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Effects of dietary astaxanthin on growth, antioxidant capacity and gene expression in Pacific white shrimp *Litopenaeus vannamei*

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Abstract

Pacific white shrimp *Litopenaeus vannamei* (1050 individuals with initial weight of 1.01 ± 0.001 g) were fed either control diet or one of six dietary astaxanthin (AX) concentration (25, 50, 75, 100, 125 and 150 mg kg^{-1}) diets for 56 days in 35 tanks (30 shrimp per tank). After 56 days of culture, shrimp-fed AX125 and AX150 diets had higher ($P < 0.05$) weight gain, specific growth rate, total antioxidant status and lower ($P < 0.05$) superoxide dismutase (SOD), catalase (CAT) than shrimp fed control diet. After low dissolved oxygen stress for 1 h, survival rate of shrimp fed AX75, AX100, AX125 and AX150 diets was higher ($P < 0.05$) than that of shrimp fed control diet. Hypoxia inducible factor-1α (HIF-1α), cytosolic manganese superoxide dismutase (cMnSOD) and CAT mRNA expression levels of shrimp fed seven diets were significantly down-regulated under hypoxia than under normoxia, but their expression levels were higher under hypoxia in shrimp fed AX-supplemented diets than in shrimp fed control diet. About 70-kDa heat-shock protein (Hsp70) mRNA expression level of shrimp fed seven diets was significantly up-regulated under hypoxia than under normoxia, but its expression level was lower under hypoxia in shrimp fed AX-supplemented diets than in shrimp fed control diet.

KEY WORDS: antioxidant capacity, astaxanthin, growth, hypoxia stress, *Litopenaeus vannamei*

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Introduction

The Pacific white shrimp *Litopenaeus vannamei* is the most commonly cultured shrimp in South China. In recent years, the deteriorating pond environments have seriously affected shrimp farming and resulted in stress-induced disease incidences mainly of viral and bacterial aetiologies (Flegel 1997; Sirirustananun *et al.* 2011). Many stress conditions like temperature at the edge of thermal windows of the species or hypoxia lead to an increased production of reactive oxygen species (ROS) (Castex *et al.* 2010; Qiu *et al.* 2011), the imbalance exists between the production of ROS and the antioxidant response to neutralize these intermediates resulting in cellular damage, which render shrimp susceptible to different diseases (Kassahn *et al.* 2009; Sheikhzadeh *et al.* 2012). Organisms have developed the antioxidant defence system, including enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx) and functional large molecules (albumin, ferritin and ceruloplasmin) and small molecules (ascorbic acid, α-tocopherol, β-carotene and uric acid), to maintain the lowest possible levels of ROS in the cell (Martínez-Álvarez *et al.* 2005; García-Triana *et al.* 2010; Sheikhzadeh *et al.* 2011), which is closely related to the immune system in response to pathogens. Therefore, it is crucial to develop the antioxidants to improve resistance and survival of shrimp in fluctuating pond environments during shrimp farming.

Astaxanthin (AX) is a red-orange coloured xanthophyll carotenoid found in high amounts in crustaceans, the flesh of salmon and trout, and other marine organisms (Chew 1995). Astaxanthin has attracted a great deal of attention due to its multiple functions and antioxidant potential, which have been reported to surpass those of β-carotene or lutein, even α-tocopherol (Naguib 2000; Kim *et al.* 2011).

Some studies demonstrated that dietary astaxanthin could increase the total antioxidant capacity (TAS) and improve growth performance of black tiger shrimp *Penaeus monodon* (Thongrod *et al.* 1995; Chien *et al.* 2003; Pan *et al.* 2003a). Dietary astaxanthin could increase the survival (Thongrod *et al.* 1995) and enhance the resistance to salinity stress (Darachai *et al.* 1998; Merchie *et al.* 1998; Chien *et al.* 2003), thermal stress (Chien *et al.* 2003), oxygen depletion (Chien *et al.* 2003) and ammonia stress (Pan *et al.* 2003b) in *P. monodon*. Dietary astaxanthin could significantly improve the low dissolved oxygen (DO) tolerance and enhance the survival rate of postlarval shrimp *L. vannamei* (Niu *et al.* 2009), and also improve the growth, survival and moult frequency of *L. vannamei* in low-salinity water (Flores *et al.* 2007). Injected astaxanthin could also significantly increase the survival of *Macrobrachium rosenbergii* challenged with *Lactococcus garvieae* and enhanced to some extent its antioxidant capacity and total haemocyte count (Angeles *et al.* 2009). Dietary astaxanthin as a powerful antioxidant could improve the growth performance and enhance the stress tolerance in aquaculture.

