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Influence of dietary biotin levels on growth and non-specific immune response in large yellow croaker, *Larimichthys crocea* R

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Abstract

A 9-week feeding experiment was conducted to determine the effect of dietary biotin levels on growth performance and non-specific immune response of large yellow croaker. Fish (6.16 ± 0.09 g) were fed twice daily to apparent satiation with diets containing 0.00 (as the basal diet), 0.01, 0.05, 0.25, 1.24 and 6.22 mg biotin kg^{-1} diet. Results showed that fish fed the basal diet had the lowest survival rate, and fish fed 0.05 mg kg^{-1} dietary biotin achieved significantly higher final weight and weight gain. Dietary biotin levels had no significant influence on carcass crude lipid, moisture and ash content, but significantly influenced the carcass crude protein. Liver biotin concentration significantly increased with the supplementation of biotin, but no tissue saturation was found within the supplementation scope of biotin. Broken-line regression analysis of weight gain showed that juvenile large yellow croaker requires a minimum dietary biotin of 0.039 mg kg^{-1} for maximal growth. The analyses of serum parameters showed that the moderate- (0.05 mg kg^{-1}) and high-dose (6.22 mg kg^{-1}) dietary biotin significantly improved both lysozyme and alternative complement pathway activities, indicating dietary biotin within a certain range could improve the non-specific immune response of large yellow croaker.

KEY WORDS: biotin, growth, large yellow croaker, *Larimichthys crocea*, non-specific immune response, requirement

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Introduction

Large yellow croaker (*Larimichthys crocea* R.) is an economically important rare fish species cultured in South China, due to its delicious meat, fast growth, efficient feed conversion and high market value. In the year 2013, the production of the cultured large yellow croaker was up to 105 230 tons in China, which increased by 10.6% than in year 2012 (China fishery statistical yearbook 2013). However, a main constraint to large yellow croaker culture is the limited supply of trash fish that is presently the main feed source for grow-out. So there is an urgent need to develop nutritionally balanced and cost-effective feeds for this fish. One of the prerequisites for developing high efficiency diet for large yellow croaker requires complete knowledge of its nutritional requirements. At present, a few studies have been published on this fish about the nutrient requirements such as dietary protein, lipid, vitamin C, methionine, lysine and phosphorus requirement (Duan *et al.* 2001; Ai *et al.* 2006, 2007; Mai *et al.* 2006a,b; Zhang *et al.* 2008a,b), and the information on its digestive physiology, optimal feeding frequency and alternative protein source for fish meal also could be found (Ma *et al.* 2005; Mai *et al.* 2005; Zhang *et al.* 2008a,b; Xie *et al.* 2011).

Biotin is a water-soluble vitamin generally included in vitamin B complex, and acts in several specific carboxylation and decarboxylation reactions. It is coenzyme for several CO_2 fixing enzymes such as propionyl-CoA carboxylase (PCC), pyruvate carboxylase (PC), methylcrotonoyl-CoA carboxylase and acetyl-CoA carboxylase, and these enzymes play indispensable roles in gluconeogenesis, fatty acid synthesis and degeneration and function of the Krebs cycle (Shiau & Chin 1999). Hence, we could infer that biotin is critical in regulation of intermediary metabolism of amino acids, carbohydrates and lipids, which is in

agreement with several observations that indicated biotin supply was involved in glucose and lipid homoeostasis (Shiau & Chin 1999; Albarracin *et al*. 2008; Larrieta *et al*. 2012).

Besides, its role as a carboxylase prosthetic group, biotin also has a wide repertoire of effects on systemic processes such as immunity (Báez-Saldaña *et al*. 1998; Báez-Saldaña & Ortega 2004; Fernandez-Mejia 2005). Several experimental evidences indicated that biotin had certain effects on the ability of the organism to mount an adequate immune response (Báez-Saldaña *et al*. 1998). Research on mice showed that biotin could have an important role in lymphocyte maturation and responsiveness to stimulation, and consequently in the capacity of the immune system to respond to an antigenic challenge (Báez-Saldaña *et al*. 1998). On weaning pig, it was reported that dietary biotin within a certain range seemed to increase the immune response to sheep red blood cells (Kornegay *et al*. 1989). Wiedmann *et al*. (2003) suggested that biotin supplementation affected gene expression in human immune cells, which was likely to modulate the response of immune cells to antigens. However, little information could be found about the effects of dietary biotin on the immune response of fish.

