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Effects of dietary supplementation with algal astaxanthin on growth, pigmentation, and antioxidant capacity of the blood parrot (*Cichlasoma citrinellum* × *Cichlasoma synspilum*)*

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Abstract An algal astaxanthin feeding trial was carried out to investigate the effects of natural astaxanthin from *Haematococcus pluvialis* as feed additives on growth, pigmenting efficacy and antioxidant capacity in blood parrot (*Cichlasoma citrinellum* × *Cichlasoma*. *synspilum*). Tissue total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT) and maleic dialdehyde (MDA) were chosen as measures of its antioxidant capacity. All fish which received an astaxanthin (from micro-algal *H*. *pluvialis*) supplemented diet with 400 mg/kg of astaxanthin, after 50 days of feeding, the astaxanthin-fed fish displayed a pink-colored skin and the control-fed fish displayed a grayish skin. For the growth, the weight gains of control-fed fish and astaxanthin-fed fish were 200% and 300%, respectively. Samples of skin and scales were used for analysis of total carotenoids and astaxanthin content, and fish feeding astaxanthin showed significantly (P<0.05) higher concentrations than the control group, indicating that the pigmentation of this fish had been significantly improved by dietary astaxanthin. Compared with the control fish, pigmented fish had lower SOD, CAT and MDA and higher TAC. It can be concluded that supplementation with dietary astaxanthin could effectively enhance growth, skin coloration and the antioxidant capacity of this fish. This study will provide a reference for application of natural astaxanthin from *H*. *pluvialis* as feed additives in blood parrot artificial breeding. Our data is also useful in ornamental fish farming, especially when the retentivity of astaxanthin in the skin and scales are involved. It is leading to the possibility of increasing the pigmentation of farmed-fish by adding the powdered form of *H*. *pluvialis* to the diet as an effective pigment.

Keyword: astaxanthin; *Haematococcus pluvialis*; pigmentation; antioxidant capacity; blood parrot

1 INTRODUCTION

Blood parrot, is a manmade cross-bred fish hybridized from male *Cichlasoma citrinellum* and female *Cichlasoma synspilum*. In recent years, blood parrot has been becoming one of the most popular ornamental fish in many countries for its bright red appearance, plump body, especially in China. Besides their body shape, fin shape and size, acceptability of ornamental fishes is affected by skin pigmentation, which plays an important role in their market price (Li et al., 2008; Yang et al., 2012). However, these fishes are animals which lack the ability to biosynthesize carotenoids de novo and they cannot achieve a red colored pigmentation unless a dietary carotenoid supplement is used. Under the conditions of artificial breeding, the color of blood parrot is very difficult to achieve the bright red level of people's needs. Feeding a diet containing the colorant not only can promote the blood parrot bright color, but also

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LI Feng and HUANG Shuiying contributed equally to this work.

Table 1 Proximate analysis of the control and pigment-supplemented diets

Proximate analysis	Control	Pigment-supplemented
Crude protein (%)	39.80	39.44
Crude fat (%)	5.56	5.67
Ash (%)	13.53	13.25
Moisture (%)	6.02	6.20
Crude fiber (%)	1.88	1.60
Lysine (%)	1.92	1.98
Astaxanthin (mg/kg)	1.60	392.60

improve its ornamental value. Now, several materials including synthetic sources (β-carotene, canthaxanthin, zeaxanthin and astaxanthin) and natural sources (yeast, algae, crustacean meal, pepper, Aztec marigold and plant) have been added to the diet of fishes and crustacea (Torrissen, 1985; Choubert and Storebakken, 1989; Vernon-Carter et al., 1996; Arredondo-Figueroa et al., 2003; Chien et al., 2003; White et al., 2003; Ponce-Palafox et al., 2006; Li et al., 2008).

Astaxanthin is commonly used in aquaculture as pigmentation sources for fish and shrimp (de la Mora et al., 2006). In addition to coloring, but also to promote animal growth, reproduction and improve animal immunity and other functions (Pei et al., 2009). Christiansen et al. (1994) found that astaxanthin was strongly influence the growth of Atlantic salmon. March et al. (1990) reported that intensity of flesh pigmentation was significantly correlated with body weight in coho salmon fish when fed a diet supplemented with astaxanthin. However, the price of astaxanthin is $200 USD per kg for a 10% active product, and the synthetic astaxanthin products in commercial diets represent between 10% and 20% of the total cost of feed (März, 2000; de la Mora et al., 2006). Therefore, research of cheaper and natural alternative sources of astaxanthin is necessary, such as *H. pluvialis*, a green unicellular freshwater alga, which contains a high amount of astaxanthin (1.5%–3.0% dry weight) (Cai et al., 2009; Li et al., 2012).

