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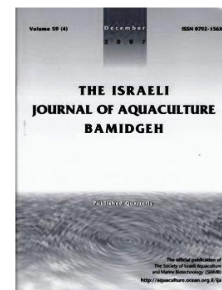

Effects of Dietary Astaxanthin on Growth Performance, Hepatic Antioxidative Activity, hsp70, and HIF-1α Gene Expression of Juvenile Golden Pompano (*Trachinotus ovatus*)

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Keywords: *Trachinotus ovatus; astaxanthin; growth; survival; immunity*

Abstract

The present study was conducted to investigate the effects of different astaxanthin (AST) levels on growth performance, hepatic antioxidative activity, heat shock protein 70 (hsp70), and hypoxia-inducible factor-1 alpha (HIF-1α) gene expression of juvenile golden pompano. Fish (mean initial body weight 5.8±0.05g) were fed six isonitrogenous and isoenergetic diets (AST-0, AST-0.005, AST-0.01, AST-0.05, AST-0.1, AST-0.2) containing various supplemented levels of astaxanthin (0%, 0.005%, 0.01%, 0.05%, 0.1%, 0.2%, respectively) in triplicate for 8 weeks. Growth performance (final body weight, FBW; weight gain, WG), and survival of fish fed diets containing astaxanthin above 0.01% were significantly higher ($P<0.05$) than of fish fed AST-0 and AST-0.005 diets; feed conversion ratio (FCR) showed the opposite trend ($P<0.05$). Fish fed 0.01% astaxanthin diet showed the highest value of survival. Hepatic antioxidant status (total antioxidant status, TAS; superoxide dismutase, SOD; carbonyl protein content) of shrimp improved significantly when dietary astaxanthin was over 0.01%. Relative expression profiles of hepatic hsp 70 mRNA and HIF-1α mRNA increased with increased dietary astaxanthin levels. The relative expression profiles of hepatic hsp 70 mRNA and HIF-1α mRNA of shrimp fed diets containing astaxanthin above 0.01% were obviously higher than those of shrimp fed AST-0 and AST-0.005 diets. Linear regression analysis on WG and HIF-1a mRNA indicated that the optimum dietary AST levels for optimal growth and immunity of juvenile pompano were 0.011% and 0.013%, respectively.

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Introduction

Various carotenoid sources such as synthetic carotenoids (astaxanthin, canthaxanthin and β-carotene), natural plant sources (yeast, bacteria and algae), and animal sources (crustacean meal) have been used as dietary supplements to enhance the pigmentation of fish and crustaceans (Zhou et al., 2016; Gholamreza et al., 2013). Besides pigmentation, increased attention is being directed toward defining the biological function of carotenoids such as increasing growth, antioxidant activity, immunostimulation, and reproduction in aquatic animals. Carotenoids also play a positive role in intermediary metabolism as well as color (Chatzifotis et al.; Talebi et al., 2013).

Marine animals such as shrimp, are unable to perform denovo synthesis of carotenoids, and must obtain them from dietary sources. They can convert synthetic canthaxanthin in feed to deposit in the body as astaxanthin (Boonyarataplin et al., 2001; Niu et al., 2012). Astaxanthin is the predominant pigment in aquatic animals (Sevdan et al., 2012; Okada et al., 1994). Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′dione, AST) is a xanthophyll carotenoid which is found in many microorganisms and marine animals, such as shrimp, crayfish, crustaceans, salmon, trout, krill, microalgae as well as yeast. Its molecular structure consists of 40 carbon atoms, divided into a central portion containing 22 carbon atoms linked with 13 conjugated double bonds and two terminal benzene rings containing hydroxyl and ketone groups, giving rise to the higher polar structure of AST compared with other carotenoids (Britton, 1995). The antioxidant activity of astaxanthin was claimed to be approximately 10 times stronger than β-carotene and 100 times greater than α-tocopherol (Shimidzu et al., 1996; Niu et al., 2009). Previous studies have demonstrated that astaxanthin maintains antioxidant status in tissues (Chien et al., 2003; Supamattaya et al., 2005). AST has also been found to promote growth performance and survival rate in Atlantic salmon (*Salmo salar)* (Storebakken and Goswami, 1996) and red porgy (*Pagrus pagrus)* (Kalinowski et al., 2011), enhances skins coloration in large yellow croaker (*Larimichthys croceus*) (Yi et al., 2014) and Atlantic salmon (*Salmo salar)* (Baker et al., 2002), anti-lipid peroxidation (Leite et al., 2010), and immune response reinforcement in *Astronotus ocellatus* (Alishahi et al., 2015).

