Aquaculture Research

Aquaculture Research, 2012, **43**, 917–928 doi: 10.1111/j.1365-2109.2011.02906.x

Dietary lysine requirement of large yellow croaker (*Pseudosciaena crocea*, Richardson 1846) larvae

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Abstract

A 30-day feeding experiment was conducted to estimate the lysine requirement of large yellow croaker larvae (2.75 ± 0.11 mg). Six isonitrogenous (509.5–519.7 g kg^{-1} crude protein) and isoenergetic (22.3–22.5 kJ g^{-1} energy) microdiets containing graded levels of lysine · HCl ranging from 24.8 to 41.0 g kg^{-1} diet in placement of glycine and glutamic acid were formulated. Mixture of crystalline amino acids (MAA) was supplemented to simulate the amino acid (AA) profiles of whole body of this larva, except for lysine. The MAA and supplemented lysine for each diet were coated with tripalmitin. Triplicate groups of 3000 fish were fed to apparent satiation by hand eight times per day. The results showed that specific growth rate (SGR), survival, body composition and the specific activity of digestive enzymes were significantly affected by dietary lysine levels ($P < 0.05$). The optimal dietary lysine requirements estimated by second-order polynomial model based on SGR and survival were 33.7 (65.5 g kg^{-1} dietary protein) and 33.4 (64.9 g kg^{-1} dietary protein) g kg^{-1} dry diet respectively. The estimated requirements for the other essential AAs were calculated by *A*/*E* ratios of whole body AA profile of this larva based on lysine requirement.

Keywords: large yellow croaker, lysine requirement, coated, growth, survival, digestive enzyme

Introduction

Fish larvae have a large growth potential, with growth rates ranging from 10% to 100% per day, which is significantly higher than that in adult stages (Houde 1989; Conceição 1997; Conceição, Dersjant-Li & Verreth 1998; Otterlei, Nyhammer, Folkvord & Stefansson Sigurd 1999). Growth at the early stage of fish is mainly in the form of protein deposition in muscle tissue (Houlihan, McCarthy, Carter & Martin 1995; Carter & Houlihan 2001). In addition, amino acids (AAs) have been proven to be a major fuel during the early life stages of several marine teleost species (Conceição, Verreth, Scheltema & Machiels 1993; Rønnestad & Fyhn 1993; Finn, Fyhn, Henderson & Evjen 1995; Finn, Rønnestad & Fyhn 1995; Sivaloganathan, Walford, Ip & Lam 1998; Parra, Rønnestad & Yúfera 1999; Rønnestad, Thorsen & Finn 1999). Therefore, fish larvae have a higher requirement of AA, and it is of utmost importance to master the requirement of each essential amino acid (EAA) in order to satisfy the high AA requirement of fish larvae and to produce AA balanced diets for fish larvae.

Lysine, which was found to be a top concentration in the carcass of many fish species (Wilson & Cowey 1985; Wilson & Poe 1985; National Research Council 1993), is generally the most limiting AA in the ingredients used to prepare fish feeds (Harris 1980; Forster & Ogata 1998; Small & Soares 2000). As an EAA, appropriate lysine content could reduce other amino acids oxidation by improving the use of other EAAs (Kerr & Easter 1995), therefore promoting a higher growth rate. In addition, lysine is also a precursor of carnitine, which plays an important role in the transport of long-chain fatty acyl groups into the mitochondria for β-oxidation (Tanphaichitr, Horne & Broquist 1971). So far, many studies of lysine requirement have been carried on either juvenile or adult stage (Small & Soares 2000; Tantikitti & Chimsung 2001; Ahmed & Khan 2004; Wang, Liu, Tian, Xie, Yang, Wang & Liang 2005; Luo, Liu, Mai, Tian, Tan, Yang, Liang & Liu 2006; Mai, Wan, Ai, Xu, Liufu, Zhang, Zhang & Li 2006; Zhou, Wu, Chi & Yang

2007; Zhou, Zhao, Jiang, Feng & Liu 2007; Zhang, Ai, Mai, Tan, Li & Zhang 2008; Deng, Dominy, Ju, Koshio, Murashige & Wilson 2010), but little is known about the lysine requirements of larval stages of marine fish.

Large yellow croaker (*Pseudosciaena crocea*) is a marine fish that has been widely cultured in China because of its delicious taste and important commercial value (300 million dollars). This natural resource has nearly been depleted due to over-fishing. Farming of large yellow croaker boomed after the success in larvae culture in the late 1980s (Lin, Zhan, Zheng, Weng & Shu 1991). In recent years, the annual production of fingerling reaches more than 300 million solely in Fujian province, the major area of large yellow croaker culture in China. There have been a number of studies on the nutrition for large yellow croaker larvae and juvenile (Duan, Mai, Zhong, Si, & Wang 2001; Mai, Yu, Ma, Duan, Gisbert, Zambonino Infante & Cahu 2005; Ai, Mai, Tan, Xu, Duan, Ma & Zhang 2006; Liu, Mai, Ai, Duan, Xu, Tan, Zhang, Ma & Liufu 2006; Mai, Zhang, Ai, Duan, Zhang, Li, Wan & Liufu 2006; Ai, Mai, Zhang, Tan, Zhang, Xu & Li 2007; Ai, Zhao, Mai, Xu, Tan, Ma, & Liufu 2008; Zhao, Ai, Mai, Tan, Xu & Ma 2008; Xie, Ai, Mai, Xu & Ma 2011). Especially, Zhang *et al.* (2008) found that the lysine requirements of juvenile large yellow croaker (initial body weight, 1.23 ± 0.02 g) were 24.8 g kg^{-1} (57.7 g kg^{-1} of dietary protein) diet [dry weight (DW)] based on growth. However, this result cannot apply to large yellow croaker larvae because of the considerable differences in nutritional requirements between juvenile and larvae stage. In addition, to know the special requirements of each EAA is urgent for developing nutritional microdiet (MD) for this larva. Therefore, the present study was designed to determine the lysine requirement of large yellow croaker larvae. Moreover, the other EAAs requirements were derived by the *A*/*E* ratios, according to lysine requirement and the AA composition of whole body of large yellow croaker larvae.