Much work has been done concerning the influences of dietary astaxanthin pigment supplementation on rainbow trout, Atlantic salmon, seabream and kuruma prawn (Gouveia et al., 1996; Bjerkeng and Berge, 2000; Gomes et al., 2002; Chien et al., 2003; Cui et al., 2009; Mukherjee et al., 2009). However, ornamental fish have received much less attention and few researches have reported the influence of pigments on the antioxidant enzymes in healthy fishes (Mansour et al., 2006; Wang et al., 2006; Cui et al., 2009; Kalinowski et al., 2011; Sheikhzadeh et al., 2012; Zhang et al., 2013). Thus, the objective of this study was to investigate the effects of natural astaxanthin from *H. pluvialis* as feed additives on growth, the pigmenting efficacy and antioxidant capacity in blood parrot (*Cichlasoma citrinellum* × *Cichlasoma*. *synspilum*). We except this study can provide a reference for application of natural astaxanthin from *H. pluvialis* as feed additives in blood parrot artificial breeding.

2 MATERIAL AND METHOD

2.1 Experimental diets

The proximate analysis was used to analysis the feed composition and the analysis result of experimental diets was displayed in Table 1. The pigment-supplemented diet was composed of the basal feed mixture as well as a dry powdered form of *H. pluvialis*, which contains astaxanthin at a target level of 400 mg/kg. The compounds were extruded through a 2-mm-diameter diet press after forming a dough by adding water and the extruded material was then air dried in the dark. The particle size of dried feed was 0.9–1.2 mm. The dried feed was stored according to the method of Wang et al. (2006).

2.2 Fish and feeding trial

The experimental blood parrot fish were held in re-circulation systems (four 500-L glass fiber tanks) maintained at 26–28°C under a 12-h light/dark photoperiod, with dissolved oxygen at $(6–7)\times10^{-6}$, pH at 6.5–7 and an NH_3 content of $(0.1–0.2)\times10^{-6}$. They were randomly assigned into two groups, two parallel groups in each group and 30 fish in each group. All fish were fed the control diet for 20 days to equalize their body astaxanthin content, and then they were fed twice daily with the two experimental diets (3% BW/day). Feces and uneaten food were siphoned off daily and 1/3 of the water was exchanged (Wang et al., 2006). The ration was adjusted at each 10-day sampling. After a 50-day feeding period, all fish received the control diet and 20 days later were sampled to check if the algal pigment had a lasting effect. Samples were freeze dried at -70°C for carotenoid and antioxidant analysis.

2.3 Carotenoid and astaxanthin analysis

Total carotenoids and astaxanthin were extracted from both skin and scale samples based on the method

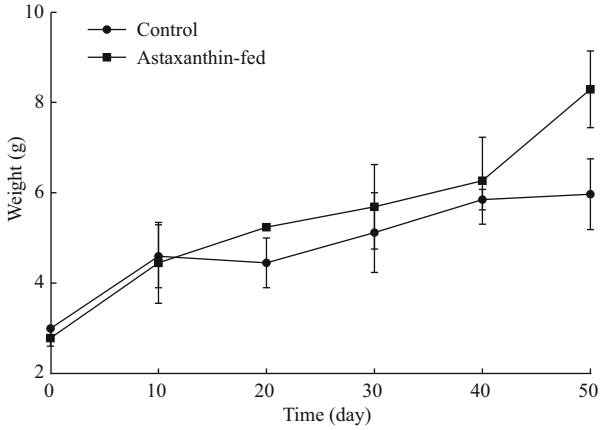

Fig.1 Growth of *Cichlasoma citrinellum* × *Cichlasoma synspilum*

of Barua et al. (1993). The samples were archived and stored at -20°C prior to high-performance liquid chromatography (HPLC).