However, the role of carotenoids in fish growth is controversial. The above studies reported a positive influence whereas other studies found no effect. Adult Japanese shrimp, *Penaeus japonicus*, fed pigment supplemented meal, showed no significant modification of growth rate at the end of the experimental period (Nègre-Sadargues et al., 1993). Some fish studies have shown that supplementation of astaxanthin lower than 300 mg/kg did not affect growth and survival of red porgy, *Pagrus pagrus,* (Kalinowski et al., 2005), characins (Wang et al., 2006), Japanese ornamental carp (Sun et al., 2012) and goldfish (Dananjaya et al., 2017). Supplementation of 50-200 mg/kg astaxanthin was reported to show no effect on the growth, while dietary 300 or 400 mg/kg astaxanthin significantly decreased the weight gain rate (WG) and increased FCR of discus fish, *Symphysodon* spp., (Song et al., 2017).

Golden pompano (*Trachinotus ovatus* Linnaeus 1758) belongs to family Carangidae, genus *Trachinotus*. It is a warm-water species (25–32^{0}C), and a carnivorous fish that preys mainly on zooplankton, small crustaceans, shellfish, and small fish (Liu and Chen, 2009). *T. ovatus*is is widely distributed in China, Japan, Australia, and other countries (Niu et al., 2013). Pompano, widely farmed owing to its high market price, and resilience to salinity and variations in temperature, is a good candidate for aquaculture (Tutman et al., 2004).

To date there have been no reports on the effects of astaxanthin in commercial diets for *T. ovatus*is. In this study, different levels of commercial astaxanthin (Carophyll Pink, 10% astaxanthin, and 10% β-carotene, DSM Nutritional Products France SAS) were added to the basal diet to investigate the effects on growth and immunity, to develop feed for *T. ovatus*is.

Materials and methods

Experimental diets and diet preparation. Dietary formula and proximate composition are given in Tables 1 & 2. Astaxanthin was added to the basal diet. Astaxanthin (Carophyll Pink, 10% astaxanthin) at 0%, 0.005%, 0.01%, 0.05%, 0.1%, and 0.2%. The method of diet preparation was the same as described by Niu et al. (2016). Briefly, all dry ingredients were finely ground, weighed, mixed manually for 5 min, and then transferred

to a Hobart mixer (A-200T Mixer Bench Model unit, Resell Food Equipment Ltd., Ottawa, Canada) to be mixed for another 15 min. Soya lecithin was added to pre-weighed fish oil, and mixed until homogenous. The oil mix was then slowly added to the Hobart mixer as mixing continued. All ingredients were mixed for a further 10 min; then distilled water (about 30–35%, v/w) was added to form a dough which was then passed through a pelletizer with a 2.5-mm-diameter die (Institute of Chemical Engineering, South China University of Technology, Guangzhou, PR China). The diets were dried until moisture was reduced to less than 10%. The dry pellets were placed in plastic bags and stored -20^{0}C until use.

Table 1 Composition of the basal diet (%).

Ingredients	
fish meal [1]	32
soybean meal [2]	30
wheat flour [2]	19
krill meal [2]	3
fish oil [2]	8
Soya lecithin [2]	2
$Ca(H_2PO_4)_2$	2
Premix-vitamine [3]	1
Premix-mineral [4]	1
Choline	0.5
Vitamin C	0.5
DL-Methionine [5]	0.4
Lys-HCl (78%) [6]	0.6
Sum	100

[1] Imported from N.E.L.T.O.Australia Pty Ltd.
[2] Zhuhai Shihai Feed Corporation Ltd, Zhuhai, China
[3] Vitamin premix (mg or g/kg diet): thiamin, 25 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; vitamin B12, 0.1 mg; vitamin K3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin, 1.20 mg; retinal acetate, 32 mg; cholecalciferol, 5 mg; α-tocopherol, 120 mg; ascorbic acid, 2000 mg; choline chloride, 2500 mg; ethoxyquin 150 mg; wheat middling, 14.012 g (Niu et al., 2013).
[4] Mineral premix (mg or g/kg diet): NaF, 2 mg; KI, 0.8 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $FeSO_4 \cdot H_2O$, 80 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $MnSO_4 \cdot H_2O$, 60 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; $Ca(H_2PO_4)_2 \cdot H_2O$, 3000 mg; NaCl, 100 mg; zoelite, 15.447 g (Niu et al., 2013);
[5] Imported from Evonik Industries AG
[6] Imported from NOVUS

Table 2 Formulation and proximate composition of each diet (% dry matter).