Materials and methods

Experimental diets

Six isonitrogenous and isoenergetic diets were formulated with graded levels of lysine (Table 1) to satisfy the nutrient requirements of large yellow croaker larvae, based on the previous studies (Liu *et al.* 2006; Ai *et al.* 2008). Low-temperature-processed white fish meal, krill meal, squid meal, hydrolysed fish meal and corn gluten meal were used as the main protein sources, the AA compositions were presented in Table 2. L-crystalline AAs were supplemented to simulate the AA pattern of large yellow croaker larvae whole body (Table 2), except for lysine. L-crystalline lysine · HCl was supplemented to the basal diets at six levels from 0.0 to 15 g kg^{-1} diet (DW) in 3.0 g kg^{-1} increments. The mixture of L-crystalline AAs and the supplemented lysine for each diet were coated with tripalmitin (Steinheim, Germany), according to the method reported by López-Alvarado, Langdon, Teshima and Kanazawa (1994). NaOH was used to adjust the pH of the diets to 7.5. The formulation and assayed composition of the experimental diets (509.5–519.7 g kg^{-1} crude protein, 22.3–22.5 kJ g^{-1} energy) are presented in Table 1.

The MD was manufactured by microbonding technology. Diets were prepared by thoroughly mixing dry ingredients with the oil and lecithin. The particle size of the formulated diets ranged from 150 to 250 μm for fish between 13 and 25 days after hatch (DAH) and 200–350 μm for fish thereafter. All formulated diets were packed in separate silver bags and stored at − 20 °C until used.

Experimental procedure

Larvae used in this study were obtained and reared at the hatchery of the Aquatic Technology Extension Station of Ningde (Fujian, China). All the larvae in the hatchery were fed with rotifers, *Brachionus plicatilis* (0.5–1.5 × 10^4 ind L^{-1}), from 3 to 8 DAH, with *Artemia nauplii* (1.0–1.5 × 10^3 ind L^{-1}) from 6 to 11 DAH and with live copepods and a commercial pellet diet (RQ Com., manufactured by Marubeni Nisshin Feed, Chuo-Ku, Japan) from 10 to 13 DAH; then, the larvae were weaned to the experimental diet. For this experiment, a total of 54 000 larvae (13 DAH), with an initial average weight of 2.75 ± 0.11 mg, were used in this study in 18 blue plastic tanks (70 × 50 × 60 cm, water volume 180 L). Each tank was stocked initially with 3000 individuals. All tanks were placed in an indoor concrete pond (800 × 400 × 160 cm). They were supplied with seawater that had been filtered through a two-grade sand filter. During the rearing period of 30 days, water temperature, pH and salinity were 23 ± 1 °C, 8.0 ± 0.2 and 23 ± 2 g L^{-1} respectively. About 150–300% of the water volume was renewed daily, and air was provided by an air stone in each tank. Larvae were reared under 14 h light:10 h dark dial cycle photoperiod. Light intensity was 8.5 W m^{-2} maxi-

Table 1 Formulation and proximate chemical composition of experimental diets (g kg^{-1} dry matter)

Ingredients	Diet 1 (24.8)	Diet 2 (27.8)	Diet 3 (30.6)	Diet 4 (33.5)	Diet 5 (37.2)	Diet 6 (41.0)
LT-white fish meal*	150.0	150.0	150.0	150.0	150.0	150.0
LT-Krill meal*	150.0	150.0	150.0	150.0	150.0	150.0
Squid meal*	150.0	150.0	150.0	150.0	150.0	150.0
Hydrolysed fish meal*	30.0	30.0	30.0	30.0	30.0	30.0
Corn gluten*	200.0	200.0	200.0	200.0	200.0	200.0
Mixed amino acids†	55.0	55.0	55.0	55.0	55.0	55.0
Fish oil	50.0	50.0	50.0	50.0	50.0	50.0
DHA oil	10.0	10.0	10.0	10.0	10.0	10.0
Soyabean lecithin	50.0	50.0	50.0	50.0	50.0	50.0
α-starch	55.0	55.0	55.0	55.0	55.0	55.0
Alginate sodium	15.0	15.0	15.0	15.0	15.0	15.0
Vitamin premix‡	15.0	15.0	15.0	15.0	15.0	15.0
Mineral premix§	15.0	15.0	15.0	15.0	15.0	15.0
Ascorbyl polyphosphate	2.0	2.0	2.0	2.0	2.0	2.0
Lycopene	0.5	0.5	0.5	0.5	0.5	0.5
Antioxidant	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride	2.0	2.0	2.0	2.0	2.0	2.0
L-lysine · HCl (mg kg^{-1})	0.0	3.0	6.0	9.0	12.0	15.0
Glutamic acid	22.5	20.8	19.1	17.4	15.8	14.1
Glycine	17.5	16.2	14.9	13.6	12.3	10.9
Proximate composition						
Lysine	24.8	27.8	30.6	33.5	37.2	41.0
Crude protein	512.8	516.6	511.5	519.7	509.5	515.5
Crude lipid	188.6	181.1	187.5	189.3	188.5	190.2
Ash	105.4	107.4	104.5	104.9	100.2	100.2
Energy (kJ g^{-1})	22.3	22.3	22.3	22.3	22.4	22.5