The HPLC system consisted of a Hitachi L-6200 pump, a Hitachi L-4250 UV-VIS detector at 470 nm, and a Hitachi D-2000 Chromato-Integrator. A silica column (Lichrosorb Si-60 5 micro 250 mm×4.6 mm column I.D., E. Merck Company) was chosen for the separation and analysis of the pigment extracts, and the method was based on Vecchi et al. (1987). The operational conditions were: mobile phase, an isocratic mixture of n-hexane/acetone (80:20 v/v); solvent flow rate, 1.5 mL/min; and a pump program, the sequence of which was 0–20 min Mixture A (acetone:n-hexane, 20:80) and 20.5–40.0 min Mixture B (100% n-heptane). Sample application was performed using an auto injector. The system-controll and the standard astaxanthin were the same as Chien et al. (2003).

2.4 Analysis of antioxidant parameters and blood protein

The pretreatment method of muscle samples was according to Puangkaew et al. (2005), and the supernatants were used for antioxidant enzyme assays.

Total antioxidant capacity (TAC), maleic dialdehyde (MDA), SOD and CAT were chosen as measures of fish antioxidant capacity. The different antioxidant parameters were analyzed using kits from Randox Laboratories (Crumlin, Co. Antrim, UK) and spectrophotometry (UV-1700PC; Macylab Instruments Inc., China). The volumes of samples used were 100 μL for SOD, 50 μL for CAT, 200 μL for TAC and MDA. Expression of activities with international enzyme units (U/L). The determine method of tissue protein was described in Wang et al. (2006).

Table 2 Effect of the feeding trial on *Cichlasoma citrinellum* × *Cichlasoma synspilum* zootechnical parameters (average values±SE)

Parameters in blood parrots	Control	Astaxanthin-fed
Body		
Initial body weight (g)	2.98±0.05	2.77±0.18
Final body weight (g)	5.97±0.79	8.30±0.86
Body weight gain (%)	200	300
Skin		
Initial astaxanthin content (mg/kg)	8.14±1.22	8.25±2.18
Final astaxanthin content (mg/kg)	1.70±0.32	14.39±1.60
Ratio of final to initial astaxanthin content	0.21	1.74
Initial total carotenoids content (mg/kg)	9.19±1.89	9.40±1.71
Final total carotenoids content (mg/kg)	5.42±0.32	17.34±2.76
Ratio of final to initial total carotenoids content	0.59	1.84
Scales		
Initial astaxanthin content (mg/kg)	13.77±1.51	15.17±0.58
Final astaxanthin content (mg/kg)	13.62±2.99	31.40±2.04
Ratio of final to initial astaxanthin content	0.99	2.07
Initial total carotenoids content (mg/kg)	15.17±0.66	15.23±1.42
Final total carotenoids content (mg/kg)	14.24±2.37	39.11±1.41
Ratio of final to initial total carotenoids content	0.94	2.56

2.5 Calculations and statistical analysis

A one-way ANOVA (Analysis of Variance) was performed to determine the main effects of astaxanthin on TAC, SOD, CAT, and MDA at each specific time point within each fish group. Duncan's multiple range test was used to compare those parameters within each main effect. A *t*-test was conducted to compare the growth, total carotenoids and astaxanthin concentrations, and the antioxidant activities between the control and astaxanthin-supplemented groups.

3 RESULT

Both the astaxanthin-supplemented and control diets were equally accepted by fish. However, fish fed with the astaxanthin-supplemented diet had a higher growth rate than control-fed fish over the course of the 50 days feeding period (Fig.1). Thus, the weight gains of control-fed fish and astaxanthin-fed fish were 200% and 300%, respectively, after the 50 days rearing (Table 2).

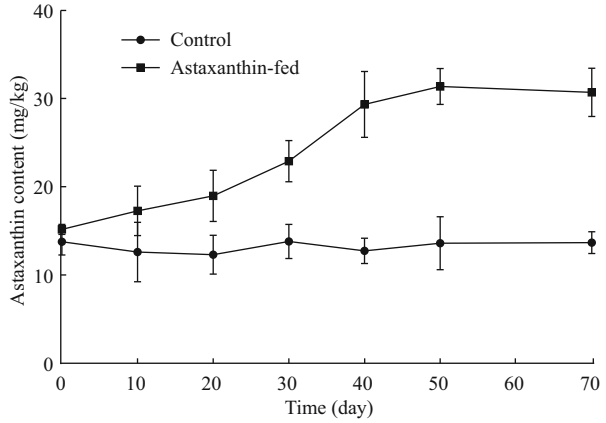

Fig.2 Astaxanthin content in scales of *Cichlasoma citrinellum* × *Cichlasoma synspilum*