Since biotin is one of the most important and expensive vitamins in aquafeed, it is necessary to quantify the minimum requirement of biotin to manufacture high-effective and less-cost commercial feeds. However, several factors have been proved to influence the need for dietary biotin in animals. For example, high dietary lipid has been shown to mask partial effects of biotin on rats, chicks and brook trout (*Salvelinus fontinalis*) (Jacobs *et al*. 1970; Marson & Donaldson 1972; Poston & McCarteney 1974), and it was likely to obscure the requirement for dietary biotin in animals. The quantitative requirement of biotin based on growth has been studied in only a few species of fish, such as, 0.1 mg kg^{-1} for lake trout (*Salvelinus namaycush*) (Poston 1976), 0.02–0.03 mg kg^{-1} for common carp (*Cyprinus carpio*) (Ogino *et al*. 1970), 0.05–0.14 mg kg^{-1} for rainbow trout (*Oncorhynchus mykiss*) (Castledine *et al*. 1978; Woodward & Frigg 1989), 2.0–2.5 mg kg^{-1} for mirror carp (*C. carpio*) (Gunther & Meyer-Burgdorff 1990), 0.06 mg kg^{-1} for hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) (Shiau & Chin 1999), 2.49 mg kg^{-1} for Asian catfish (*Clarias batrachus*) (Shaik Mohamed *et al*. 2000), 0.25 mg kg^{-1} for Indian catfish (*Heteropneustes fossilis*) (Shaik Monhamed 2001), 0.046 mg kg^{-1} for Japanese seabass (*Lateolabrax japonicus*) (Li *et al*. 2010), 0.15 mg kg^{-1} for Jian carp (Zhao *et al*. 2012) and 0.5 mg kg^{-1} for zebrafish (Yossa *et al*. 2014).

To our knowledge, no information is available about dietary biotin requirement of large yellow croaker at present. Meanwhile, its immune response to dietary biotin level is also unreported. Therefore, the present investigation was undertaken to determine the effect of different dietary biotin levels on growth and non-specific immune response for large yellow croaker.

Materials and methods

Experimental diets

Six isonitrogenous and isoenergetic diets were formulated with graded levels of biotin. Formulation and proximate composition of the basal diet is shown in Table 1. Vitamin-free casein (Sigma, Chemical, St. Louis, MO, USA) and gelatin (Sigma, Chemical) were used as protein source. Dextrin (Shanghai Chemical Co., Shanghai, China) was used as carbohydrate source. Menhaden fish oil (Food grade) and soybean oil (Food grade) were used as lipid source. Amino acids (Shanghai Chemical Co.) mixture was added to simulate the whole body amino acids pattern of

Table 1 Formulation and proximate composition of the experimental diets

Ingredients	Content (g kg^{-1} dry matter)
Casein (Vitamin free)	320
Gelatin	80
Dextrin	285
Menhaden fish oil	70
Soybean oil	40
Amino acid mixture[1]	85
Lecithin	20
Sodium alginate	10
α-Cellulose	30
Mineral premix[2]	40
Vitamin premix (Biotin free)[3]	20
Proximate analysis (n = 3)	
Crude protein	431
Crude lipid	126

[1] Amino acid premix (g kg^{-1} diet): Aspartic acid, 17.8 g; Threonine, 4.8 g; Alanine, 14.2 g; Arginine, 9.5 g; Cystine, 0.3 g; Valine, 4.3 g; Methionine, 5.2 g; Phenylalanine, 3.4 g; Isoleucine, 5.3 g; Leucine, 8.2 g; Lysine, 11.7 g.