*White fish meal and corn gluten, obtained from Cishan Fisheries (Shandong, China), crude protein, 677 and 550 g kg^{-1} dry matter, respectively, crude lipid, 90 and 33 g kg^{-1} dry matter, respectively; Krill meal and squid meal, obtained from Jinhaiyun (Zhejiang, China), crude protein, 648 and 501 g kg^{-1} dry matter respectively, crude lipid, 57 and 48 g kg^{-1} dry matter, respectively; Hydrolyzed fish meal, obtained from Chaoxing Halobios (Zhejiang, China), crude protein, 634 g kg^{-1} dry matter, crude lipid, 78 g kg^{-1} dry matter.

†Mixed amino acids (g kg^{-1} diet): arginine 6.3; histidine 3.0; isoleucine 3.7; methionine 5.6; threonine 6.5; valine 5,9; alanine 3.5; aspartic acid 144; serine 54; phenylalanine 0.39; and tyrosine 0.11.

‡Composition of vitamin premix (IU or g kg^{-1} diet): retinal palmitate, 30 000 00 IU; cholecalciferol, 12 000 00 IU; DL-α-tocopherol acetate, 40.0 g kg^{-1}; menadione, 8.0 g kg^{-1}; thiamin-HCl, 5.0 g kg^{-1}; riboflavin, 5.0 g kg^{-1}; D-calcium pantothenate, 16.0 g kg^{-1}; pyridoxine-HCl, 4.0 g kg^{-1}; meso-inositol, 200.0 g kg^{-1}; D-biotin, 8.0 g kg^{-1}; folic acid, 1.5 g kg^{-1}; *para*-aminobenzoic acid, 5.0 g kg^{-1}; niacin, 20.0 g kg^{-1}; cyanocobalamin, 0.01 g kg^{-1}; and ascorbyl polyphosphate (contained 25% ascorbic acid), 100.0 g kg^{-1}.

§Composition of mineral premix (g kg^{-1} diet): $Ca(H_2PO_4)_2 \cdot H_2O$, 675.0; $CoSO_4 \cdot 4H_2O$, 0.15; $CuSO_4 \cdot 5H_2O$, 5.0; $FeSO_4 \cdot 7H_2O$, 50.0; KCl, 50.0; KI, 0.1; $MgSO_4 \cdot 2H_2O$, 101.7; $MnSO_4 \cdot 4H_2O$, 18.0; NaCl, 80.0; $Na_2SeO_3 \cdot H_2O$, 0.05; and $ZnSO_4 \cdot 7H_2O$, 20.0.

LT, low temperature.

mum during daytime at the water surface. The foreign matter floating on the water surface was skimmed every 15 min with a polyvinylchloride pipe and accumulations of feed and faeces at the tank bottoms were siphoned twice daily.

From 13 to 42 DAH, the fish were manually fed with the experimental diets to satiation each time (eight times per day, Xie *et al.* 2011).

Sampling and dissection

At the beginning of the experiment, three hundred larvae at 13 DAH were randomly sampled to monitor wet body weight. At the end of the experiment, fish survival was determined by counting individuals remaining in each tank. All fish were deprived of food for 1 day before sampling to empty their guts (Xie *et al.* 2011). Two hundred of specimens were randomly sampled from each tank for weight and length analysis. Samples of 50 individuals were randomly collected from each tank for enzymatic assays were immediately frozen in liquid nitrogen and then were stored at − 80 °C until use. The remaining fish from each tank were collected and stored at − 20 °C for subsequent analysis.

The larvae (42 DAH) were dissected as described by Cahu and Zambonino Infante (1994) and Ma, Cahu, Zambonino, Yu, Duan, Le Gall and Mai (2005),

Table 2 Amino acid composition of the ingredients, experimental diets and whole body of *Pseudosciaena crocea* larvae (g kg^{-1} dry matter)