Hypoxic conditions can appear because of natural factors, such as seasonal and daily temperature fluctuation, or by many anthropogenic factors, as for example, a bloom of algae caused by inputs of organic pollutant (Tenney 1979; Belão *et al.* 2011). Hypoxia exposure enhanced the generation and the release of mitochondrial ROS and caused oxidative stress (Pialoux *et al.* 2009; Jusman *et al.* 2010). Chronic hypoxia increased the transcription of genes in the mitochondrial genome and up-regulated the expression of genes encoding proteins involved in metabolism in grass shrimp *Palaemonetes pugio* (Brouwer *et al.* 2007; Li & Brouwer 2007, 2009a,b). Blue crabs (*Callinectes sapidus*) collected from Pensacola Bay Florida showed significant down-regulation of cMnSOD gene expression at a diurnally hypoxic marsh site (Brown-Peterson *et al.* 2005), and hypoxic conditions also significantly decreased the expression level of HIF-1α mRNA in *C. sapidus* (Hardy *et al.* 2012). After *L. vannamei* were subjected to hypoxia, lactate dehydrogenase (LDHvan-1) transcript increased 2.5-fold in gills, while the LDHvan-2 transcript decreased 14-fold in muscle (Sonanez-Organis *et al.* 2012), and hypoxia also increased ATPase beta transcripts in *L.* vannamei (Martinez-Cruz *et al.* 2011). Vitamin E treatment attenuated the mitochondrial oxidative damage induced by acute and severe hypoxia exposure, the administration of antioxidants before exposure to hypoxia may be beneficial to counteract mitochondrial alterations resulting from oxidative stress (Magalhães *et al.* 2005).

The aim of this study was to further improve understanding of the antioxidant mechanism of dietary astaxanthin in *L. vannamei* by evaluating the expression of antioxidant enzyme (SOD and CAT), 70-kDa heat-shock protein (Hsp70) and hypoxia inducible factor-1α (HIF-1α) mRNAs under normoxia or hypoxia stress. Moreover, we also examined the growth performance and antioxidant parameters after administration of astaxanthin. The liver-specific marker enzymes such as aspartate transaminase (AST) and alanine transaminase (ALT) were also determined to evaluate the toxicity of dietary astaxanthin to hepatopancreas of *L. vannamei*.

Materials and methods

Diet formulation and preparation

The experimental diets were formulated for this study as described in Table 1. The animals were only fed with the basal diet (without astaxanthin) as control group, the basal diet supplemented with astaxanthin (Lucantin® Pink 10%; BASF SE, Ludwigshafen, Germany) at levels of 25, 50, 75, 100, 125 and 150 mg kg^{-1} as treatment groups, which were designated as AX25, AX50, AX75, AX100, AX125 and AX150, respectively. All ingredients were thoroughly mixed with soybean oil, and water was added to produce pellets of approximate 1.2 mm in diameter. Subsequently, the pellets were dried in the dark to prevent the degradation of astaxanthin. The dried pellets were stored at −20 °C in the dark until used. Finally, the concentrations of astaxanthin were, respectively, measured with high-performance liquid chromatography (HPLC) (Agilent 1200; Agilent Technologies, Santa Clara, CA, USA) to further verify the dietary astaxanthin levels of the seven experimental diets.

Animals and experimental design

White shrimp (*L. vannamei*) with mean initial weight of 1.01 ± 0.001 g (mean ± SD) were obtained from the shrimp farm of Evergreen South Ocean Science and Tech Co. Ltd, Zhanjian, China. Shrimp were fed with the basal diet for 1 week to acclimate the experimental environments. Shrimp were cultured in 35 indoor tanks (500 l of each) with water depth of 1.5 m and divided into seven groups and five tanks within each group with density of 30. Shrimp were fed with various experimental diets three times daily at 08 : 00, 16 : 00 and 20 : 00 for 56 days. The daily feeding rate was 50–80 g kg^{-1} of body weight and was

Table 1 Formulation and chemical proximate composition of the experimental diets

Ingredients	Dietary treatments						
	Control	AX25	AX50	AX75	AX100	AX125	AX150
Ingredients (g kg^{-1})							
Fish meal	300	300	300	300	300	300	300
Soybean meal	200	200	200	200	200	200	200
Peanut meal	164	164	164	164	164	164	164
Wheat flour	218.5	218.5	218.5	218.5	218.5	218.5	218.5
Beer yeast	50	50	50	50	50	50	50
Lecithin	10	10	10	10	10	10	10
Fish oil	10	10	10	10	10	10	10
Soybean oil	10	10	10	10	10	10	10
Phospholipids (purity 97%, pc-60)	10	10	10	10	10	10	10
Vitamin premix[1]	10	10	10	10	10	10	10
Mineral premix[2]	10	10	10	10	10	10	10
Vitamin C	1	1	1	1	1	1	1
Choline chloride (50%)	5	5	5	5	5	5	5
Astaxanthin (10%)	0	0.25	0.50	0.75	1.00	1.25	1.50
Cellulose	1.50	1.25	1.00	0.75	0.50	0.25	0
Proximate composition (g kg^{-1})							
Moisture	90	90	87	94	91	87	90
Crude protein	424	420	423	418	425	420	426
Crude lipid	72	75	76	76	78	77	77
Ash	84	85	83	83	83	84	83
Astaxanthin (mg kg^{-1})	0.66	24.39	47.37	74.81	100.55	129.09	153.51

[1] Vitamin mix (kg^{-1} of diet): vitamin A, 300 000 IU; riboflavin, 500 mg; pyridoxine HCL, 400 mg; cyanocobalamin, 1.2 mg; thiamin, 20 mg; menadione, 40 mg; folic acid, 130 mg; biotin, 10 mg; α-tocopherol, 3000 IU; *myo*-inositol, 8000 mg; calcium pantothenate, 760 mg; nicotinic acid 200 mg; choline chloride 8000 mg; vitamin D, 40 000 IU. (Modified from Wang *et al.* 2006).
[2] Mineral mix (kg^{-1} of diet): $ZnSO_4$ $7H_2O$, 4 g; $CaCO_3$, 215 g; KCL, 90 g; KI, 0.04 g; NaCl 40 g; $CuSO_4$ $5H_2O$ 3 g;; $CoSO_4$ $7H_2O$, 0.02 g; $FeSO_4$-$7H_2O$, 20 g; $MnSO_4$ H_2O, 3 g; $MgSO_4$ $7H_2O$, 124 g; $Ca(HPO_4)_2$ $2H_2O$, 500 g;. (Modified from David & Gatlin 1996).

adjusted according to prior feeding responses. During the experimental period, the pH, water temperature, salinity, dissolved oxygen and ammonia–nitrogen were 7.8–8.2, 28–29 °C, 24–27%, 8.0–9.0 mg L^{-1} and 0.06–0.08 mg L^{-1}, respectively.