	AST-0	*AST-0.005*	*AST-0.01*	*AST-0.05*	*AST-0.1*	*AST-0.2*
Basal diet	100	99.995	99.99	99.95	99.9	99.8
Astaxanthin	0	0.005	0.01	0.05	0.1	0.2
Proximate composition *						
Moisture	8.28	8.12	8.18	8.20	8.18	8.20
Crude protein	40.63	40.45	40.65	40.39	40.54	40.62
Crude lipid	13.10	13.06	13.29	13.19	13.16	13.16
Ash	9.52	9.47	9.57	9.48	9.52	9.55

* Values (moisture, crude protein, crude lipid, ash) are expressed as means and S.E.M. of three replicates.

Experimental procedures. The feeding trial of 56 days was conducted at an experimental station in South China Sea Fisheries Research Institute of CAFS (Sanya, Hainan). The fish were obtained from a commercial farm near Hongsha Bay, Sanya, Hainan province, China. Prior to the feeding trial, the fish were reared in floating sea cages (3.0 m ×3.0 m ×3.0 m), and fed the control diet (D1) for 2 weeks to acclimate to the experimental diet and conditions. At the start of the experiment, the fish were fasted for 24 h and weighed after being anesthetized with eugenol (1:10,000) (Shanghai Reagent Corp., China). Juvenile pompano (*Trachinotus ovatus*) of similar size (initial body weight approx. 5.88 g) were randomly allotted into 18 sea cages (1.0 m × 1.0 m × 1.5 m), three cages per treatment, each cage stocked with 20 fish. The fish were hand

fed twice daily at 08:30 and 16:30 h respectively, with small amounts to prevent waste of pellets. During feeding, the fish always swim to the surface to ingest the diet but never come to the surface after reaching satiation. Feed consumption for entire experimental period was recorded. Water temperature during the experiment fluctuated between 28-31^{0}C, salinity was 28-31 g/L, and dissolved oxygen was approximately 8.33 mg/L. At the end of experiment, the fish were fasted for 24 h and all the fish from each cage were weighed.

Sampling and preservation. Before the feeding trial, 18 juveniles were randomly sampled and used for analyses of whole-body composition. At the end of the 56-day experiment, 10 fish from each cage were randomly collected for proximate analysis, 4 fish were analysed for whole-body composition, and 6 were anesthetized with eugenol and dissected to retrieve muscle and liver samples. The latter were frozen immediately in liquid nitrogen and stored at -70°C for further analysis.

Diet and fish samples composition analysis. Diets and fish samples were analysed in triplicate for proximate composition. Moisture, crude protein, crude lipid, and ash were determined using standard methods (AOAC, 2001). Moisture was determined by drying in an oven at 105^{0}C for 24 h; crude protein (N×6.25) was analysed by the Kjeldahl method after acid digestion (1030-Auto-analyzer, Tecator, Höganäs, Sweden); crude fat was determined by ether-extraction method by Soxtec System HT (Soxtec System HT6, Tecator, Sweden); crude ash by incineration in a muffle furnace at 550^{0}C for 24 h.

Determination of antioxidant enzyme activity. TAS is a quantitative measurement which represents the total contribution from a wide range of antioxidant molecules (Prior and Cao, 1999). Using TAS to detect the actual antioxidant status in crustaceans has previously been limited to evaluation of the effects of astaxanthin (Pan et al., 2003; Niu et al., 2012). Superoxide dismutase (SOD) is a major antioxidant enzyme, which is responsible for scavenging reactive oxygen species and protecting mechanisms within tissue injury following the radical process and phagocytosis (Pan et al., 2003). To measure TAS and SOD activities, 20 and 25 μl of supernatant samples were used and determined spectrophotometrically at 600 and 505 nm, respectively, with a U-2000 spectrophotometer (Hitachi, Japan) at 37^{0}C using ELISA kits (Randox Laboratories Ltd.) according to the manufacturer's instructions. The TAS and SOD activities were expressed as U/mg protein.

Malondialdehyde and carbonyl protein assays. For an index of lipid peroxidation, we used the formation of Thiobarbituric acid reactive substances (TBARS) during an acid heating reaction, which is a widely adopted method previously described by Draper and Hadley (1990). Results were expressed as malondialdehyde (MDA) equivalents (nmol/mg protein). For protein carbonylation, supernatants were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP) dissolved in 2 M HCl to allow DNTP to bind to the carbonyl groups (Levine et al., 1994). Blanks were run with HCl only. Afterwards, proteins were precipitated with 6% trichloroacetic acid (TCA) and centrifuged for 10 min at 11000 g. The protein pellets were washed three times with ethanol/ethylacetate (1:1), re-suspended in 6 M guanidine hydrochloride, 50% formic acid, incubated at 37°C until complete re-suspension. The carbonyl content was measured spectrophotometrically (Synergy HT, Bioteck) in the resulting suspensions at 370 nm (molar extinction coefficient 22,000 M^{-1} cm^{-1}). Results are expressed as nanomoles of DNPH incorporated mg/protein. The total protein content was determined for each sample according to Lowry et al. (1951).