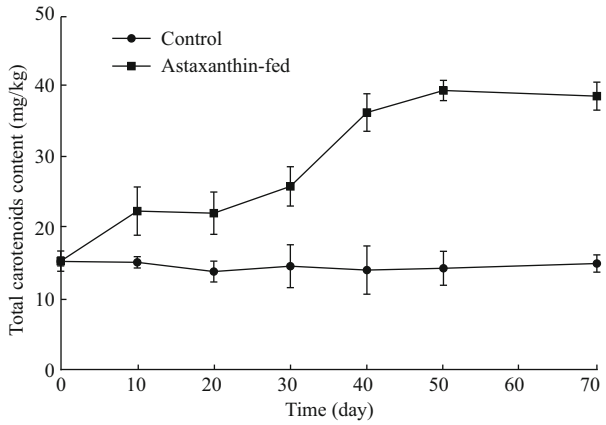

Fig.3 Total carotenoids content in scales of *Cichlasoma citrinellum* × *Cichlasoma synspilum*

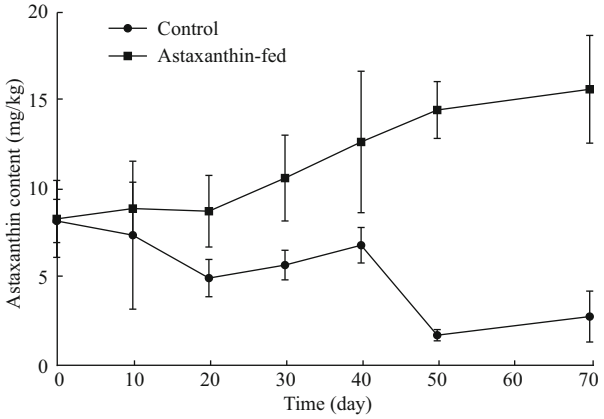

Fig.4 Astaxanthin content in skin of *Cichlasoma citrinellum* × *Cichlasoma synspilum*

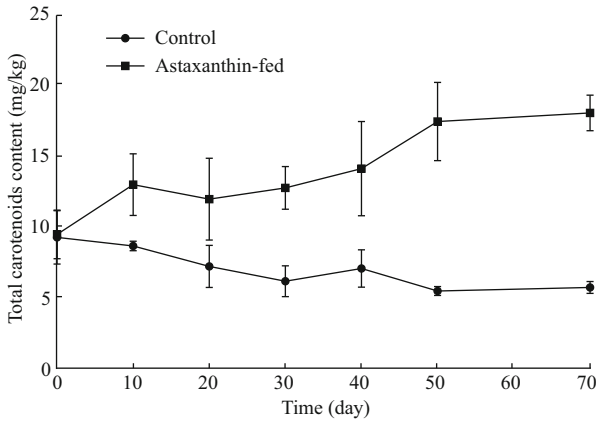

Fig.5 Total carotenoids content in skin of *Cichlasoma citrinellum* × *Cichlasoma synspilum*

After the 50 days rearing, fish fed with the diet containing astaxanthin pigment had significantly higher (P<0.05) concentrations of astaxanthin and total carotenoids (P<0.05) compared to the scales and skin of the control fish (Figs.2–5). Furthermore, compared with astaxanthin-fed fish, the control-fed fish had 87.9% less skin astaxanthin and 67.9% less total carotenoid contents (Figs.4–5), together with 52.2% less scale astaxanthin and 63.3% less total carotenoid contents (Figs.2–3).

In the second rearing period, after all fish had received the control diet for 20 days, neither the concentrations of astaxanthin nor total carotenoids in the fish fed previously with a pigment-supplemented diet showed any significant decrease (Fig.6d).

The TAC, total SOD, CAT and MDA activities in fish tissue are shown in Fig.7. The enzymes activities were significantly affected by dietary supplementation with astaxanthin. Total SOD, CAT and MDA activities of fish in the groups whose diets were supplemented with astaxanthin were lower than those of the fish in the control group. However, the control fish had a lower TAC than the fish fed an astaxanthin-supplemented diet (Fig.7). However, no significant trends were observed between enzyme activities and time.