At the end of the experiment, shrimp were fasted for 24 h and then counted and weighed. Survival rate (SR), final body weight (FBW), weight gain (WG), specific growth rate (SGR) and feed conversion ration (FCR) were calculated according to the study described by Yang *et al.* (2004) and Huai *et al.* (2009) as follows: WG % = 100 × (FBW–IBW)/IBW, SGR = 100 × (ln final body weight – ln initial body weight)/total number of experimental days, FCR = dry feed intake (g)/wet weight gain (g), SR% = 100 × final shrimp number/initial shrimp number.

Assessments of immune parameters

Haemolymph of six shrimp from each tank was drawn from the ventral sinus, respectively, with a 1-mL sterile syringe containing 750 μL of precooled anticoagulant solution (0.114 M trisodium citrate, 450 mM NaCl, 10 mM KCl, 10 mM HEPES at pH 7.4) and then pooled together and immediately stored in liquid nitrogen until used.

TAS, SOD and CAT activities of haemolymph were determined spectrophotometrically at 520, 550 and 240 nm (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA), respectively. The antioxidant enzyme detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). One unit of SOD activity was defined as the amount of tissue extracts that inhibited the rate of xanthine reduction at 25 °C by 50%, and the specific activity was expressed as SOD units (mg $protein^{-1}$). One unit of CAT activity was defined as the amount of enzyme that catalysed the decomposition of 1.0 mmol of H_2O_2 per min. AST and ALT levels were measured by an automatic blood analyzer (Hitachi 7170A; Hitachi Ltd., Tokyo, Japan) in a professional laboratory (Sun Yat-Sun University of Medical Sciences, Guangzhou, China).

Body composition and astaxanthin content

Three shrimp of each tank were used to analyse moisture (DM, 105 °C, 16–18 h), crude protein (N × 6.25 semi-micro-Kjeldahl; Kjeltec-Auto System, Tecator,

HOganas, Sweden), crude lipid (diethyl ether extraction method, Soxtec System HT6, Tecator) and ash (precombustion on a hot plate followed by 3–4 h at 550 °C) according to the method described by Huai *et al*. (2009).

According to the methods of Schwartz & Patroni (1985) and Chien & Shiau (2005), the shell of three shrimp of each tank was dissected, weighed and freeze-dried, respectively. The freeze-dried shell was ground and placed into a 50-mL polypropylene centrifuge tube. Twenty milliliter of acetone was added into each tube as solvent, and the mixture was homogenized (Polytron PT-MR-3000; PT. Hartono Istana Teknologi, Indonesia) at 12 000 × g for 1 min and then were centrifuged (Hitachi 18 PR-52; Hitachi Ltd.) at 12 700 × g for 5 min. The pellet was resuspended and centrifuged with additional 20 mL of acetone until the acetone extract was clear. The pooled acetone extracts were transferred to a 250-mL separatory funnel, partitioned with 30 mL *n*-hexane, which was washed three times with 10% NaCl to remove residual acetone, and then the extract was reduced to 10 mL using a rotary evaporator. Subsequently, the extract was filtered through a 0.2-Am Millipore filter and stored in three 2-mL brown vials (Schwartz & Patroni 1985; Weber *et al*. 2007). Finally, astaxanthin contents were determined using high-performance liquid chromatography (Agilent 1200; Agilent technologies). The standard of the chromatographically pure astaxanthin was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). The HPLC condition was adjusted according to the method of Yuan *et al*. (1996): The pigment extract was filtered through 0.45-pm filters and separated by using a Grace-Smart RP-18 5 μ (250 × 4.6 mm) column at room temperature. The mobile phase consisted of 75% methanol and 25% acetonitrile (V/V). The flow rate was set at 1.0 mL min^{-1}. The pigment extracts were injected with a Rheodyne 7725 valve with a 20 μL loop. The UV-VIS absorbance detector was set at 476 nm. Chromatographic peaks were identified by comparing retention times against known standards.

Low dissolved oxygen stress test

At the end of the experiment, the haemolymph of three shrimp from each drum was sampled to immediately store in liquid nitrogen until used to examine the expression levels of cMnSOD, CAT, Hsp70 and HIF-1α mRNAs under normoxia from shrimp fed the experimental diets. Then, other 18 shrimp from each tank were randomly selected and transferred to 20-L plastic drums with 10 L seawater. Low DO conditions were maintained by stopping the water flow and aeration and overlying a plastic sheet on the surface of each drum. During the test, the level of DO was monitored using the YSI Dissovled Oxygen Analyzer (YSI-550A; YSI Incorporated, Yellow Springs, OH, USA). The DO was 8.7 mg L^{-1} at the beginning and then linearly decreased to 0.8 mg L^{-1} and finally maintained at this level. After low DO stress at 1 h, shrimp were observed in each drum respectively, and shrimp were regarded dead when heart of shrimp were not beat. Moreover, the haemolymph of three shrimp from each drum was sampled to immediately store in liquid nitrogen to determine the expression levels of cMnSOD, CAT, Hsp70 and HIF-1α mRNAs under hypoxia.