Relative expression analysis of Hsp70 mRNA and HIF-1α mRNA. Heat shock protein 70 (Hsp 70) is an evolutionarily highly conserved molecular chaperone that is an important part of cell machinery for protein folding, and helps to protect cells from stress (Joly et al., 2010). To understand the antioxidant molecular mechanism of astaxanthin, in this study, the expression level of Hsp70 mRNA and hypoxia inducible factor-1α (HIF-1α) were measured by real-time quantitative PCR in fish fed the six different experimental diets.

Total RNAs were extracted using RNeasy Mini Kit (QIAGEN Cat: no. 74104) according to the manufacturer's instructions, and treated with DNase I (QIAGEN Cat: no. 79254) to remove contaminated DNA. Subsequently, the first-strand cDNA was synthesized based on manufacturer's instruction of PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa DRR037S) using total RNA as template. cDNA mix was diluted to 1:5 and stored at -80^{0}C for subsequent real-time Quantitative RT-PCR. All primers of β-actin (5'-

TACGAGCTGCCTGACGGACA-3', 5'- GGCTGTGATCTCCTTCTGCA-3'), Hsp 70 (5'-TTGAGGAGGCTGCGCACAGCTTGTG-3', 5'-ACGTCCAGCAGCAGCAGGTCCT-3') and HIF-1a (5'-TGCTTTGGACTCTGACCATCT-3', 5'-ATGACAGTGGCTTGGGTTTC-3') were produced by Takara Biotechnology (Dalian) Co., Ltd. (Takara Dalian), and the reaction conditions were also optimized.

Real-time quantitative RT-PCR was performed in a total volume of 20μl containing 10μl of 2×SYBR Green Real-time PCR Master Mix (TaKaRa DRR041A), 1μl of cDNA, 0.16μM of each primer and 8.2μl of double-distilled water. Real-time quantitative polymerase chain reaction (RT-PCR) program consisted of denaturation step at 95^0C for 2 min, followed by 40 amplification cycles of 15 s denaturation at 95^0C, 15 s annealing at 58^0C, 30 s extension at 72^0C. Fluorescence readings were performed at the end of each cycle. To analyze Hsp70 mRNA expression level, the comparative CT method (2–ΔΔCT method) was used. The CT for the target amplified Hsp70 and the CT for the internal control β-actin were determined for each sample. Differences in the CT for the target and the internal control, called ΔCT, were calculated to normalize the differences in the amount of total cDNA added to each reaction and the efficiency of the RT-PCR. The control group was used as the reference sample, called the calibrator. The ΔCT for each sample was subtracted from the ΔCT of the calibrator, the difference was called ΔΔCT. PoGal mRNA expression level was calculated by 2-ΔΔCT, and the value stood for an n-fold difference relative to the calibrator.

Calculations and statistical analysis. The following variables were calculated:

Weight gain (WG) (%) = $100 \times (W_f - W_i) / W_i$

Survival (%) = $100 \times N_t / N_0$

Feed conversion ratio (FCR) = dry feed intake / $(W_t - W_0)$

Where W_f and W_i were mean final and initial fish body weights; N_t is number of fish at the end of the trials and N_0 at the start; W_t (g) is total final body weight and W_0 (g) total initial body weight.

All data are presented as means ±S.E.M. and subjected to one-way analysis of variance (ANOVA) to test the effects of experimental diets using the software of the SPSS for windows (ver 16.0, U.A.S). Duncan's new multiple range test was used to resolve the differences among treatment means (Duncan, 1995). Statistical significance was examined at $P<0.05$ unless otherwise noted. Linear regression model ($Y=a+bX$) (Robbins et al., 1979) was employed to estimate the optimum astaxanthin requirement.

Results

Growth performance. Growth performance of pompano juveniles presented in Table 3 was significantly affected by diet treatments ($P<0.05$). Growth performance (FBW and WG) of fish fed diets containing 0.01%-0.2% astaxanthin were significantly higher than those of fish fed diets containing 0% and 0.005% astaxanthin ($P<0.05$); no significant differences were found in growth performance among AST-0.01, AST-0.05, AST-0.1 and AST-0.2 diets treatments ($P>0.05$). Results showed that FCR was in the range of 1.22-1.75, FCR of fish fed diets containing 0.01%-0.2% astaxanthin were significantly lower than that of fish fed the basal diet ($P<0.05$) but there was no significant difference with fish fed AST-0.005 diet ($P>0.05$). Survival of fish fed AST-0.01 diet showed the highest value and was significantly higher ($P<0.05$) than that of fish fed the basal diet but there was no significant difference ($P>0.05$) in fish fed other diets.