4 DISCUSSION

4.1 Growth and feed utilization

Many factors can affect fish growth. These factors include the ecological factors (light, temperature, aeration and pH) (Boeuf and Le Bail, 1999; Boeuf and Payan, 2001) and the diet supplied to fish. Our results showed that a positive effect was observed on the growth of fish fed with an astaxanthin supplemented diet. In the study by Christiansen et al. (1995), dietary astaxanthin was found to make a good impact on both the growth and survival of Atlantic salmon (*Salmo salar* L.). Kalinowski et al. (2011) also observed dietary astaxanthin influenced positively the growth of red porgy, the final body

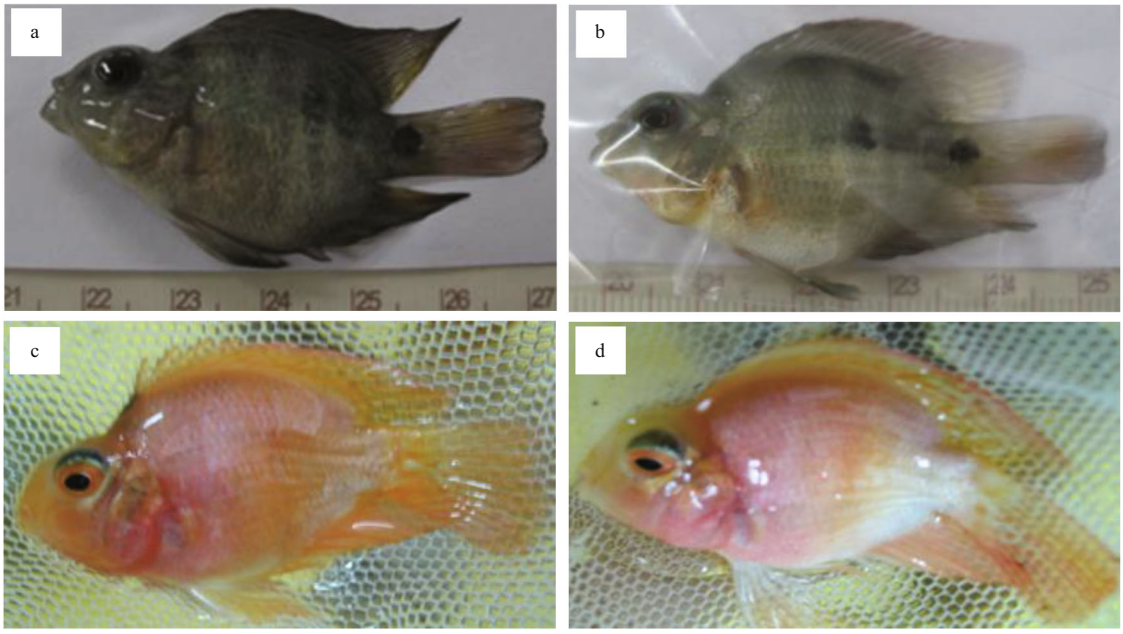

Fig.6 Pigmentation of *Cichlasoma citrinellum* × *Cichlasoma synspilum* with astaxanthin from *Haematococcus pluvialis*

a. the body color before the experiment; b. the body color of control group after 70 days rearing; c. the body color of astaxanthin-fed group after 50 days rearing; d. the body color of astaxanthin-fed group after 70 days rearing.