[2] Mineral premix (mg 100 g^{-1} permix): NaF, 20.0 mg; KI, 8.0 mg; $CoCl_2{\cdot}6H_2O$ (1%), 500.0 mg; $CuSO_4{\cdot}5H_2O$, 100.0 mg; $FeSO_4{\cdot}H_2O$, 800.0 mg; $ZnSO_4{\cdot}H_2O$, 300.0 mg; $MnSO_4{\cdot}H_2O$, 150.0 mg; $MgSO_4{\cdot}7H_2O$, 12000.0 mg; Ca $(H_2PO_4)_2{\cdot}H_2O$, 75000.0 mg; NaCl, 1000.0 mg; Zoelite, 10 122 mg.

[3] Vitamin premix (mg kg^{-1} diet): B_1, 25 mg; B_2, 45 mg; B_6, 20 mg; B_{12}, 0.1 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg; A, 32 mg; D, 5 mg; E, 120 mg; K_3, 10 mg; C, 2000 mg; inositol, 800 mg; choline chloride, 2500 mg; antioxidant, 150 mg; wheat middling, 14 013 mg.

large yellow croaker fingerling. Biotin (Sigma) was added to the test diets at the expense of small amount of cellulose, and the biotin concentrations of the diets were determined by HPLC (Lahely *et al.* 1999) to be 0.00 (as the basal diet), 0.01, 0.05, 0.25, 1.24 and 6.22 mg kg^{-1} diet.

All the dry ingredients were grounded into fine powder through 220 μm mesh and thoroughly mixed with biotin, then the mixture was mixed with menhaden fish oil and soybean oil, and then cold water was added to produce a stiff dough. The dough was pelleted with an experimental diet machine (F-26 (II), South China University of Technology, China) and dried for about 12 h in a ventilated oven at 45 °C. After drying, the diets were broken up and sieved into proper pellet size. The sizes of pellets were 2.0 × 3.0 mm and 3.0 × 5.0 mm. All the diets were sealed in bags and stored at −20 °C until used.

Experimental procedure

Experimental fish were obtained from a commercial farm in Ningbo, Zhejiang province, China. Prior to the feeding trial, the fish were reared in seawater in a concrete pond (4.5 × 3.0 × 1.8 m) with continual aeration, and fed the basal diet for 2 weeks to acclimate to the experimental diet and the rearing conditions. At the start of the experiment, the fish were fasted for 24 h and weighed after being anesthetized with eugenol (purity >98%) (1 : 10 000) (Shanghai Reagent Corp, Shanghai, China). Juvenile large yellow croaker with similar size (6.16 ± 0.09 g) were randomly allocated into 18 flow-through fibreglass tanks (120 L) filled with 100 L of water (three tanks per treatment), and these tanks were arranged in a randomized block design to avoid confounding factors. Each tank was stocked with 15 fish and provided with continual aeration from an air blower. The fish were fed by hand twice daily at 6:30 and 18:30. To prevent the waste of dietary pellets, fish were slowly hand-fed little by little to apparent satiation on the basis of visual observation of fish feeding behaviour. The feeding trial lasted for 9 weeks, from weeks 1–4, 2.0 × 3.0 mm pellets were fed; thereafter, 3.0 × 5.0 mm pellets were fed until the end of the experiment. During the experimental period, feed consumption was recorded daily. The number and weight of dead fish were recorded and the photoperiod was set to a 12L:12D cycle. Water used in this study was from a nearby water source and the water flow rate through each tank was 1 L min^{-1}. Water temperature ranged from 27.0 to 30.0 °C, salinity from 25.0 to 28.0‰, pH from 8.0 to 8.1 and dissolved oxygen content was approximately 7 mg L^{-1} during the experiment.