Table 3 Growth performance and survival of golden pompano fed diets with different levels of astaxanthin.

Treatments	*AST-0*	*AST-0.005*	*AST-0.01*	*AST-0.05*	*AST-0.1*	*AST-0.2*
IBW (g)	5.87±0.01	5.87±0.01	5.89±0.01	5.88±0.01	5.87±0.02	5.87±0.02
FBW (g)	77.57±2.77 ^{a}	80.21±6.43 ^{a}	90.79±0.86 ^{b}	91.05±0.22^b	90.22±0.64^b	90.05±2.02^b
WG (%)	1221±48.38^a	1266±106.9 ^{a}	1442±13.70^b	1447±6.47^b	1437±7.76 ^{b}	1434±31.27^b
FCR	1.75±0.31 ^{b}	1.66±0.05 ^{ab}	1.22±0.03 ^{a}	1.27±0.08 ^{a}	1.26±0.10 ^{a}	1.23±0.07 ^{a}
Survival (%)	71.67±9.28 ^{a}	88.33±3.33^{ab}	96.67±3.33 ^{b}	88.33±7.26^{ab}	91.67±6.01^b	88.33±1.67^{ab}

$^{a, b, c, d}$ Values are means ± S.E.M. of three replicates and values in the same row with different letters are significant different ($P < 0.05$).

When linear regression analysis was used as shown in Fig. 1 the optimal requirement of dietary astaxanthin levels for maximal growth of juvenile pompano was 0.011%.

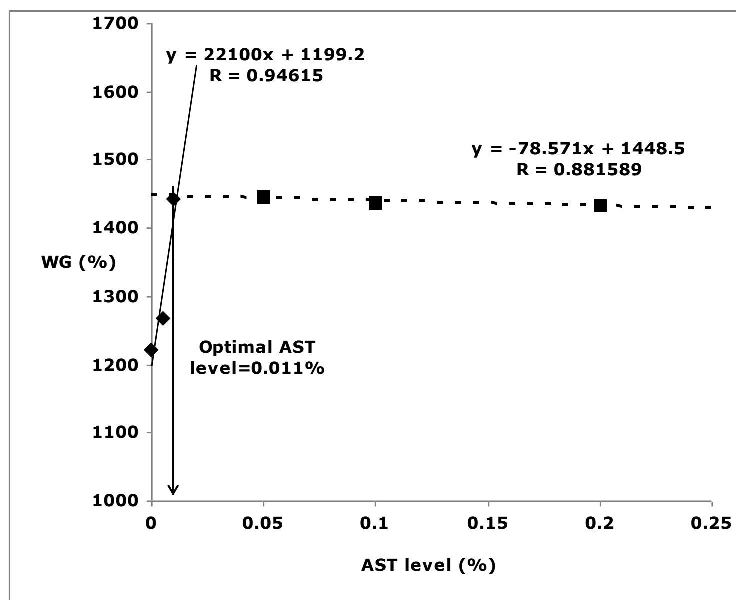

Figure 1 Relationship between dietary AST level and weight gain (WG) of golden pompano (*Trachinotus ovatus.*) fed the six diets for 56 days

Whole body and muscle composition. Whole body and muscle composition of fish is shown in Table 4. No significant differences (P>0.05) were found in whole body moisture, crude protein, lipid, and ash content among all dietary treatments. Muscle lipid content decreased with increase in dietary astaxanthin; muscle lipid content of fish fed AST-0.1 and AST-0.2 diets was significantly lower (P<0.05) than that of fish fed the basal diet but there was no significant difference (P>0.05) in fish fed AST-0.005, AST-0.01, and AST-0.05 diets. No significant differences (P>0.05) were found in muscle moisture, crude protein, and ash among all diet treatments.

Table 4 Whole-body and muscle composition (% dry weight) of golden pompano fed diets with different levels of astaxanthin.

