, the NEFA content increased significantly compared with the diet containing astaxanthin alone. This conclusion is consistent with our results of the carotenoid content and the redness values.

### Conclusion

This study suggests that the addition of alfalfa saponins to diets containing astaxanthin may regulate lipid metabolism and improve utilization of astaxanthin in blood parrot fish. The results indicate that the role of alfalfa saponins is worthy of further development and application. Determination of the optimal dosage requires further study.

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### References

- Barbosa M., Morais R., and G. Choubert**, 1999. Effect of carotenoid source and dietary lipid content on blood astaxanthin concentration in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 176, 331-341.
- Boonyaratpalin M., Thongrod S. Supamattaya K., Britton G. and L. Schlipalius**, 2001. Effects of  $\beta$ -carotene source, *Dunaliella salina*, and astaxanthin on pigmentation, growth, survival and health of *Penaeus monodon*. *Aquacult. Res.*, 32, 182-190.
- Brown A. C., Mcgraw KJ., and E. D. Clotfelter**, 2013. Dietary carotenoids increase yellow nonpigment coloration of female convict cichlids, *Amantitlania nigrofasciata*). *Physiol Biochem Zool*, 86, 312-22.
- Choubert G., Cravedi J.-P. and M. Laurentie**, 2009. Effect of alternate distribution of astaxanthin on rainbow trout (*Oncorhynchus mykiss*) muscle pigmentation. *Aquaculture*, 286, 100-104.
- Cie**, 1976. Official Recommendations on Uniform Colour Space, Colour Difference Equations and Metric Colour Terms. Suppl. No. 2 to CIE Publication No.15, Colorimetry. Commission International del' Eclairage, Paris.
- Cookson F. and S. Fedoroff**, 1968. Quantitative relationships between administered cholesterol and alfalfa required to prevent hypercholesterolaemia in rabbits. *British J Exp. Pathol.*, 49, 348.
- Huang B. F., Xu Z. W. and J. B. Luo**, 2008. The Effects of Protein Levels on the Growth and Deposition of Chilli Pigment in Red *Carassius auratus*. *Freshwater Fish.*, 38, 49-52.
- Ilsley S., Miller H. and C. Kamel**, 2005. Effects of dietary quillaja saponin and curcumin on the performance and immune status of weaned piglets. *J. Anim. Sci.*, 83, 82-88.
- Johnson E. A. and G.-H. An**, 1991. Astaxanthin from microbial sources. *Crit. Rev. Biotechnol.*, 11, 297-326.
- Lacaille-Dubois M. A., Hanquet B., Cui Z. H., Lou Z. C. and H. Wagner**, 1999. A New Biologically Active Acylated Triterpene Saponin from *Silene fortunei*. *J Nat. Prod.*, 62, 133-136.
- Leng X. J. and X. Q. Li**, 2006. The recent advance of aquatic animal pigmentation. *Chinese J. Fish.*, 30, 138-143.
- Lim G. B., Lee S. Y., Lee E. K., Haam S. J. and W. S. Kim**, 2002. Separation of

astaxanthin from red yeast *Phaffia rhodozyma* by supercritical carbon dioxide extraction. *Biochem. Engin. J.*, 11, 181-187.

**Liu J. H., Wang H. L. and W. N. Wang**, 2005. Stability of Total Pigment in Grass Goldfish (II)—Effects of pH, Vitamins and Organic Compounds. *Fish. Sci.*, 24, 7-9.

**Meng D. Y. and X. F. Yin**, 1999. The clinical significance of non-esterified fatty acids and metabolic characteristics of body fat. *Clin. Biochem. Lab. Med. Int. J.*, 20, 156-157.

**Miao F., Lu D., Li Y. and M. Zeng**, 2006. Characterization of astaxanthin esters in *Haematococcus pluvialis* by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Anal. Biochem.*, 352, 176-181.

**Peng B. A., Gao Y. G., Wang C. Z., Wang Y. H., Zhang X. X. and M. P. Han**, 2011. Effects of fresh alfalfa levels and alfalfa meal in the diet on the production performance of sows. *Acta Prataculturae Sinica*, 20, 219-228.