At the end of the trial, the fish were fasted for 24 h and anaesthetized with eugenol (purity >98%) (1 : 10 000). Then, the fish in each cage were weighed and counted. Five fish per cage were randomly selected for proximate analysis. Then, blood was sampled from the caudal veins of other five fish per cage using ethylenediaminetetraacetic acid (EDTA)-containing Vacutainers (Huabo Medical Instrument Co., Ltd, Shandong, China). Plasma was separated from the blood via centrifugation at 2054 *g* for 10 min at 4 °C and stored at −80 °C until use. After blood sampling, the liver of five fish were pooled into 10 mL tubes, frozen in liquid N2 and then stored at −80 °C for liver biotin contents assay.

Proximate analyses

Proximate analyses on feedstuffs, diets and fish were performed according to the standard methods of AOAC (1995). Liver lipid contents were determined according to the method of Folch *et al.* (1957). Biotin contents of the diet and fish liver were determined by using the method of Lahely *et al.* (1999).

Measurement of lysozyme activity

Lysozyme activity was measured following the method of Ellis (1990). Briefly, 0.05 mL serum was added to 1.4 mL of a suspension of *Micrococcus lysodeikticus* (Sigma) (0.2 mg mL^{-1}) in a 0.1 M sodium phosphate buffer (pH 6.8). The reaction was carried out at 25 °C and absorbance was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer. Each unit of activity is defined as the amount of sample causing a decrease in absorbance of 0.001 per minute.

Measurement of alternative complement pathway activity

Alternative complement pathway activity (ACH50) was determined and calculated using the method of Yano (1992). Briefly, a series of volumes of the diluted serum ranging from 0.1 to 0.25 mL were dispensed into test tubes and the total volume made up to 0.25 mL with barbitone buffer in the presence of ethyleneglycol-bis (2-aminoethoxy)-tetraacetic acid (EGTA) and Mg^{2+} (0.1 mol L^{-1}), then 0.1 mL of rabbit red blood cells (RaRBC) (2 × 10^8cells mL^{-1}) was added to each tube. After incubation for 90 min at 22 °C, 3.15 mL 0.9% NaCl was added. Following this, the sample was centrifuged at 1600 ***g*** for 5 min at 4 °C to eliminate unlysed RaRBC. The optical density of the supernatant was measured at

414 nm. The volume of serum producing 50% haemolysis (ACH50) was determined and the number of ACH50 U mL^{-1} was obtained for each group.

Calculations and statistical analyses

The following variables were calculated:

Survival rate (%) = $N_F/N_I \times 100$
Weight gain (WG) (%) = $(W_F - W_I)/W_I \times 100$
Feed efficiency ratio (FER) = $(W_F - W_I)/F_T$
Protein efficiency ratio (PER) = $(W_F - W_I)/P_T$
Feed intake (FI) = $F_T/[(W_I + W_F)/2 \times T]$
Hepatosomatic index (HSI) (%) = $W_L/W_B \times 100$

where N_I and N_F were initial and final number of fish; W_I and W_F were initial and final weight of fish in g; W_L and W_B were liver and body weight of fish in g; F_T was total feed intake in dry basis in g; P_T was protein intake in dry basis in g; T was the experimental duration in day.

All statistical evaluations were performed by using the software SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Data from each treatment, passed homogeneity, were subjected to a one-way analysis of variance (ANOVA) according to Tukey's multiple range test. The level of significance was chosen at $P < 0.05$. The broken-line model (Robbins *et al.* 1979) was used to estimate the optimal requirements of dietary biotin for large yellow croaker. Values presented were treatment means with standard error of the mean (SEM).

Results

Deficiency syndrome

After 5 weeks of the feeding trial, fish fed the basal diet began to show the biotin deficiency syndrome, which characterized by lower survival rate, poor growth, anorexia and atrophy. However, fish fed biotin-containing diets did not show any deficiency syndrome.