Treatments	*AST-0*	*AST-0.005*	*AST-0.01*	*AST-0.05*	*AST-0.1*	*AST-0.2*
Whole body						
Moisture	64.82±3.90	66.17±0.60	65.70±1.63	65.81±0.44	66.18±0.52	65.43±1.48
Crude protein	50.53±1.49	50.45±4.49	51.26±2.40	51.67±1.21	52.48±0.34	51.44±3.04
Lipid	34.89±0.15	35.32±0.24	34.50±3.16	31.66±4.16	34.26±0.42	31.79±2.47
Ash	10.87±0.73	10.62±1.31	11.27±0.71	11.46±0.41	11.43±0.77	11.22±0.87
Muscle						
Moisture	71.95±0.66	70.43±1.15	69.57±3.69	72.61±0.58	70.37±1.96	69.79±1.81
Crude protein	71.23±1.47	67.50±2.34	66.70±5.40	71.83±0.37	67.53±2.53	66.66±3.41
Lipid	26.36±2.24 [b]	24.21±2.91 [ab]	23.08±1.91 [ab]	22.64±0.95 [ab]	21.00±2.50 [a]	20.42±1.61 [a]
Ash	6.93±1.29	5.99±0.40	5.87±0.90	6.45±0.71	6.00±0.84	5.62±0.34

[a, b] Values are means ± S.E.M. of three replicates and values in the same row with different letters are significant different (P < 0.05).

Hepatic antioxidant status. The hepatic antioxidant status of fish is shown in Table 5. Hepatic TAS activity increased when dietary astaxanthin was increased, hepatic TAS activity of fish fed AST-0.05, AST-0.1, and AST-0.2, diets was significantly higher (P<0.05) than that of fish fed AST-0, AST-0.005, and AST-0.01, diets. On the contrary, hepatic SOD activity decreased in relation to increasing levels of dietary astaxanthin. Hepatic SOD activity of fish fed the basal diet was significantly higher (P<0.05) than that of fish fed AST-0.1 and AST-0.2 diets but no significant difference (P>0.05) wasa observed in fish fed AST-0.005, AST-0.01, and AST-0.05 diets. The hepatic carbonyl protein content decreased with increased dietary astaxanthin, the hepatic carbonyl protein contents of fish fed AST-0.1 and AST-0.2 diets were significantly lower (P<0.05) than that of fish fed AST-0, AST-0.005 and AST-0.01 diets but there was no significant difference (P>0.05) in fish fed AST-0.05 diet. No significant difference (P>0.05) was found in hepatic MDA content among all diets treatments.

Table 5 Hepatic antioxidant status of golden pompano fed diets with different levels of astaxanthin.

Treatments	*AST-0*	*AST-0.005*	*AST-0.01*	*AST-0.05*	*AST-0.1*	*AST-0.2*
TAS (U/mg protein)	0.1±0.06[a]	0.14±0.04[a]	0.14±0.05[a]	0.24±0.07[b]	0.33±0.01[c]	0.36±0.11[c]
SOD (U/mg protein)	261.21±3.36[c]	255.38±23.46[bc]	225.17±23.41[abc]	243.68±24.58[abc]	219.26±16.88[ab]	212.01±18.51[a]
Carbonyl protein (nmol/mg protein)	2.28±0.25[b]	2.00±0.65[b]	2.1±0.48[b]	1.88±0.69[ab]	1.38±0.37[a]	1.31±0.33[a]
MDA (nmol/mg protein)	0.32±0.05	0.30±0.03	0.28±0.04	0.30±0.03	0.30±0.04	0.28±0.03

[a, b, c] Values are means ± S.E.M. of three replicates and values in the same row with different letters are significant different (*P*<0.05).

Expression profile of Hsp70 mRNA and HIF-1α mRNA. The expression of Hsp70 mRNA and HIF-1α mRNA of fish is shown in Table 6. Hsp 70 mRNA in hepatic profile of fish fed AST-0.1 and AST-0.2 diets was significantly higher (*P*<0.05) than that of fish fed AST-0 and AST-0.005 diets but there was no significant difference (*P*>0.05) in fish fed AST-0.01 and AST-0.05 diets. Similarly, the expression of HIF-1α mRNA in the hepatic profiles of fish fed AST-0.1 and AST-0.2 diets were significantly higher (*P*<0.05) than that of fish fed AST-0, AST-0.005 and AST-0.01 diets but there was no significant difference (*P*>0.05) in fish fed AST-0.05 diet.

Table 6. The relative gene expression level of hepatic Hsp 70 and HIF mRNA of golden pompano fed diets with different levels of astaxanthin.

Treatments	*AST-0*	*AST-0.005*	*AST-0.01*	*AST-0.05*	*AST-0.1*	*AST-0.2*
Hsp 70	1±0.00 [a]	1.19±0.34 [a]	5.63±0.87 [ab]	5.31±1.16 [ab]	7.35±0.9 [b]	8.60±1.11 [b]
HIF-1a	1±0.00 [a]	1.38±0.23 [ab]	2.73±0.53 [ab]	3.05±0.53 [bc]	4.70±0.93 [c]	4.76±0.59 [c]

Hsp 70: Heat shock protein 70
HIF-1a: Hypoxia-inducible factor-1a
[a, b, c] Values are means ± S.E.M. of three replicates and values in the same row with different letters are significant different (*P* < 0.05).