Amino acids	Fish meal	Krill meal	Hydrolysed fish meal	Squid meal	Corn gluten	Diet 1 (24.8)	Diet 2 (27.8)	Diet 3 (30.6)	Diet 4 (33.5)	Diet 5 (37.2)	Diet 6 (41.0)	Whole body of larvae*
EAA												
Arginine	48.9	49.5	32.5	36.3	17.9	29.7	29.5	29.0	29.0	28.7	29.1	30.1
Histinine	15.6	14.5	9.8	10.1	10.6	9.2	9.0	8.9	8.9	8.9	8.8	11.0
Isoleucine	32.7	26.4	17.8	18.7	22.7	18.2	18.3	18.3	17.4	17.4	17.5	19.9
Leucine	57.8	48.1	35.2	35.0	87.9	41.2	41.7	39.6	39.2	39.4	40.5	36.3
Lysine	59.4	51.7	37.0	30.9	9.2	24.8	27.8	30.6	33.5	37.2	41.0	37.2
Methionine	19.7	16.7	10.9	9.0	13.5	14.2	14.3	13.9	14.5	14.6	14.0	14.6
Phenylalanine	27.4	26.0	15.6	14.0	34.1	19.1	18.8	18.2	18.4	18.9	18.2	18.7
Threonine	33.4	25.6	20.4	19.2	18.8	20.2	20.2	21.6	19.6	20.7	20.6	21.6
Valine	37.9	29.4	25.3	19.0	26.7	23.7	23.4	23.7	23.6	23.5	23.7	24.1
NEAA												
Alanine	41.8	38.1	46.1	23.0	48.5	28.1	28.5	27.8	25.2	28.0	27.2	29.5
Aspartic acid	74.7	65.6	46.7	43.9	33.9	46.3	46.8	45.3	45.4	44.7	45.4	48.0
Cystine	3.5	3.1	3.4	2.3	4.3	9.1	9.3	7.0	9.9	6.7	8.3	9.1
Glutamic acid	105.1	97.3	79.2	109.0	157.4	85.9	84.2	80.5	76.3	76.8	77.9	67.8
Glycine	45.8	31.0	72.1	30.5	18.5	37.0	35.8	33.2	29.2	28.1	26.5	33.4
Serine	34.7	22.0	21.5	19.7	28.4	19.0	19.5	19.0	18.7	18.6	20.1	22.4
Tyrosine	25.1	21.3	8.3	13.0	28.2	4.6	5.6	5.1	7.0	7.6	5.0	14.9

Values are the mean of triplicate samples.
*Whole body of larvae: whole body of large yellow croaker larvae.

in order to separate pancreatic and intestinal segments (IS). Dissection was conducted on a glass plate maintained at 0 °C.

Analytical methods

Wet body weight of three groups (100 larvae per group) per treatment was measured after removing water with filter paper. The chemical composition of diets and fish was determined following the standard procedures (Association of Official Analytical Chemists 1995). The samples of diets and fish larvae were dried to a constant weight at 105 °C to determine the dry matter content. Protein was determined by the Kjeldahl method; lipid by ether extraction using a Soxhlet; and ash by combustion at 550 °C. Gross energy of each diet was determined using a Oxygen Bomb Calorimeter (Parr 1281, Parr Instrument Company, Moline, IL, USA).

Samples of feed ingredients and experimental diets were freeze-dried and 0.02 g of samples was used for AA analysis. The samples were hydrolyzed with 15 mL of 6 N HCl at 110 °C for 24 h, then filtered and added to ultrapure water (from Milli-Q system, Millipore, Billerica, MA, USA) in a 50 mL volumetric flask. A 2 mL solution was then transferred to a glass bottle and dried in a Binder Oven (VD23, Binder Company, Germany). Thereafter, 2 mL of ultrapure water was then added to the bottles and dried in the Binder Oven repeatedly three times, and then 2 mL of loading buffer was added to dissolve the remains. The supernatant was analysed by the ninhydrin method with an automatic AA analyzer (Biochrom 30, GE, Biochrom Ltd, Cambridge, UK), equipped with a sodium exchange column (μ-2345). The column temperature was 37–135 °C. Ultraviolet detection was performed at a wavelength of 440 nm (for proline) and 570 nm (for other AAs).

For fish at 42 DAH, the dissected samples, 0.2–0.3 g pancreatic segments (PS), were homogenized in 2 mL of cold (0 °C) ultrapure water and centrifuged at 3300 *g* for 3 min, and the supernatant was collected for further assay. Furthermore, 0.2–0.3 g IS were homogenized to purify brush border membranes (BBMs) according to a method developed for intestinal scraping (Crane, Boge & Rigal 1979) and adapted to IS (Cahu & Zambonino Infante 1994). Before $CaCl_2$ solution was added, 1 mL of homogenate was diverted for intestinal enzyme assays. This homogenate was then centrifuged at 3300 *g* for 3 min, and the supernatant was used for enzyme assays. Trypsin was assayed according to Holm, Hanssen, Krogdahl and Florholmen (1988). Leucine-aminopeptidase N (AN) and alkaline phosphatase (AP) activities were assayed according to Maroux, Louvard and Baratti (1973) and Bessey, Lowry and Brock (1946) respec-

tively. Enzyme activities are expressed as specific activities (mU. . .mg protein $^{-1}$). Protein was determined according to Bradford (1976), using bovine serum albumin (Sigma A-2153, Sigma Aldrich, St. Louis, MO, USA) as a standard.