Survival rate and growth performance

Survival rate (%), final weight (FW), weight gain (WG, %), feed efficiency ratio (FER) and protein efficiency ratio (PER) of large yellow croaker were shown in Table 2. The survival rate of fish fed the basal diet (82.2%) was the lowest among the six dietary treatments while no significant difference was found among them. FW and WG significantly increased to the highest with increasing dietary biotin level up to 0.05 mg kg^{-1} diet ($P < 0.05$), and thereafter leveled off when the biotin level between 0.05 and 6.22 mg kg^{-1}. The broken-line regression was used to estimate the optimal dietary biotin level: $Y = 331.8 + 4220 (X - 0.039)$ ($R^2 = 0.8743$) and ($X - 0.039 = 0$) when $X > 0.039$, which may indicate the biotin requirement of large yellow croaker was estimated to be 0.039 mg kg^{-1} diet based on WG (Fig. 1). FER and PER both significantly increased with increasing dietary biotin level up to 0.05 mg kg^{-1} diet ($P < 0.05$), and thereafter, no significant differences were observed.

Carcass composition and liver biotin content

Carcass composition and liver biotin content of large yellow croaker were shown in Table 3. Dietary biotin level had no significant influence on carcass crude protein, crude lipid, moisture and ash content but significantly affected the liver biotin concentration. Liver biotin concentration significantly increased with increasing dietary biotin level ($P < 0.05$) and no tissue saturation was found within the experimental biotin scope.

Table 2 Initial weight (g), survival rate (%), final weight (g), weight gain (%), feed efficiency ratio (FER) and protein efficiency ratio (PER) of large yellow croaker fed experimental diets with graded biotin levels for 9 weeks[1]

Dietary biotin levels (mg kg^{-1})	Initial weight (g)	Survival rate (%)	Final weight (g)	Weight gain (%)	FER	PER
0.00 (the basal diet)	6.2 ± 0.19	82.2 ± 2.22	13.3 ± 0.17^{c}	115.9 ± 2.74^{c}	0.4 ± 0.01^{c}	0.8 ± 0.02^{c}
0.01	6.2 ± 0.19	91.1 ± 2.22	23.1 ± 0.36^{b}	274.8 ± 5.77^{b}	0.6 ± 0.01^{b}	1.3 ± 0.02^{b}
0.05	6.2 ± 0.19	88.9 ± 2.22	28.5 ± 0.12^{a}	363.1 ± 1.98^{a}	0.8 ± 0.01^{a}	1.8 ± 0.03^{a}
0.25	6.2 ± 0.19	95.6 ± 2.22	25.2 ± 0.47^{b}	309.6 ± 7.60^{b}	0.7 ± 0.01^{a}	1.7 ± 0.02^{a}
1.24	6.2 ± 0.19	93.3 ± 3.85	25.2 ± 0.99^{b}	309.5 ± 16.01^{b}	0.7 ± 0.01^{a}	1.7 ± 0.03^{a}
6.22	6.2 ± 0.19	93.3 ± 3.85	24.4 ± 0.61^{b}	295.5 ± 9.87^{b}	0.7 ± 0.02^{a}	1.7 ± 0.04^{a}
ANOVA						
F value		2.759	94.147	94.258	187.595	190.378
P value		0.070	0.000	0.000	0.000	0.000

ANOVA, one-way analysis of variance.
[1] The average index of five fish per tank was used as a unit. Values are means ± SEM of three replicate groups ($n = 3$); Means with different letter in the same column differ significantly ($P < 0.05$).

Non-specific immune related parameters

Three treatments, the basal diet (0.00 mg biotin kg^{-1} diet), moderate-dose diet (0.05 mg biotin kg^{-1} diet) and high-dose diet (6.22 mg biotin kg^{-1} diet), were chosen to investigate the effect of dietary biotin level on non-specific immune response of large yellow croaker. With the increasing of dietary biotin level, the lysozyme activity and alternative complement pathway activity both showed an increasing trend, and the highest value of lysozyme activity (171.0 U mL^{-1}) and alternative complement pathway activity (153.5 U mL^{-1}) were observed in moderate- and high-dose treatments, respectively. The lysozyme activity of moderate-dose treatment was significantly higher than that of the basal diet treatment (130.7 U mL^{-1}) ($P < 0.05$), but was comparable to high-dose treatment (165.3 U mL^{-1}). Meanwhile, the alternative complement pathway activities in moderate- (146.5 U mL^{-1}) and high-dose treatments were both significantly higher than it in the basal diet treatment (95.3 U mL^{-1}) ($P < 0.05$) (Table 4).