When linear regression analysis was used to estimate the expression profiles of HIF-1α mRNA, (Fig. 2) the dietary astaxanthin level requirement for maximal immunity of juvenile pompano was 0.013%.

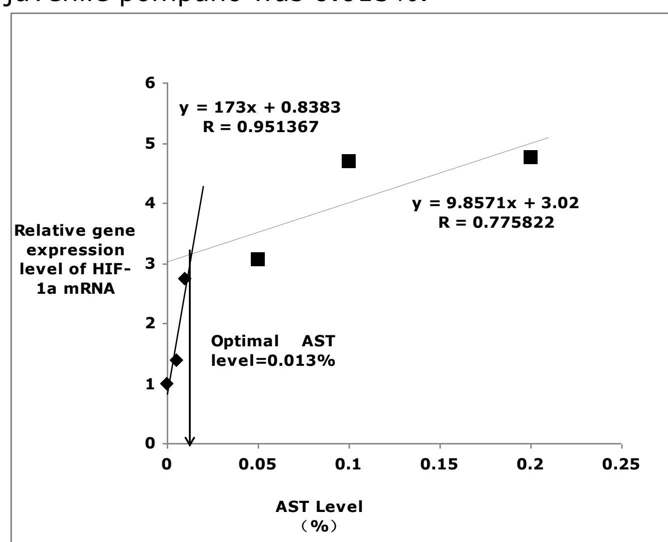

Figure 2 Relationship between dietary AST level and relative gene expression level of HIF-1a mRNA of golden pompano (*Trachinotus ovatus.*) fed the six diets for 56 days

Discussion

Effects of astaxanthin on growth performance, survival and tissue composition. Carotenoids are reported to improve growth performance of fish as it is believed that they exert a positive influence on intermediary metabolism in aquatic animals (Segner et al., 1989). These enhance nutrient utilization that ultimately results in improving growth (Amar et al., 2001). It has been suggested that other possible mechanisms may adjust the intestinal flora by breaking down indigestible feed components to extract more nutrients and stimulate production of enzymes transporting fats for growth instead of

storage (James et al., 2006). Astaxanthin was found to enhance lipid utilization in whole fish and liver, providing more energy and consequently enhancing growth performance (Kalinowski et al., 2011). In the present experiment, supplementation of 0-0.005% showed no significant effects on growth performance (FBW and WG) and feed utilization (FCR), while dietary 0.01-0.2% astaxanthin significantly increased growth performance (FBW and WG) and feed utilization (FCR) (see Table 3). Therefore, for growth, supplementation levels of astaxanthin in diet of golden pompano should not be lower than 0.01%. This result agrees with previous studies on Atlantic salmon (Christiansen and Torrissen, 1996), red porgy (Kalinowski et al. 2011), large yellow croaker (Li et al., 2014) and Astronotus ocellatus (Alishahi et al., 2015). However, the effect of carotenoids on fish growth is controversial. Some earlier studies have reported that dietary astaxanthin showed no significant influence on growth and flesh composition of fish (Yi et al., 2014; Tejera et al., 2007; Zhang et al., 2012; Pham et al., 2014). In the present experiment, muscle lipid content decreased in fish fed diets with supplemental astaxanthin compared with fish fed diets without supplemental astaxanthin (Table 4). The effectiveness of carotenoids in terms of deposition and physiological function is species-specific in fish and not all fish species possess the same pathways for the metabolism of carotenoids (Kop and Durmaz, 2008). The mechanisms related to these findings have not yet been clearly elucidated. Our latest research results showed that dietary astaxanthin increases the apparent digestibility coefficient of the diet and further promotes the expression of insulin-like growth factors (IGFs), moreover, as members of the family of transforming growth factors β, myostatin is affected by dietary astaxanthin (unpublished data).