Calculations

$$\text{Specific growth rate (SGR, \% day}^{-1}) = ((\text{Ln}W_f - \text{Ln}W_i)/d) \times 100$$

where W_f is the final wet body weight, W_i is the initial wet body weight and d is the experimental period in days:

$$\text{Survival rate}\,(\%) = N_{final}/N_{initial} \times 100$$

where N_{final} is the number of fish larvae in each tank at the end of this experiment and $N_{initial}$ is the number of fish larvae in each tank at the beginning of this experiment:

$$A/E \text{ ratio} = [(\text{amount of each EAA}) / (\text{total amount of EAA including tyrosine and cystine})] \times 1000$$

The requirement of EAA (R_x) other than lysine (R_{lys}) was calculated from A/E ratios using the following formula (Forster & Ogata 1998):

$$R_x = R_{lys} \times [(A/E)_x/(A/E)_{lys}].$$

Statistical analyses

One of broken-line model ($Y = L - U\,(R - X)$, if $X > R$ then $Y = L$), two slope broken-line model ($Y = L - U\,(R - X)U_2\,(X - R)$, if $X > R$ then $Y = L - U_2\,(X - R)$; if $X < R$ then $Y = L - U\,(R - X)$) and second-order polynomial model ($Y = a + bx + cx^2$) were used for estimating the lysine requirement.

Results are given as mean ± SE All data were subjected to one-way analysis of variance (ANOVA) using the software program SPSS 13.0. Tukey's honest significant difference test (Tukey HSD test) was chosen as a multiple comparison test and the significance level of 0.05 was used.

Results

Survival and growth

The survival of larvae fed the diet with 24.8 g kg^{-1} lysine was the lowest, which was significantly lower than that of 30.6, 33.5 and 37.2 g kg^{-1} lysine treatments ($P < 0.05$, Table 4). Larvae fed the diet with 30.6 g kg^{-1} lysine had the highest survival result, although no significant differences were observed among 30.6, 33.5 and 37.2 g kg^{-1} lysine treatments ($P > 0.05$).

The results of growth were similar to survival. The SGR of larvae fed the diet with 24.8 g kg^{-1} lysine was significant lower than that in other treatments ($P < 0.05$). Fish larvae fed the diet with 30.6 g kg^{-1} lysine had the best SGR, significantly higher than that in other treatments, except for 33.5 g kg^{-1} lysine group ($P < 0.05$) (Table 3).

Body composition

Whole body protein and lipid of fish larvae were significantly affected by dietary lysine levels ($P < 0.05$, Table 4). The whole body protein (46.8–52.9 g 100 g^{-1}) increased with increasing lysine levels (24.8–33.5 g kg^{-1}, DW) and decreased from 37.2 to 41.0 g kg^{-1} lysine levels in diets. The larvae fed with 33.5 g kg^{-1} lysine had the highest protein content, significantly higher than that in 24.8, 27.8, 30.6 and 41.0 g kg^{-1} lysine treatments ($P < 0.05$). The lipid content (13.0–14.3 g kg^{-1}) showed an opposite tendency to body protein content. The larvae fed with 30.6 g kg^{-1} lysine had the lowest lipid content, significantly lower than that in 24.8, 37.2 and

Table 3 Effects of lysine levels on growth and survival of *Pseudosciaena crocea* larvae (42 DAH, mean ± SE, $n = 3$)

Treatments	Initial weight (mg)	Final weight (mg)	Survival (%)	SGR (% day^{-1})
Diet 1 (24.8)	2.75 ± 0.11	32.09 ± 1.45^{a}	18.37 ± 0.51^{a}	8.18 ± 0.15^{a}
Diet 2 (27.8)	2.75 ± 0.11	46.64 ± 0.58^{bc}	20.83 ± 0.54^{ab}	9.44 ± 0.04^{bc}
Diet 3 (30.6)	2.75 ± 0.11	64.68 ± 2.28^{d}	24.41 ± 0.85^{c}	10.52 ± 0.12^{d}
Diet 4 (33.5)	2.75 ± 0.11	59.16 ± 2.13^{d}	24.30 ± 0.41^{c}	10.22 ± 0.12^{d}
Diet 5 (37.2)	2.75 ± 0.11	51.23 ± 0.55^{c}	21.69 ± 0.30^{bc}	9.75 ± 0.04^{c}
Diet 6 (41.0)	2.75 ± 0.11	42.16 ± 0.61^{b}	20.15 ± 0.79^{ab}	9.10 ± 0.05^{b}

Values with the same superscript showed no significant differences determined by the Tukey test ($P > 0.05$).
SGR, specific growth rate.

Table 4 The effects of lysine levels on body composition of *Pseudosciaena crocea* larvae (42 DAH, mean ± SE, $n = 3$)

Treatments	Protein (g kg^{-1})	Lipid (g kg^{-1})	Moisture (g kg^{-1})
Diet 1 (24.8)	46.8 ± 0.8^{a}	14.3 ± 0.5^{b}	890.4 ± 25.5
Diet 2 (27.8)	47.6 ± 0.7^{a}	13.6 ± 0.7^{ab}	877.7 ± 17.9
Diet 3 (30.6)	49.3 ± 0.5^{b}	13.0 ± 0.3^{a}	892.3 ± 31.2
Diet 4 (33.5)	52.9 ± 0.9^{c}	13.5 ± 0.4^{a}	908.1 ± 33.7
Diet 5 (37.2)	51.7 ± 1.1^{bc}	14.1 ± 0.9^{b}	907.3 ± 29.8
Diet 6 (41.0)	48.4 ± 1.0^{ab}	14.1 ± 0.6^{b}	898.4 ± 36.7

Values with the same superscript showed no significant differences determined by the Tukey test ($P > 0.05$).