Expression of cMnSOD, CAT, Hsp70 and HIF-1α mRNAs

Total RNAs were extracted using RNeasy Mini kit (Cat: no. 74104; QIAGEN, Shanghai, China) according to the manufacturer's instructions and treated with DNase I (Cat: no. 79254; QIAGEN) to remove contaminated DNA. Total RNAs were quantified at 260 and 280 nm to evaluate their concentration and purity and also analysed using 2% agarose gel electrophoresis to assess whether they were degraded. Total RNAs with clear ribosomal bands and high RNA ratios ($A_{260}/A_{280} \geqq 1.8$) were used for further experiments. The first-strand cDNA was synthesized based on manufacture's instruction of PrimeScript™ RT reagent kit (Perfect Real-Time) (TaKaRa DRR037S; Takara Bio, Tokyo, Japan) with total RNA as template. cDNA mix was diluted to 1 : 5 and stored at −80 °C for subsequent real-time quantitative RT-PCR.

Primers of each gene were designed based on published white shrimp *L. vannamei* cDNA using Primer 3 software (http://primer3.wi.mit.edu/) (Table 2). All primers were produced by Shanghai Sangon Biological Engineering Technology and Service CO., Ltd. (Shanghai, China), and the reaction conditions were also optimized. Real-time quantitative RT-PCR was performed in total volume of 20 μL containing 10 μL of 2 × SYBR Green Real-time PCR Master Mix (TaKaRa DRR041A; Takara Bio), 50 ng of cDNA, 0.16 μM of each primer and 8.2 μL of double-distilled water. Real-time quantitative RT-PCR program consisted of denaturation step at 95 °C for 2 min, followed by 40 amplification cycles of 15 s denaturation at 95 °C, 15 s annealing at 56–58 °C, 30 s extension at 72 °C. After PCR amplification, melt-curve analysis was conducted to confirm that there was only one amplified product. Data analysis of the real-time PCR was performed with Mastercycler ep realplex PCR System Software. The comparative C_T method ($2^{-\Delta\Delta}C_T$

Table 2 Primers for quantitative real-time PCR study

Gene name	GenBank number	Reference	Primer sequence (5′–3′)	Annealing temperature (°C)	Product (bp)
Cytosolic manganese superoxide dismutase (cMnSOD)	DQ005531	Gómez-Anduro *et al.* (2006)	(F)ATCACTCACGGACTGGTTCC (R)GAGAGAAACGCCCTTGTGAC	59	219
Catalase (CAT)	AY518322	Tavares-Sánchez *et al.* (2004)	(F)GCCCGTACAAGGAACTACCA (R)TGACGTTCTGCCTCATTCAG	58.5	230
Hypoxia inducible factor-1α (HIF-1α)	FJ807918	Sonanez-Organis *et al.* (2009)	(F)CTGTCGATTCCAGACTTGCT (R)CAACAAGAGTGGCTCCCTTA	57	164
70-kDa heat-shock protein (Hsp70)	AY645906	Zhou *et al.* (2010)	(F) CCTCCAGGACTTCTTCAACG (R)GGTCACGTCCAACAGCAAC	58	135
β-actin	AF300705y	Sun *et al.* (2007)	(F)GTGCCCATCTACGAGGGATA (R)TAGGACTTCTCCAGCGAGGA	56.5	233

method) was used. The C_T for test gene and the C_T for β-actin were determined for each sample. Differences in the C_T for test gene and β-actin, called ΔC_T, 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 ΔC_T for each sample was subtracted from the ΔC_T of the calibrator, and the difference was called $\Delta\Delta C_T$. Expression level of test gene could be calculated by $2^{-\Delta\Delta}C_T$, and the value stood for an n-fold difference relative to the calibrator.

Statistical analysis

All the data in figure and table were presented as means ± standard deviation of five tanks. The data were statistically analysed by statistical software SPSS 17.0 (SPSS, Chicago, IL, USA). One-way ANOVA was used to determine whether significant difference existed among the experimental groups. Duncan's multiple range tests were followed for individual comparisons. In addition, the effect of dietary astaxanthin on weight gain of *Litopenaeus vannamei* was estimated by a second-order polynomial. A two-way ANOVA was conducted to examine whether dietary astaxanthin level, hypoxia stress, and their interaction affected the expression levels of cMnSOD, CAT, Hsp70 and HIF-1α mRNAs. A probability (P) value of < 0.05 was considered significant.

Results

Growth performance

Final wet body weight (FBW), weight gain (WG), special growth ratio (SGR), feed conversion rate (FCR) and survival rate (SR) were summarized in Table 3. After feeding trial for 56 days, FBW, WG and SGR of shrimp fed AX125 and AX150 diets were significantly higher ($P < 0.05$) than those of shrimp fed control diet. The data of WG were examined using regression statistical model, and it appeared that growth was increased with the level of astaxanthin, $y = 1.1667\ x^2 + 6.0238x + 1012.7$, $R^2 = 0.815$. (Fig. 1).