**Pu J., Bechtel P. J. and S. Sathivel**, 2010. Extraction of shrimp astaxanthin with flaxseed oil: Effects on lipid oxidation and astaxanthin degradation rates. *Biosystems Engin.*, 107, 364-371.

**Sun Y., Long R. C., Zhang T. J., Yang Q. C. and H. Zhou**, 2013. Advances in the study of alfalfa saponin. *Acta Prataculturae Sinica*, 22, 274-283.

**Sevdan Yilmaz, Sebahattin Ergün, and Ergün, Nergiz Soytas** 2013. Enhancement of Growth Performance, and Pigmentation in red *Oreochromis mossambicus* Associated with Dietary Intake of Astaxanthin, Paprika and Capsicum. *Isr. J. Aquacult.-Bamidgeh*, IJA\_65.2013.825,1-7

**Torrissen O., Hardy R., Shearer K., Scott T. and F. Stone**, 1990. Effects of dietary canthaxanthin level and lipid level on apparent digestibility coefficients for canthaxanthin in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 88, 351-362.

**Wang C. Z., Wang Y. H., Shi Y. H., Yan X. B., He Y. and W. N. Fan**, 2011. Effects of alfalfa saponins on the lipid metabolism, antioxidation and immunity of weaned piglets. *Acta Prataculturae Sinica*, 20, 210.

**Wang X. D., Marini R. P., Hebuterne X., Fox J. G., Krinsky N. I. and R. M. Russell**, 1995. Vitamin E enhances the lymphatic transport of  $\beta$ -carotene and its conversion to vitamin A in the ferret. *Gastroenterology*, 108, 719-726.

**Wang X. K., Shi Y. H., Wang C. Z., Chen M. L. and D. D. Yuan**, 2012. Effects of Alfalfa Saponins on Cholesterol Metabolism and Its Related Gene Expression in Hyperlipidemic Rats. *Chinese J. Anim. Nutr.*, 24, 983-990.

**Wang X. X. and L. Shao**, 2008. Research status of ornamental fish body color. *Reservoir Fish.*, 28, 57-59.

**Wathne E., Bjerkeng B., Storebakken T., Vassvik V. and A. B. Odland**, 1998. Pigmentation of Atlantic salmon (*Salmo salar*) fed astaxanthin in all meals or in alternating meals. *Aquaculture*, 159, 217-231.

**Yagiz Y., Kristinsson H. G., Balaban M. O., Welt B. A., Raghavan S. and M. R. Marshall**, 2010. Correlation between astaxanthin amount and  $a^*$  value in fresh Atlantic salmon (*Salmo salar*) muscle during different irradiation doses. *Food Chem.*, 120, 121-127.

**Yalinkilic O. and H. Enginar**, 2008. Effect of X-Radiation on Lipid Peroxidation and Antioxidant Systems in Rats Treated with Saponin-containing Compounds. *Photochem. Photobiol.*, 84, 236-242.

**Yang H. Y., Mu X. D., Luo D., Hu Y. C., Song H. M., Liu C. and J. R. Luo**, 2012. Sodium taurocholate, a novel effective feed-additive for promoting absorption and pigmentation of astaxanthin in blood parrot (*Cichlasoma synspilum* ♀ × *Cichlasoma citrinellum* ♂). *Aquaculture*, 350, 42-45.

**Young A., Morris P. C., Huntingford F. A. and R. Sinnott**, 2006. Replacing fish oil with pre-extruded carbohydrate in diets for Atlantic salmon, *Salmo salar*, during their entire marine grow-out phase: Effects on growth, composition and colour. *Aquaculture*, 253, 531-546.

**Yuan C., Jin Z. Y., Xu X. M., Zhuang H. N. and W. Y. Shen**, 2008. Preparation and stability of the inclusion complex of astaxanthin with hydroxypropyl- $\beta$ -cyclodextrin. *Food Chem.*, 109, 264-268.