Table 5 The effects of dietary lysine levels on specific activities of digestive enzymes of *Pseudosciaena crocea* larvae (42 DAH, mean ± SE, $n = 3$)

Treatments		Diet 1 (24.8)	Diet 2 (27.8)	Diet 3 (30.6)	Diet 4 (33.5)	Diet 5 (37.2)	Diet 6 (41.0)
PS	Trypsin*	55.90 ± 0.96^{c}	46.11 ± 1.09^{b}	32.44 ± 1.38^{a}	36.81 ± 0.54^{a}	38.89 ± 1.27^{ab}	34.87 ± 1.79^{a}
IS	Trypsin*	29.88 ± 0.62^{cd}	33.14 ± 0.98^{d}	24.71 ± 0.94^{b}	31.14 ± 1.19^{cd}	27.63 ± 1.52^{bc}	18.24 ± 1.10^{a}
Trypsin-IS/trypsin-PS		0.53 ± 0.01^{a}	0.72 ± 0.04^{b}	0.76 ± 0.06^{b}	0.85 ± 0.02^{b}	0.71 ± 0.02^{b}	0.53 ± 0.04^{a}
Specific activities of digestive enzymes in purified brush border membrane of intestine							
Leucine-aminopeptidase†		184.52 ± 2.70^{a}	183.68 ± 5.80^{a}	193.80 ± 5.98^{ab}	207.30 ± 4.14^{ab}	219.16 ± 7.80^{b}	192.16 ± 5.87^{a}
Alkaline phosphatase‡		105.38 ± 3.30^{a}	139.96 ± 2.36^{cd}	128.01 ± 3.60^{bc}	145.53 ± 2.75^{d}	152.37 ± 2.50^{d}	124.87 ± 2.38^{b}

Values with the same superscript showed no significant differences determined by the Tukey test ($P > 0.05$).
*The units of enzyme activity is mU mg^{-1} · protein.
†The units of enzyme activity is mU mg^{-1} · protein.
‡The units of enzyme activity is U mg^{-1} · protein.
PS, pancreatic segments; IS, intestinal segments; trypsin-IS, trypsin of intestinal segment; trypsin-PS, trypsin of pancreatic segment.

41.0 g kg^{-1} lysine treatments ($P < 0.05$). There was no significant difference in body moisture content (877.7–908.1 g kg^{-1}) among dietary treatments ($P > 0.05$).

Special activities of digestive enzymes

The ratio of trypsin specific activities between IS and PS significantly increased from 24.8 to 33.5 g kg^{-1} lysine, and then decreased from 33.5 to 41.0 g kg^{-1} lysine ($P < 0.05$, Table 5). The ratios in 24.8 and 41.0 g kg^{-1} lysine groups were significantly lower than that in other treatments ($P < 0.05$).

The specific activities of AN and AP both showed a similar trend to the ratio between trypsin-IS and trypsin-PS. Lower enzyme activities of AN and AP were observed in lysine-deficient and excessive treatments (24.8 and 41.0 g kg^{-1}).

Estimation of requirements

In this experiment, the second-order polynomial model had a higher R^2-value (0.9145 and 0.8778 for growth and survival respectively) than that of the broken-line model (0.604 and 0.471 for growth and survival respectively).The two slope broken-line model gave a higher R^2-value (0.96) on growth data than the second-order polynomial model, but it had a lower R^2-value (0.858) on survival. In order to unify the statistical method in this experiment, the second-order polynomial regression curves [$y = -0.0266x^2 + 1.7904x - 19.759$, ($R^2 = 0.9145$); $y = -0.0755x^2 + 5.0503x - 60.486$ ($R^2 = 0.8778$)] were used to estimate the optimal point for the maximal growth or maximum survival (Figs 1 and 2) respectively. The results showed the breakpoints were at 33.7 g kg^{-1} (65.5 g kg^{-1} dietary protein) on growth and 33.4 g kg^{-1} (64.9 g kg^{-1} dietary protein) on survival, giving the least mean square error respectively.

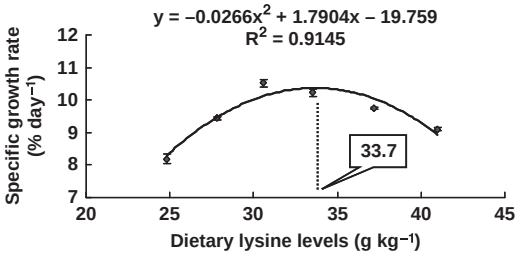

Figure 1 Relationship between dietary lysine levels and specific growth rate of *Pseudosciaena crocea* larvae was analysed by the second-order polynomial model.

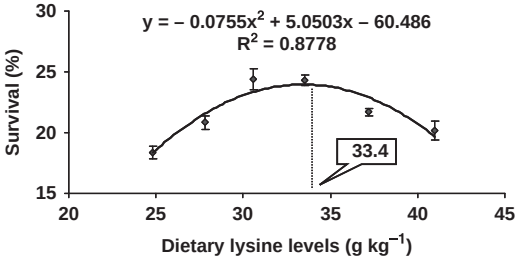

Figure 2 Relationship between dietary lysine levels and survival of *Pseudosciaena crocea* larvae was analysed by the second-order polynomial model.