FCR of shrimp fed AX125 and AX150 diets was significantly lower ($P < 0.05$) than that of shrimp fed control diet. SR of shrimp among all experimental groups had no significant difference ($P > 0.05$).

Body composition and astaxanthin content

The body compositions of shrimp fed the experimental diets were presented in Table 4. The astaxanthin contents of shrimp shell were shown in Fig. 2. The crude protein, crude lipid and ash of shrimp had no significant difference ($P > 0.05$) between the control and experimental groups. The astaxanthin content of shrimp shell in experimental groups was significantly higher than that of control group. Among experimental groups, the astaxanthin contents of shrimp shell in AX25 diet and AX50 diet were significantly less than those of other AX-supplemented diets.

Antioxidant parameters

The antioxidant parameters were presented in Table 5. TAS activity of the shrimp fed AX125 and AX150 diets was significantly higher than that of shrimp fed control and other AX-supplemented diets ($P < 0.05$). SOD and CAT activities of shrimp fed AX75, AX100, AX125 and AX150 diets were significantly lower ($P < 0.05$) those that of shrimp fed control diet. AST and ALT activities were not significantly different between the control and treatment groups ($P > 0.05$).

Table 3 Growth parameters and survival rate of *Litopenaeus vannamei* fed with different astaxanthin levels over the 56 days feeding trial

	Dietary treatments						
Parameters	Control	AX25	AX50	AX75	AX100	AX125	AX150
FBW[1](g)	11.3 ± 0.22^{a}	11.7 ± 0.26^{ab}	11.7 ± 0.19^{ab}	11.4 ± 0.31^{ab}	11.8 ± 0.11^{ab}	12.3 ± 0.42^{b}	12.3 ± 0.25^{b}
WG[2](%)	1009 ± 20.28^{a}	1046 ± 25.09^{ab}	1054 ± 18.18^{ab}	1032 ± 20.60^{ab}	1064 ± 11.25^{ab}	1109 ± 42.17^{b}	1107 ± 23.47^{b}
SGR[3]	4.25 ± 0.02^{a}	4.35 ± 0.04^{ab}	4.37 ± 0.03^{ab}	4.33 ± 0.05^{ab}	4.36 ± 0.01^{ab}	4.48 ± 0.06^{b}	4.45 ± 0.04^{b}
FCR[4]	1.51 ± 0.06^{a}	1.44 ± 0.03^{abc}	1.49 ± 0.05^{ab}	1.44 ± 0.03^{abc}	1.41 ± 0.01^{abc}	1.39 ± 0.02^{bc}	1.35 ± 0.02^{c}
SR[5](%)	98.7 ± 1.34	97.3 ± 1.94	94.0 ± 2.21	95.3 ± 2.00	98.0 ± 1.34	93.3 ± 2.99	95.3 ± 0.83

Values are mean (n = 5) ± standard deviation. Mean values in the same row for each type of experiment with different letters are significantly different ($P < 0.05$).

[1] FBW (g shrimp^{-1}): final body wet weight (g shrimp^{-1}).

[2] WG (%): weight gain = 100 × (final body weight – initial body weight)/ initial body weight.

[3] SGR: specific growth ratio = 100 × (ln. final body weight – ln. initial body weight)/ total number of experimental days.

[4] FCR: feed conversion ratio = dry feed intake (g) / wet weight gain (g).

[5] SR: survival rate (%) = 100 × (final shrimp number)/(initial shrimp number).

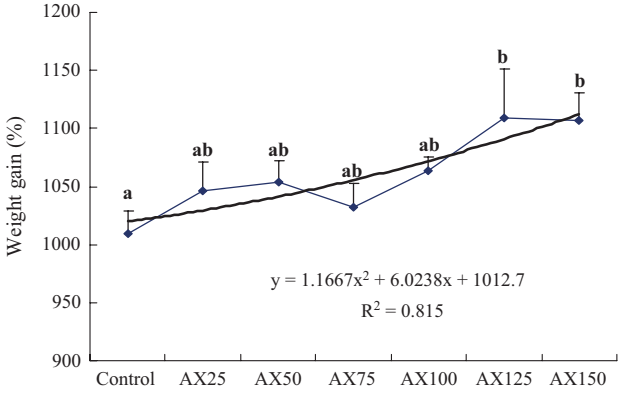

Figure 1 Relationship between weight gain and dietary astaxanthin level for *Litopenaeus vannamei* as described by second-order polynomial regression. The significant difference was superscripted by the different letters ($P < 0.05$) (n = 5).

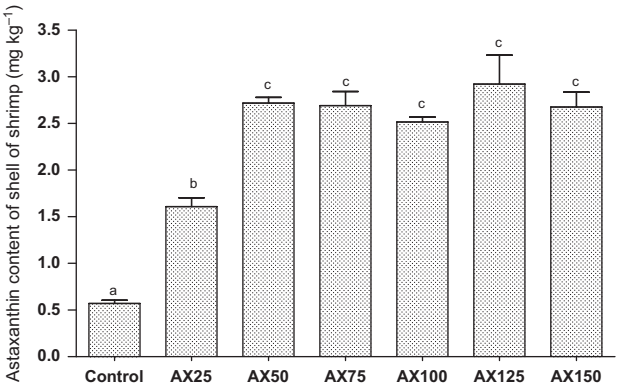

Figure 2 The astaxanthin content of *Litopenaeus vannamei* shell after the 56 days feeding trial. The significant difference was superscripted by the different letters ($P < 0.05$). (n = 5).