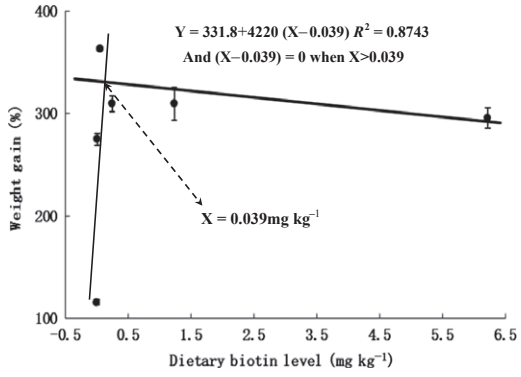

Figure 1 Relationship between dietary biotin level and weight gain (%) of large yellow croaker fed experimental diets for 9 weeks as fitted by broken-line regression analysis, and showed that the optimal dietary biotin level is 0.039 mg kg^{-1} diet.

Discussion

In rats (Jacobs *et al.* 1970), chicks (Marson & Donaldson 1972) and trout (Poston & McCarteney 1974), high dietary lipid has been reported to obscure effects of biotin deficiency. In this study, menhaden fish oil, soybean oil and lecithin were used as the main lipid source, and the crude lipid of diet was kept at 12.6%, which was just the right amount to meet the need of large yellow croaker (Ai *et al.* 2004a). Therefore, in this study, it was biotin deficiency that caused syndromes such as heavy mortality, poor growth, anorexia, atrophy and dark skin colour appeared after the basal diet used for 5 weeks. An early study also reported that pacific salmonids fed biotin-deficient diets showed skin disorders, atrophy, convulsions, lesions in the colour and loss of appetite (Halver 1989). Shaik Mohamed *et al.* (2000) reported that the Asian catfish appeared anorexia, dark skin colour and convulsions syndrome after fed biotin-free diet for 7 weeks, and shortly afterwards, he also found Indian catfish developed severe deficiency signs after feeding a biotin-free diet for 6 weeks characterized by convulsions, heavy mortality, listlessness, poor feed intake and feed conversion, dark skin colour, tetanus and weight loss (Shaik Monhamed 2001). All of the above studies have revealed the essentiality of dietary biotin for normal growth

Table 3 Carcass composition, liver lipid and biotin content of large yellow croaker fed experimental diets with graded biotin levels for 9 weeks (wet weight)1

Dietary biotin levels (mg kg^{-1})	Crude protein (%)	Crude lipid (%)	Moisture (%)	Ash (%)	Liver lipid (%)	Liver biotin content (μg g^{-1})
0.00 (the basal diet)	13.9 ± 0.11^{b}	5.0 ± 0.19	77.6 ± 0.56	4.6 ± 0.08	10.6 ± 0.49^{b}	0.0 ± 0.00^{f}
0.01	14.5 ± 0.12^{ab}	5.4 ± 0.18	76.7 ± 0.82	4.6 ± 0.09	12.5 ± 0.34^{a}	0.5 ± 0.08^{e}
0.05	14.2 ± 0.24^{ab}	5.4 ± 0.15	76.7 ± 0.58	4.6 ± 0.04	13.2 ± 0.27^{a}	1.3 ± 0.11^{d}
0.25	14.4 ± 0.13^{ab}	5.3 ± 0.12	75.1 ± 1.24	4.5 ± 0.08	13.7 ± 0.19^{a}	3.6 ± 0.09^{c}
1.24	14.7 ± 0.15^{a}	5.3 ± 0.06	76.5 ± 0.69	4.7 ± 0.05	13.3 ± 0.44^{a}	5.2 ± 0.08^{b}
6.22	14.5 ± 0.05^{ab}	5.4 ± 0.17	76.5 ± 0.86	4.6 ± 0.06	13.2 ± 0.45^{a}	5.9 ± 0.09^{a}
ANOVA						
F value	3.350	0.868	0.893	0.545	9.043	928.404
P value	0.040	0.530	0.516	0.740	0.001	0.000

ANOVA, one-way analysis of variance.