Table 6 Estimated amino acids requirements based on lysine requirement and amino acid compositions of whole *Pseudosciaena crocea* larvae body

Essential amino acids	Estimated requirements (g kg^{-1} dry weight)	Estimated requirements (g kg^{-1} dietary protein)	*A/E* ratios
Arginine	27.3	52.3	131.5
Histinine	33.7	64.5	162.2
Isoleucine	18.0	34.5	86.9
Leucine	32.9	63.0	158.4
Lysine	33.7	64.5	162.2
Methionine+cystine	14.9	28.5	71.8
Phenylalanine+tyrosine	29.5	56.5	142.1
Threonine	19.6	37.5	94.3
Valine	21.8	41.7	104.9

A/E = [(amount of each EAA)/(total amount of EAA including tyrosine and cystine)] × 1000.

The requirement of EAA (R_x) other than lysine (R_{lys}) was calculated from A/E ratios using the formula:

$$R_x = R_{lys} \times [(A/E)_x/(A/E)_{lys} lys]$$

.

The estimated requirements for the other EAAs calculated from A/E ratios of whole body AA profile of *P. crocea* larvae based on the lysine requirement are presented in Table 6.

Discussion

Supplementing crystalline AAs in fish feed is likely to become an increasingly common practice to make up for the lack of one or more dietary EAAs (Segovia-Quintero & Reigh 2004). However, many studies obtained poor results in several species, such as rainbow trout (Rumsey & Ketola 1975; Kaushik & Luquet 1980), common carp (Aoe, Masuda, Abe, Saito, Toyoda & Kitamura 1970; Pongmaneerat, Watanabe, Takeushi & Satoh 1987) and tilapia (Mazid, Tanaka, Katayama, Simpson & Chichester 1978; Viola, Angeoni & Lahav 1994) when using crystalline amino acids. There are two main important reasons as follows: (1) rapid absorption rate of purified FAAs, which may result in excessive AA catabolism and reduced utilization efficiency (Lovell 1991); and (2) the leaching loss in aquatic environments (López-Alvarado *et al.* 1994; Zarate & Lovell 1997). These two aspects could be accomplished by coating the FAA with a digestion-resistant material that could slow the releasing and absorbing rapid (Cowey & Walton 1988; Villamar & Langdon 1993; De la Higuera, Garzón, Hidalgo, Peragón, Cardenete & Lupiáñez 1998) and reduce the leaching loss (López-Alvarado *et al.* 1994; Segovia-Quintero & Reigh 2004). Therefore, tripalmitin was chosen to coat the FAA in this experiment, based on the results from López-Alvarado *et al.* (1994), Segovia-Quintero and Reigh (2004) and Deng, Mai, Ai, Zhang, Wang and Tan (2007).

Data of an optimum requirement of each EAA are a prerequisite to the formulation of nutritionally adequate cost-effective diets for fish culture. In the present study, the optimal lysine levels for maximum growth and survival were 33.7 (65.5 g kg^{-1} dietary protein) and 33.4 (64.9 g kg^{-1} dietary protein) g kg^{-1} diet (DW) respectively. These results of the present study were higher than that reported for juvenile large yellow croaker (56.5–57.7 g kg^{-1} dietary protein) (Zhang *et al.* 2008). This could be mainly attributed to two factors: (1) the considerable differences in growth rate between juvenile (SGR, 2.76–3.27% day^{-1}) and larvae (SGR, 8.18–10.52% day^{-1}) of large yellow croaker; and (2) the weak capacity to digest manufactured diet as a result of the lower developed digestive system of this larvae (Ma *et al.* 2005). Else, the lysine requirement of this croaker larvae was higher than those reported for other commonly cultured fish species at juvenile or adult stages (32.0–62.0 g kg^{-1} dietary protein) (Wilson 2002). These results further indicate that higher AA content in the diet is required for fish larvae (Conceição, Grasdalen & Rønnestad 2003; Rønnestad, Tonheim, Fyhn, Rojas-García, Kamisaka, Koven, Finn, Terjesen, Barr & Conceição 2003). The wide variations about lysine requirement among fish species can be attributed to differences in species, feeding regime, culture conditions, dietary protein and AA sources, the reference AA compositions, dietary energy contents, diet formulation (Kim, Kayes & Amundson 1992; Akiyama, Oohara & Yamamoto 1997; Ruchimat, Masumoto, Hosokawa, Itoh & Shimeno 1997; Forster & Ogata 1998; Simmons, Moccia, Bureau, Sivak &

Herbert 1999; De Silva, Gunasekera & Gooley 2000; Mai, Zhang *et al.* 2006), as well as to differences in the response criteria and in the mathematical models (Rodehutscord, Becker, Pack & Pfeffer 1997).