Table 4 Whole body composition of *Litopenaeus vannamei* fed with different astaxanthin levels over the 56 days feeding trial

	Dietary treatments						
Body composition	Control	AX25	AX50	AX75	AX100	AX125	AX150
Moisture (g kg^{-1} wet basis)	714 ± 0.36	723 ± 0.18	728 ± 0.62	725 ± 0.54	724 ± 0.25	730 ± 0.27	719 ± 0.41
Crude protein (g kg^{-1} dry basis)	683 ± 0.08	683 ± 0.11	687 ± 0.24	687 ± 0.21	686 ± 0.19	685 ± 0.23	684 ± 0.19
Crude lipid (g kg^{-1} dry basis)	65.3 ± 0.18	65.7 ± 0.11	64.7 ± 0.04	66.0 ± 0.10	64.9 ± 0.02	64.8 ± 0.09	64.7 ± 0.17
Ash (g kg^{-1} dry basis)	113 ± 0.17	114 ± 0.08	114 ± 0.19	116 ± 0.19	115 ± 0.14	115 ± 0.18	115 ± 0.22

Values are mean (n = 5) ± standard deviation. Mean values in the same row for each type of experiment with different letters are significantly different ($P < 0.05$).

Table 5 Haemolymph antioxidant capacity parameters of *Litopenaeus vannamei* fed with different astaxanthin levels over the 56 days feeding trial

	Dietary treatments						
Haemolymph parameters	Control	AX25	AX50	AX75	AX100	AX125	AX150
TAS[1] (M haemolymph)	4.80 ± 0.31^{a}	5.37 ± 0.27^{ab}	5.71 ± 0.58^{ab}	5.84 ± 0.36^{ab}	5.95 ± 0.52^{ab}	6.08 ± 0.22^{b}	6.25 ± 0.15^{b}
SOD[2] (unit mg^{-1} protein)	92.9 ± 3.35^{a}	88.2 ± 2.02^{ab}	84.2 ± 2.85^{abc}	81.6 ± 4.60^{bc}	77.1 ± 3.31^{c}	76.6 ± 2.85^{c}	73.9 ± 2.80^{c}
CAT[3] (unit mg^{-1} protein)	4.28 ± 0.47^{a}	3.84 ± 0.22^{ab}	3.96 ± 0.42^{ab}	2.87 ± 0.50^{b}	2.90 ± 0.37^{b}	2.82 ± 0.27^{b}	2.74 ± 0.21^{b}
AST[4] (unit mg^{-1} protein)	235 ± 8.77	285 ± 23.4	285 ± 18.0	285 ± 48.9	268 ± 24.6	273 ± 7.48	288 ± 36.4
ALT[5] (unit mg^{-1} protein)	200 ± 50.2	242 ± 66.5	226 ± 48.8	304 ± 83.1	211 ± 61.1	256 ± 41.8	282 ± 23.6

Values are mean (n = 5) ± standard deviation. Mean values in the same row for each type of experiment with different letters are significantly different (P < 0.05).
[1] TAS (per M haemolymph): total antioxidant status.
[2] SOD (unit mg^{-1} protein): superoxide dismutase.
[3] CAT (unit mg^{-1} protein): catalase.
[4] AST (unit mg^{-1} protein): aspartate transaminase.
[5] ALT (unit mg^{-1} protein): alanine transaminase.

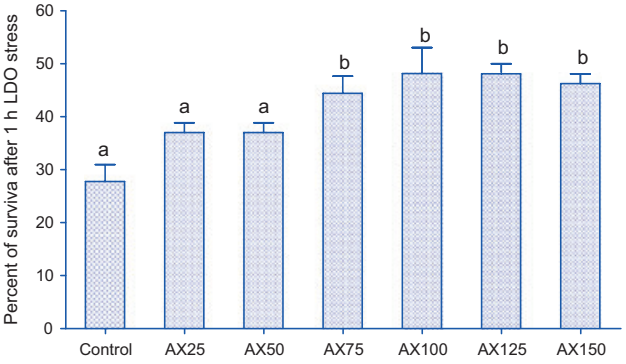

Figure 3 The survival rate (%) of *Litopenaeus vannamei* after low dissolved oxygen stress. Significant difference was indicated with the different letters (P < 0.05). (n = 5).

Low DO stress test and expression of cMnSOD, CAT, Hsp70 and HIF-1α mRNAs

After low DO stress, SR of shrimp fed the experimental diets was calculated and shown in Fig. 3. SR of shrimp fed AX75, AX100, AX125 and AX150 diets were significantly higher (P < 0.05) than that of shrimp fed control diet.

Hsp70 and HIF-1α mRNAs expression levels had no significant difference (P > 0.05) between shrimp fed AX-supplemented diets and shrimp fed control diet under normoxia. cMnSOD mRNA expression level of shrimp fed AX-supplemented diets was significantly decreased (P < 0.05) under normoxia compared with that of shrimp fed control diet, and its expression level was not significantly different (P > 0.05) among shrimp fed other AX-supplemented diets under normoxia. CAT mRNA expression level of shrimp fed AX75, AX100, AX125 and AX150 diets was significantly lower (P < 0.05) than that of shrimp fed control and AX25 diets (Fig. 4).