[1] All the carcasses of five fish has been dried off and then smashed by grinder. The liver sample used in this study was separated from another five fish. Values are means ± SEM of three replicate groups ($n = 3$); Means with different letter in the same column differ significantly ($P < 0.05$).

Table 4 LA and ACH50 of large yellow croaker fed experimental diets with graded biotin levels for 9 weeks[1]

Dietary biotin levels (mg kg^{-1})	LA (U mL^{-1})	ACH50 (U mL^{-1})
0.00 (the basal diet)	130.7 ± 10.91[b]	95.3 ± 6.33[b]
0.05 (moderate-dose diet)	171.0 ± 6.24[a]	146.5 ± 7.31[a]
6.22 (high-dose diet)	165.3 ± 7.31[a]	153.5 ± 11.46[a]
ANOVA		
F value	6.807	13.364
P value	0.029	0.006

LA, lysozyme activity; ACH50, alternative complement pathway activity; ANOVA, one-way analysis of variance.
LA(U mL^{-1}) = 2500 × (A1−A2), where A1 means the absorbance at 0.5 min, while A2 means the absorbance at 4.5 min. ACH50 (U mL^{-1}) = n $2k^{-1}$, where n mean the dilute times of serum, and k means the volume of serum led to 50% haemolysis.
[1] Values are means ± SEM of three replicate groups (n = 3); Means with different letter in the same column differ significantly ($P < 0.05$).

in varied fish species, and that feeding fish a biotin-free diet after a certain time probably would result in biotin deficiency syndrome.

Dose–response experiments with increasing supply of nutrient are accepted in principle as a method for determining dietary nutrient requirements. Nutrient requirements in fish can be estimated by either broken-line regression analysis (Robbins *et al.* 1979) or polynomial regression analysis (Zeitoun *et al.* 1976). Comparisons between broken-line regression model and second-order polynomial regression model have been made before the analysis of optimal dietary biotin requirement on the basis of WG. The sum of squares about regression (SSR) and the coefficient of estimation (R^2) have been calculated. The results indicated that the broken-line model is more suitable to describe the relationship between dietary biotin level and WG of large yellow croaker. Hence, the requirement of juvenile large yellow croaker for dietary biotin was estimated to be 0.039 mg kg^{-1} by using the broken-line regression model. The result was comparable to the data of 0.02–0.03 mg kg^{-1} for common carp (Ogino *et al.* 1970), 0.05 mg kg^{-1} for rainbow trout (Woodward & Frigg 1989), 0.046 mg kg^{-1} for Japanese seabass (Li *et al.* 2010) and 0.06 mg kg^{-1} for hybrid tilapia (Shiau & Chin 1999). Whereas it was much lower than those of 0.1 mg kg^{-1} for lake trout (Poston 1976), 2.0–2.5 mg kg^{-1} mirror carp (Gunther & Meyer-Burgdorff 1990), 2.49 mg kg^{-1} for Asian catfish (Shaik Mohamed *et al.* 2000) and 0.25 mg kg^{-1} for Indian catfish (Shaik Monhamed 2001). The discrepancy observed in the requirements for biotin among fish species might be due to the differences in diet formulation, fish size and age and genetic diversity. Besides, the model used to analyse the dose–response relationship also influences the estimate of requirements, and broken-line model generally gives lower estimates of requirements compared to nonlinear models (Baker 1986).