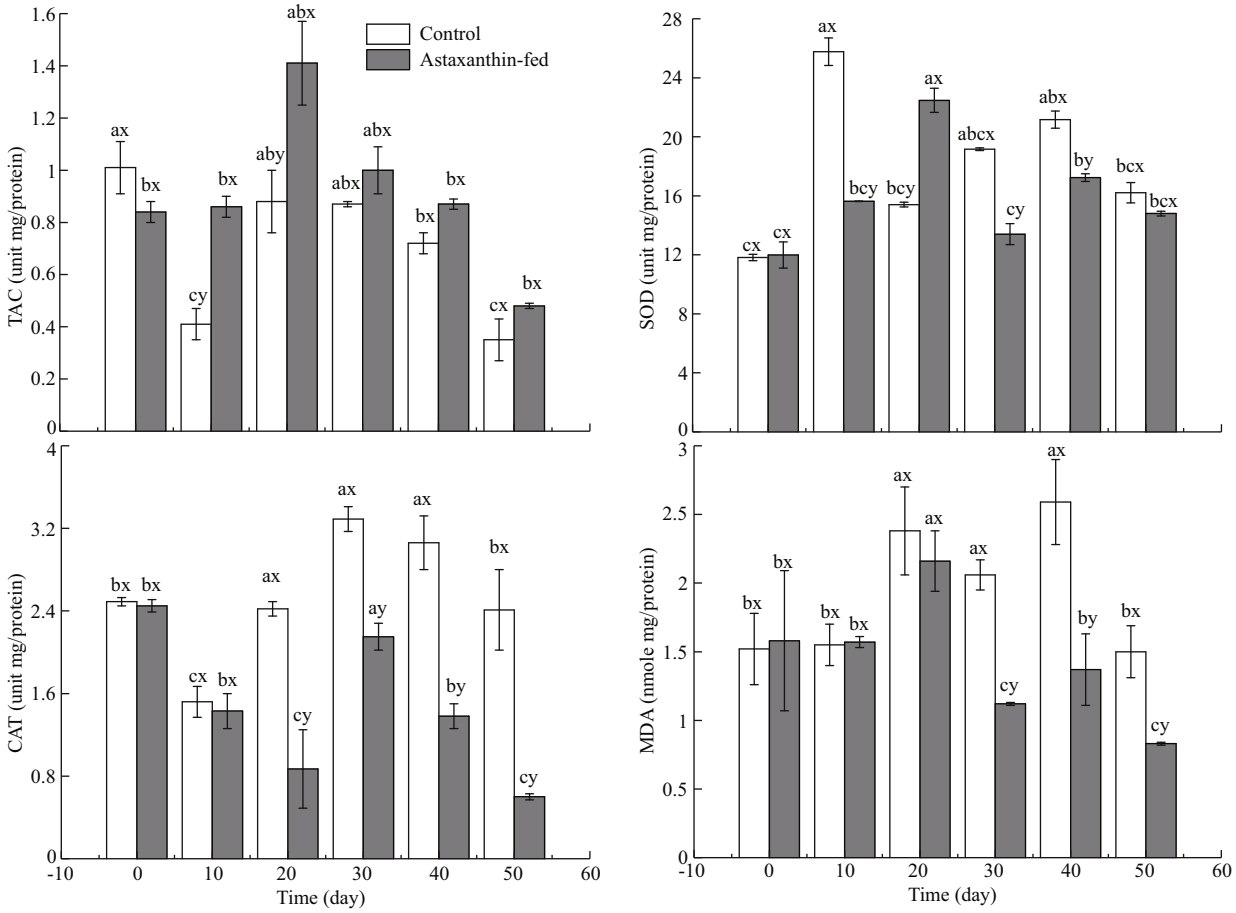

Fig.7 TAS, SOD, CAT and MDA contents of astaxanthin-fed group and control group at each specific time point

Values are means±SE. Values with the letters “a”, “b”, and “c” indicate significant differences among different time points within fish from the same dietary treatment. The letters “x” and “y” indicate significant differences between astaxanthin-fed group and control group at the same time point. Significance level is set at $P<0.05$.

weight, weight gain and eviscerated weight achieved significantly higher values. However, in the study by Gouveia et al. (2003), growth parameters, namely weight gain, sustainable growth rate and feed efficiency are not affected by dietary treatments with astaxanthin (*H. pluvialis*). Wang et al. (2006) also found that there are no differences in growth between characins (*Hyphessobrycon callistus*) in treatment groups fed with astaxanthin, β carotenoid, a 1:1 mixture of astaxanthin and β carotenoid, and the control group. Torrissen et al. (1990) reported that when the fat content of fish feed is 4.1%–23.0%, the digestibility of astaxanthin increases with the increasing concentration of fat. Therefore, various factors should be considered, such as culture conditions, absorption, metabolism and so on, to improve the utilization of astaxanthin by the body and to achieve the best effects.

4.2 Pigmentation

The carotenoid pigment astaxanthin is widely used as dietary supplements in diets for aquaculture industry as a method for inducing the typical red color of the flesh (Torrissen, 1985; Skrede and Storebakken, 1986; Choubert and Storebakken, 1989; Gomes et al., 2002; Gouveia et al., 2003). The pigmenting efficacy of astaxanthin has been observed in several studies. Previous study indicated that rainbow trout were pigmented faster with astaxanthin than with canthaxanthin, and the mean retention coefficient for astaxanthin was 1.3 times higher than for canthaxanthin (Choubert and Storebakken, 1989). For goldfish the best coloring obtained and red hue was maximum when using a diet containing astaxanthin-rich *H. pluvialis* (Gouveia et al., 2003). In our study, feeding 400 mg/kg of astaxanthin can make the content of astaxanthin and total carotenoid in scales and skin of blood parrot increased, and the astaxanthin coloring effect is very significant compared with the control group (Fig.6).