After 1 h low DO stress, Hsp70 mRNA expression level of shrimp fed the experimental diets all was significantly up-regulated (P > 0.05) under hypoxia than under normoxia, but its expression level of shrimp fed AX50, AX75, AX100, AX125 and AX150 diets were lower under hypoxia than that of shrimp fed control diet. HIF-1α mRNA expression level of shrimp fed all experimental diets was significantly down-regulated (P < 0.05) under hypoxia than under normoxia, but its expression level of shrimp fed AX150 diet was significantly higher (P < 0.05) than that of shrimp fed control and AX25 diets under hypoxia. cMnSOD mRNA expression level of shrimp fed all experimental diets was significantly down-regulated (P < 0.05) under hypoxia than under normoxia, and its expression level of shrimp fed AX150 diet was significantly higher (P < 0.05) than that of shrimp fed control and AX25 diets under hypoxia. CAT mRNAs expression level of shrimp fed all experimental diets also was significantly down-regulated (P < 0.05) under hypoxia than under normoxia, and its expression level of shrimp fed AX100, AX125 and AX150 diets were significantly (P < 0.05) higher than that of shrimp fed control diet under hypoxia (Fig. 4).

Discussion

Carotenoids, which could enhance nutrient utilization and might ultimately improve growth, play an important role in the intermediary metabolism of aquatic animals (Segner *et al.* 1989; Amar *et al.* 2001; Niu *et al.* 2011). The previous studies demonstrated that dietary astaxanthin could significantly improve the growth performance of

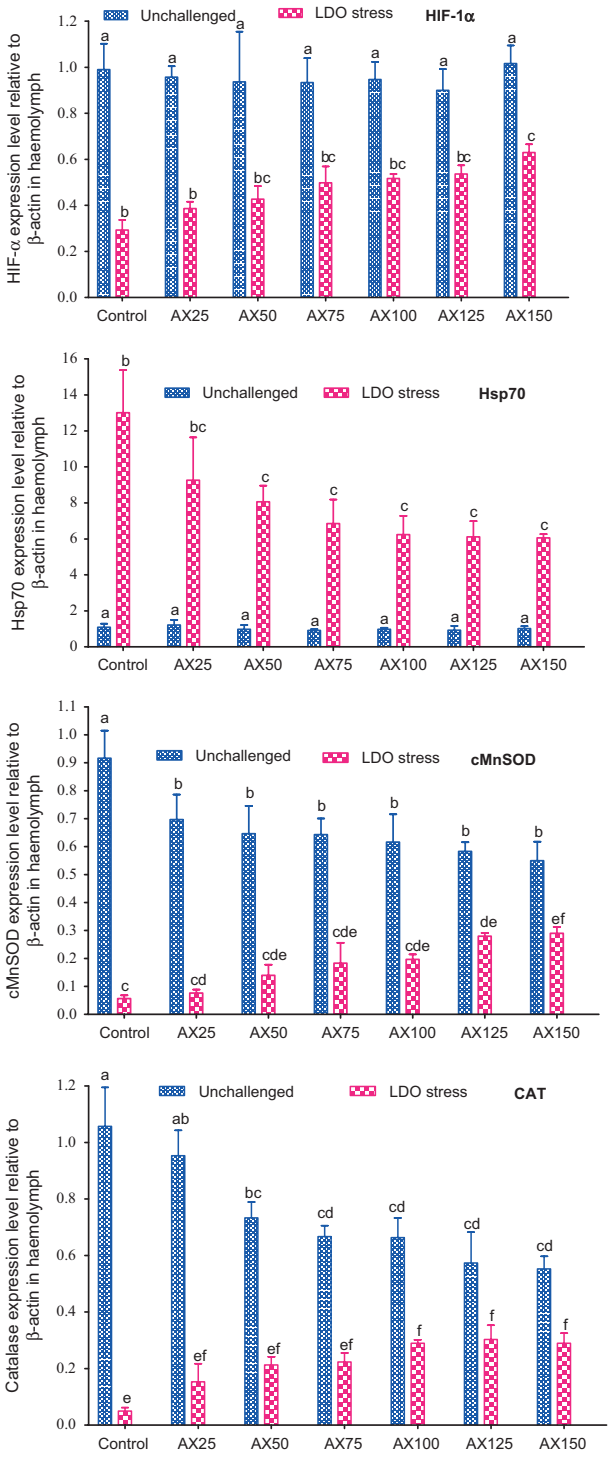

Figure 4 HIF-1α, Hsp70, cMnSOD and CAT mRNAs expression profiles of *Litopenaeus vannamei* fed with all experimental diets were examined under normoxia and hypoxia by real-time quantitative RT-PCR. Actin gene was used as an internal control to calibrate the cDNA template for all the samples. Vertical bars represented the mean ± SE Significant difference was indicated with the different letters ($P < 0.05$). ($n = 5$).

P. monodon (Thongrod *et al.* 1995) and could also enhance the growth rate and shorten the moulting cycles of postlarval stages of *M. japonicus* (Petit *et al.* 1997). The present study indicated that dietary astaxanthin levels of 125 and 150 mg diet kg^{-1} could significantly improve the growth performance of *L. vannamei*. However, Chien & Shiau (2005) suggested that dietary synthetic astaxanthin could not significantly improve the growth performance of *M. japonicus*. Yamada *et al.* (1990) and Négre-Sadargues *et al.* (1993) also indicated that dietary carotenoids had no significant effects on the growth of *P. japonicus*. So the effects of dietary astaxanthin on the growth of aquatic animals are still not well understood nowadays, and the functional mechanism of dietary astaxanthin need to be further studied.