When fish were fed an AA imbalanced diet, the absorbed dietary AA that do not match the profile for protein synthesis will be deaminated and used in energy production, gluconeogenesis or lipogenesis (Ballantyne 2001). The AA imbalanced diets have been shown to increase AA oxidation (Kim, McMillan & Bayley 1983; Kaczanowski & Beamish 1996). A balanced dietary AA profile increases the AA retention and may improve growth and nitrogen utilization (Aragão, Conceição, Dinis & Fyhn 2004). In the present study, lower protein and higher lipid in fish fed with lysine-deficient (24.8 g kg^{-1}, DW) and excessive diets (41.0 g kg^{-1}, DW) may be attributed to imbalanced AA profile caused by the lysine supplemented, which could increase oxidation of all the FAAs as reported in adult fish (Tacon & Cowey 1985) and which could accelerate AA to be used for lipogenesis. One the other hand, lysine serves along with methionine as a precursor to carnitine, which is involved in the transportation of long-chain fatty acyl groups into the mitochondria for β-oxidation (Walton, Cowey & Adron 1984). Lower protein and higher lipid in fish fed lysine-deficient diets may be explained by reduced β-oxidation of fatty acids, resulting in a lower utilization of lipid as an energy source, increasing the utilization of protein as an energy source (Zhang *et al.* 2008).

In the present study, the growth and survival were significantly affected by dietary lysine levels; the lowest growth and survival were both found in lysine-deficient treatment (24.8 g kg^{-1}, DW). These results indicate that lysine is essential for large yellow croaker larvae, and this species is able to utilize L-crystalline lysine. The lower growth of larvae in lysine-deficient (24.8 and 27.8 g kg^{-1}, DW) and excessive treatments (37.2 and 4.10 g kg^{-1}, DW) may be attributed to the imbalance of dietary AAs, which caused lower protein retention in fish body. Meanwhile, lysine deficiency could cause loss of appetite, resulting in low diet intake and in reduced growth (Borlongan & Benitez 1990; Khan & Jafri 1993; Murthy & Varghese 1997; Mai, Zhang *et al.* 2006; Zhou, Wu *et al.* 2007; Zhang *et al.* 2008). Some studies showed similar results of reduced growth in higher dietary lysine treatments (Walton *et al.* 1984; Choo, Smith, Cho & Ferguson 1991; Murthy & Varghese 1997; Ahmed & Khan 2004; Deng *et al.* 2010). However, some studies found no significant differences in growth performance between excessive lysine treatment and appropriate lysine group (Ruchimat *et al.* 1997; Forster & Ogata 1998; Luo *et al.* 2006; Zhou, Wu *et al.* 2007), although slight decreased tendencies were reported in several studies (Cheng, Hardy & Usry 2003; Wang *et al.* 2005; Mai, Zhang *et al.* 2006; Zhang *et al.* 2008). The differences in fish species and life stages could account for the different results. In addition, the growth and survival could be correlated to the development of the larvae digestion system.

Many studies have focused on the development of digestive function in fish larvae (Cahu & Zambonino Infante 1994, 1995a, b; Kolkovski 2001; Ma *et al.* 2005; Mai *et al.* 2005). Larvae undergo major developmental changes in their digestive functions, which can be affected by components present at low concentration in the gut (Cahu & Zambonino Infante 1994). Two main stages are considered crucial in the maturation process of the digestive function, which are the achievement of pancreas secretion function and the onset of BBM enzymes in intestine (Cahu & Zambonino Infante 1995b; Kurokawa & Suzuki 1996; Ribeiro, Zambonino Infante, Cahu & Dinis 1999; Buchet, Zambonino Infante & Cahu 2000; Lazo, Dinis, Holt, Faulk & Arnold 2000; Ma *et al.* 2005). In this experiment, the ratio between trypsin-IS and trypsin-PS, which reflects the secretion activity of the exocrine pancreas (Cahu & Zambonino Infante 1994), was significantly influenced by the dietary lysine levels, and the results showed that optimal lysine supplementation could strengthen the pancreatic secretion. Both AP and AN are mainly located in cell membranes, and the variation in their activities during larval development could reflect globally the maturation process in cells of the intestinal membrane (Cahu, Zambonino-Infante, Escaffre, Bergot & Kaushik 1998). In this experiment, the strong increases of AP and AN activity indicated that the dietary lysine levels significantly affected the development of intestinal membrane cells. Therefore, the significant improvement in the survival of larvae fed with appropriate lysine levels can be associated with a strong increase in enzymatic activities of BBM (Cahu & Zambonino Infante 1995b). On the basis of the analysis of digestive enzymes in this experiment, an appropriate lysine level is beneficial to switch from a primary to an adult mode of digestion for larvae.

Conclusion

Results of this experiment indicated that lysine is essential for large yellow croaker larvae and that this

larva is able to efficiently utilize crystal L-lysine. The optimum dietary lysine level for this fish larva was estimated to be 33.7 (65.5 g kg^{-1} dietary protein) and 33.4 g kg^{-1} (64.9 g kg^{-1} dietary protein) based on growth and survival respectively. The estimated requirements for the other EAAs calculated from *A/E* ratios based on the lysine requirement will provide useful information to formulate more cost-effective and AA balanced diets for this fish larva.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (Grant No.: 31072222 and 30400335), the National 863 High-Tech Program (Grant No.: 2004AA603610) and the Program for New Century Excellent Talents in University (NCET–07–0776). We also thank Jin Gao, Jikang Shentu, Qingfei Li and Rui Ma for their help during the experiment.

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