Astaxanthin is the major carotenoid in tissues of blood parrot. This was especially evident in our results: the total carotenoid content of scales in control-fed fish was steady at a low value for 70 days rearing time, and the ratio of astaxanthin to total carotenoid was maintained at a high value at the same time. It is indicated that this fish could reach its full efficiency in astaxanthin deposition without a lag, however, the pathway is unclear. Since the weight and surface area of the fish increase faster than the uptake of carotenoid, carotenoid concentration is diluted with the growth of the animal (Meyers and Latscha, 1997). This dilution effect was also observed in other studies (Menasveta et al., 1993; Pan et al., 1999). In our study, the weight of the control-fed fish was increased by 200%. The carotenoid dilution factor in the skin was 33%, which could partly explain the low ratios of final to initial skin astaxanthin (21%) and carotenoid (59%) contents. In contrast, after the fish were fed with an astaxanthin diet, the astaxanthin and total carotenoids of skin were increased respectively by 174% and 184%. It is showed that when astaxanthin was supplemented in the diet, the astaxanthin deposition in scales was more sensitive than in skin by comparing the ranges of increment in scale astaxanthin (207%) with skin astaxanthin (174%).

Since the level of dietary carotenoid required to maintain body astaxanthin varies with the target tissue and there are no data concerning the dietary concentration of carotenoid reported for this fish, our data may be useful in ornamental fish farming, especially when the retentivity of astaxanthin in the skin and scales are involved. It is leading to the possibility of increasing the pigmentation of farmed-fish by adding the powdered form of *H. pluvialis* to the diet as an effective pigment. Furthermore, lowering the production costs of pigment enhancement has been a dominant preoccupation, but with this new method, the commercial production costs are more affordable.

4.3 Antioxidant capacity

Free radicals are produced during normal aerobic metabolism and their negative effects can be neutralized by an antioxidant defense system under normal physiological conditions. As higher vertebrates, fishes possess two major antioxidant defense systems, including the non-enzymatic system (vitamins and other molecules such as glutathione, etc.) and the superoxide dismutase (SOD) or catalase (CAT) enzymatic system, to scavenge H_2O_2 and lipid hydroperoxides as well as protect the cells from damage by ROS (Winston and Di Giulio, 1991; Martínez-Álvarez et al., 2005; Liu et al., 2010; Halliwell and Gutteridge, 2015). As an indicator of the status of overall antioxidant defenses against reactive oxygen species and reactive oxygen intermediates, TAC expresses well antioxidant capacity, including enzymatic and non-enzymatic systems, which are in a dynamic balance process. Thus, the higher TAC value in the fish fed with astaxanthin supplement compared with the control

suggested a higher antioxidant capacity.

The activity of SOD in tissue and the level of MDA (which is the lipid superoxide) reflect the competence of clearing the free radicals and the severity of cell impairment. CAT is also a primary enzyme for radical scavenging, which is involved in the protective mechanisms within tissue injury following oxidative processes and phagocytosis. Therefore, these three enzymes have been chosen as antioxidant parameters to indicate the status of the organisms affected by dietary astaxanthin supplements (Winston and Di Giulio, 1991). Commonly, higher SOD, MDA and CAT activities suggest that more radicals need to be scavenged (Andersen et al., 1998; Ross et al., 2001). In our study, significantly lower SOD and CAT activities in the fish in treatment group were detected, it indicates that the feed may be having good sequestering activity of free radicals. Thus, it can be concluded that higher TAC value in the fish in treatment group caused by the increase antioxidant capacity of non-enzymatic systems. This can be attributed to astaxanthin's long conjugated double bond-system with relatively unstable electron orbital, which scavenges the oxygen radicals in the cells (Stanier et al., 1971) and thus reduces cellular damage and enhances resistance.

5 CONCLUSION

According to our results, fish that eat astaxanthin has higher growth efficiency than fish in the control group, so we have reason to believe that the addition of astaxanthin in the feed favors the growth of blood parrot fish, although the mechanism is still unclear. In addition, the red color of blood parrot fish can be increased very significant by fed astaxanthin, which was once again confirmed in our experiments. Moreover, the higher TAC value and lower SOD, CAT, MDA values in the blood parrot fish fed with the astaxanthin supplement compared with the control fish, indicated that dietary astaxanthin could increase the antioxidant capacity of healthy fish.

6 DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

7 ACKNOWLEDGMENT

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