The previous studies demonstrated that dietary astaxanthin could enhance a variety of stress tolerance of aquatic animals. Pan *et al.* (2003a,b) and Chien & Shiau (2005) indicated that the dietary astaxanthin could increase the survival rate of *P. monodon* after the thermal, osmotic, ammonia or low DO challenge. Chien & Shiau (2005) also suggested that *M. Japonicus* fed algae and synthetic astaxanthin diets had longer survival time than the control under the 0.5 mg L^{-1} DO condition. In the present study, after *L. vannamei* were suffered from low DO stress, SR of shrimp fed AX-supplemented diets (dietary astaxanthin concentration > 75 mg diet kg^{-1}) were significantly higher ($P < 0.05$) than that of shrimp fed control diet. These studies suggested that dietary astaxanthin could increase the resistance of shrimp against the environmental stresses and enhance the survival rate of shrimp, which was very important to shrimp farm. However, little was known about the precise antioxidant mechanisms of dietary astaxanthin.

TAS is an overall indicator of the antioxidant status of an individual, as the value of TAS increases, the antioxidant defence against free radical reaction also increases (Chien *et al.* 2003; Castillo *et al.* 2006). In the present study, TAS activities of shrimp fed AX-supplemented diets were significantly higher ($P < 0.05$) in dose-dependent manner than that of shrimp fed control diet. SOD and CAT are two major antioxidant enzymes, which are responsible for scavenging reactive oxygen species (ROS) and protecting mechanisms within tissue damaged by radical process and phagocytosis (Chien *et al.* 2003). The higher SOD and CAT values, the more superoxide radicals need to be reacted. In the present study, SOD and CAT activities of shrimp fed AX-supplemented diets were lower in dose-dependent manner than that of shrimp fed control diet, and significantly lower ($P < 0.05$) in AX75, AX100, AX125

and AX150 groups. Wang *et al*. (2006) also indicated that SOD activity of *Hyphessobrycon callistus* significantly decreased with the increase in dietary carotenoid concentrations, and SOD activity of AX-fed group was lowest among various types of dietary carotenoid. These studies suggested that the astaxanthin had stronger O_2 quenching activity than SOD and CAT, and dietary astaxanthin could relieve the oxidative stress and keep the healthy status of individuals.

The previous studies have demonstrated that hypoxia could regulate the expression levels of HIF-1α Hsp70, cMnSOD and CAT mRNAs in shrimp (Tavares-Sánchez *et al*. 2004; Sonanez-Organis *et al*. 2009; Zhou *et al*. 2010; García-Triana *et al*., 2010). In this study, their expression levels were examined among shrimp fed all experimental diets. HIF-1α is a transcription factor that regulates dozens of genes involved in the response to hypoxia (Harris 2002; Treinin *et al*. 2003; Leiser & Kaeberlein 2010), these molecular responses then cascade into a series of biochemical and physiological adjustments, enabling the animal to survive better under hypoxic conditions (Wu 2002). After *L. vannamei* were fed the experimental diets, the expression levels of HIF-1α mRNA were similar under normoxia in all experimental diets. Its expression levels significantly decreased under hypoxia than under normoxia in shrimp fed all experimental diets, but its expression levels were higher in shrimp fed AX-supplemented diets than in shrimp fed control diet, which indicated that dietary astaxanthin could partially alleviate the hypoxia stress response in *L. vannamei* by enhancing the efficiency or utility of the oxygen transportation. Hsp70 functions as a chaperone and assists to repair and protect cellular proteins from stressor-induced damage and minimize protein aggregation (Franzellitti & Fabbri 2005). Hypoxic conditions represent high-stress levels that can lead to the induction of genes involved in the cellular response such as heat-shock proteins (Woo *et al*. 2011; Xu *et al*. 2011). The expression levels of Hsp70 mRNAs were significantly up-regulated under hypoxia than under normoxia in shrimp fed all experimental diets, but its expression levels under hypoxia was lower in shrimp fed AX-supplemented diets than in shrimp fed control diet, which also indicated that dietary astaxanthin could partially alleviate the hypoxia stress response. cMnSOD is an antioxidant enzyme that dismutates superoxide anion (O_2^-) into a hydrogen peroxide molecule and oxygen, protecting against cellular and genetic damage generated by O_2^-. Shrimp subjected to hypoxia had lower cMnSOD transcripts and SOD activity, and reoxygenation reverted the effect of hypoxia increasing the levels of cMnSOD transcripts and SOD activity in gills and hepatopancreas, which indicated that cMnSOD was very important in the redox system regulation of *L. vannamei* (García-Triana *et al*. 2010). In this study, shrimp fed AX-supplemented diets had lower expression levels of cMnSOD mRNAs than that fed control diet under normoxia, and its expression levels significantly decreased under hypoxia than under normoxia. On the other hand, shrimp fed AX-supplemented diets had higher expression levels than that fed control diet under hypoxia. CAT is also an important antioxidant enzyme, its expression profiles was similar to that of cMnSOD mRNAs. These results suggested that dietary astaxanthin could partially alleviate oxidative stress by the relatively higher mRNA expression levels of antioxidant enzymes in *L. vannamei*, which could help to reduce damage by reactive oxygen species to the tissue cells.

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