Effects of astaxanthin on antioxidant capacity. The effect of different treatments on antioxidant balance (Table 5) showed that the antioxidative capacity (TAS activity) of fish increased with increasing levels of dietary astaxanthin (S.S. Kim et al. 2012). Higher TAS values were reported for fish fed diets with supplemental astaxanthin than in fish fed the basal diet, as the astaxanthin exhibited strong antioxidant properties. The enhancing effect of carotenoids on total antioxidant status was supported by Wang et al. (2006) who showed that diets with high levels of fat-soluble antioxidants, such as synthetic astaxanthin and vitamin E, increased antioxidant capacity. TAS is an overall indicator of antioxidant status by representing the enzyme and nonenzyme levels of original antioxidants in the body (Xiao et al., 2004). As values increase, antioxidant defense against free radical reaction and reactive oxygen intermediates increases (Chien et al., 2003). The present beneficial effect of carotenoids on antioxidant capacity was in accordance with previously published research which has found that carotenoids alleviate oxidative damage and repress free radical production from cellular metabolism and various stresses (Chien et al., 2003; Pan et al., 2010; Niu et al., 2014). A relationship between dietary astaxanthin concentration and antioxidant status in liver and muscle was observed in Atlantic salmon, indicating that carotenoids may enhance fish health (Christiansen and Torrissen, 1996).

The need for endogenous antioxidant enzymes, such as total superoxide dismutase (SOD) for the protection against O_2 was reduced. Superoxide dismutase (SOD), a cytosolic enzyme specific for scavenging superoxide radicals, is the first enzyme to react against oxygen radicals and important endogenous antioxidants for protection against oxidative stress (Winston and Di Giulio, 1991). If levels of dietary fat-soluble antioxidants, such as astaxanthin and vitamin E are high, it has been shown that there is a reduced need for endogenous antioxidant enzymes, such as total SOD (Lygren et al. 1999). Carotenoids possess good singlet oxygen quenching properties and may serve as antioxidants in systems containing unsaturated fatty acids by quenching free radicals (Martinez-Alvarez et al., 2005). More superoxide radicals need to be reacted when SOD values increase (Yang et al., 2010). Liver SOD activity significantly decreased with dietary astaxanthin supplementation in olive flounder (*Paralichthys olivaceus*) (Pham et al., 2014), large yellow croaker (*Pseudosciaena crocea*) (Li et al., 2014), and rainbow trout (*Oncorhynchus mykiss*) (Zhang et al., 2012). In this present study, SOD was significant lower in fish fed diets with supplemental astaxanthin, implying that astaxanthin can eliminate reactive oxygen species and prevent SOD production in fish tissues.

Malondialdehyde (MDA) is a product of lipid peroxidation, through crosslinking with the nucleophilic groups of proteins, nucleic acids, and amino phospholipids. Accumulation of MDA leads to cell toxicity, accelerating cell and tissue damage (Buege and Aust, 1978). However, the present results showed no significant difference in hepatic MDA content, which meant that the breeding period was good. The use of carbonyl protein as a biomarker of oxidative damage to protein, is relatively new for fish (Parvez and Raisuddin, 2005) and is not commonly used for shrimp. Oxidative modification of protein is one of the many consequences of oxidative stress (Stadtman, 1986), and the assay of carbonyl groups in protein provides a convenient technique for detecting and quantifying oxidative stress (Levine et al., 1994). In general, these all lower oxidative stress levels in fish fed astaxanthin-containing diets (0.005%-0.2% AST) compared to the basal diet (0% AST).

Effects of astaxanthin on hepatic gene expression of Hsp 70 and HIF-1α. Heat shock proteins (Hsp) constitute a family of ubiquitous proteins encoded by genes. Hsp 70 is an evolutionarily highly conserved molecular chaperone that is an important part of cell machinery for protein folding, and can assist in repair and protection of cellular proteins from stressor-induced damage and minimize protein aggregation (Franzellitti and Fabbri, 2005; Woo et al., 2011). In the present experiment, results demonstrated that dietary astaxanthin significantly enhanced the expression level of Hsp 70 mRNA of golden pompano, suggesting that their immune status was better than with the basal diet treatment. It has been proposed that HIF-1α may contribute to protection during early intervals and/or moderate hypoxia or against severe and/or prolonged hypoxia (Heidbreder et al. 2003). HIF-1α was found to be a transcription factor that regulates dozens of genes involved in the response to hypoxia; these molecular responses then cascade into a series of biochemical and physiological adjustments, enabling the animal to survive better under hypoxic conditions (Treinin et al. (2003). In the present experiment, expression levels of HIF-1α mRNA of fish fed astaxanthin-containing diets were higher than in fish fed the basal diet, which also indicated that dietary astaxanthin could improve the sensitivity of antioxidant capacity of fish by enhancing the efficiency or utility of the oxygen transportation.

Conclusion

In conclusion, growth trials and immunological response data suggest that dietary astaxanthin supplementation can improve growth performance and hepatic antioxidant capacity by eliminating the reactive oxygen species. The effect of astaxanthin on both growth performance and survival of golden pompano showed that the level of astaxanthin supplemented in the diet should be between 0.011%~0.013%.

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