Large yellow croaker might also have a non-negligible intestinal microflora contributing some biotin for the lower requirement, since it is well established that gut bacterial flora is a significant source of a range of vitamins to the ruminant and human (Hill 1997) and Kashiwada *et al.* (1971) isolated water-soluble vitamin-synthesizing bacteria from the intestine in common carp. Wu *et al.* (2002) found that the SGR, daily increment in shell length, viscera biotin concentration and visceral pyruvate carboxylase and acetyl-CoA carboxylase activities of the antibiotic diet group were obviously depressed in juvenile abalone *Haliotis discus hannai* Ino. They pointed out that the intestinal microflora probably contributes to biotin nutrition for this animal. Further research is necessary to identify whether the intestinal microorganisms can synthesize biotin and whether it is a significant source of biotin in large yellow croaker.

Shaik Monhamed (2001) reported that the body protein and lipid of Indian catfish were significantly lower when fed the biotin-free diet compared with the fish fed the biotin-supplemented diets. However, in this study, dietary biotin level did not significantly affect the carcass crude protein, crude lipid, moisture and ash of large yellow croaker, though the carcass crude protein and crude lipid both showed the minimum value when fed the biotin-free diet. Besides, in this study, the liver biotin concentration increased significantly as dietary biotin level increased and no tissue saturation was found in this species. This result was similar to research on hybrid tilapia (Shiau & Chin 1999) and Asian catfish (Shaik Mohamed *et al.* 2000), but was not in agreement with that by Shaik Monhamed (2001), who reported that the carcass biotin content of Indian catfish increased significantly as the dietary supplementation level increased (up to 0.26 mg kg^{-1}), and then reached to a plateau when the dietary supplementation levels increased further to 4.5 mg kg^{-1}. This probably indicated that, as body pool of biotin, liver and carcass seemed to have different storage abilities in fish species, and the liver did not reached to the maximal biotin storage in this study.

Biotin deficiency has been proved to affect immune function in both humans and experimental animals (Báez-Saldaña *et al.* 1998). Wolf (1995) and Cowan *et al.* (1979) reported that syndromes caused by biotin deficiency were closely related to the lack of several carboxylases. In

rodents (Zempleni *et al.* 2009), guinea pigs (Petrelli *et al.* 1981) and cultured cells from mouse spleen (Kung *et al.* 1979), biotin was demonstrated to play essential roles in the normal function of a variety of immune cell, such as antibody synthesis, thymocyte maturation, lymphocyte generations. However, little information about the effects of biotin on fish immune response is available. It is customary to divide the immune system into the innate (non-specific) and the acquired (specific) immune system. Fish belongs to the lower vertebrates, and an increasing body of evidence from fish showed that these are combinational systems (Magnadóttir 2006). However, the specific immune mechanisms of fish species are quite imperfect, since most of them only have a single immune globulin type (IgM) and lack of the main immune globulin (IgG) that exists in higher animals' humoral immunity, and they cannot generate antibody from secondary reaction. There are several examples of the innate immune parameters of fish being more active and effective (Sunyer & Tort 1995; Zarkadis *et al.* 2001). In this study, the lysozyme activity (LA) and alternative complement pathway activity (ACH50) of moderate-dose treatment (0.05 mg biotin kg^{-1} diet) and high-dose treatment (6.22 mg biotin kg^{-1} diet) were both significantly higher than biotin-free treatment (the basal diet), indicating that dietary biotin with certain range could improve the non-specific immune response of large yellow croaker. The relationship between the dose of dietary biotin and immune function and the mechanism that biotin increased the non-specific immune response of large yellow croaker will be studied in further research.

In conclusion, dietary biotin significantly influenced the growth performance and non-specific immune response of large yellow croaker. The requirement of juvenile large yellow croaker for dietary biotin was estimated to be 0.039 mg kg^{-1} by using the broken-line regression model, and dietary biotin with certain range could improve the non-specific immune response of large yellow croaker. Further study is needed to determine the relationship between the dose of dietary biotin and immune function and the mechanism that dietary biotin affected the non-specific immune response of large yellow croaker.

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