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Effects of dietary amino acid patterns on growth and protein metabolism of large yellow croaker (*Larimichthys crocea*) larvae

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ABSTRACT

This study was conducted to evaluate the effects of dietary amino acids (AA) patterns on growth, survival, activities of digestive enzymes and aminotransferases and target of rapamycin (TOR) gene expression of large yellow croaker (*Larimichthys crocea*) larvae. The control diet was produced using intact protein as the only protein source. Four isonitrogenous and isolipidic semi-purified diets were formulated with crystalline-AA replacing approximately 40% fish meal protein-bound nitrogen. The AA patterns of these diets were adjusted according to the overall AA pattern of large yellow croaker egg protein (LEP), large yellow croaker larvae whole-body protein (LLP), large yellow croaker muscle protein (LMP) and white fishmeal protein (WFP), respectively. The test diets and live copepod were fed to triplicate groups of larvae (initial body weight 3.15 ± 0.15 mg) five times (6:00, 8:30, 12:30, 14:30, and 17:00) daily for 30 days. The results showed that specific growth rate (SGR) of fish fed the WFP diet was significantly higher than LEP or LLP diet ($P < 0.05$). At the end of the growth trial, there was no significant difference in survival rate among larvae fed LEP, LLP, WFP and the control diet ($P > 0.05$), whereas the highest value was recorded in larvae fed the LLP diet, followed by WFP, the control, LEP and LMP diet, respectively. Whole-body crude protein content was significantly higher in larvae fed the WFP diet compared to the LEP or LLP diet ($P < 0.05$). Larvae fed the live copepod had significantly higher whole-body crude protein and lipid contents than that in larvae fed artificial microdiets ($P < 0.05$). Whole-body moisture content was not significantly affected by dietary treatments ($P > 0.05$). The specific activities of digestive enzymes and the ratio "pancreatic enzyme in intestinal segment/pancreatic enzyme in pancreatic segment" were significantly higher in fish fed the WFP diet than fish fed LLP and LEP diet ($P < 0.05$). Specific activities of alanine and aspartate aminotransferases were significantly higher in fish fed the WFP diet compared to the other treatments ($P < 0.05$). No significant differences were observed in larvae body TOR gene expression among dietary treatments ($P > 0.05$). Results of this study indicated that white fishmeal protein amino acid pattern was a more suitable amino acid pattern in diets of large yellow croaker larvae compared to the amino acid pattern of LEP, LLP, and LMP.

1. Introduction

The transition stage from endogenous nutrition to exogenous feeding is the most critical phase for marine fish larvae. Once the intraembryonic yolk sac reserves were depleted, the digestive tract becomes critical in ensuring steady supply of dietary amino acids (AA) to the metabolic pathways of the growing larval tissues (Rønnestad et al., 2003; Sanderson and Kupferberg, 1999). At the early stages, fish have high AA requirements, which is partly due to their high growth rates compared to adult stages (Conceição, 1997) and partly due to the need of more AA providing energy for fish larvae (Parra et al., 1999). Furthermore, it has been found that the food of fish larvae in the natural environment (phyto- and zooplankton) contains high free amino acids (FAA) levels (Helland et al., 2003). Hence, the content and quality of the FAA pool in the food are probably essential during the critical period when the larvae convert from endogenous to exogenous feeding (Fyhn, 1993). However, the requirements of all the essential amino acids (EAA) are known only for a limited number of fishes (National Research Council, 1993; Lall and Anderson, 2005; Mambrini and Kaushik, 1995; Tibaldi and Kaushik, 2005; Wilson and Hardy, 2002). Especially for fish larvae, the data are much more absent. Due to the absence of amino acid requirements data for some species, different EAA profiles (whole fish egg, whole fish larvae, fish muscle or fish meal protein and so on) have been used as indicative of dietary EAA profiles.

In fish, the optimum dietary EAA pattern represents the proper balance of amino acids required for optimal protein retention (Boisen et al., 2000; Conceição et al., 2003). AA imbalances between dietary and larval body protein AA profiles can increase AA oxidation and decrease food conversion efficiencies. Consequently, the imbalance of dietary AA negatively affects the growth of fish (Fauconneau et al., 1992). In addition to meeting EAA requirements and optimizing EAA profiles, care should also be given to the ratio of EAA/NEAA

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(non-essential amino acids) when formulating diets for fish larvae (Peres and Oliva-Teles, 2006). NEAA should not exceed a particular proportion of dietary protein for optimum performance (Green et al., 2002).

Amino acids are not only important substrates for the synthesis of proteins and other nitrogenous compounds, but also are involved in the regulation of major metabolic pathways (Jobgen et al., 2006) and are considered as signaling molecules. Amino acids regulate protein synthesis by activating the target of rapamycin (TOR), and the central in TOR signaling pathway is TOR protein, which is a conserved serine/threonine kinase, and can relay a permissive nutritional signal to downstream targets which modulate the initiation and elongation phases of translation (Wullschleger et al., 2006). Some studies on rainbow trout (Lansard et al., 2010) and Jian carp (Chen et al., 2012) indicated that nutrition status could regulate the TOR signaling pathway in fish as in mammal.

Large yellow croaker (*L. crocea*) is a marine fish that has been widely cultured in south China due to its delicious taste and important commercial value. The natural resource of large yellow croaker has nearly been depleted due to over-fishing. Farming of large yellow croaker boomed after the success of large yellow croaker larval culture in the late 1980s (Lin et al., 1991). There have been a number of studies on the nutrition for large yellow croaker larvae (Ai et al., 2008; Liu et al., 2006; Ma et al., 2005; Mai et al., 2005; Wang et al., 2010; Xie et al., 2011, 2012; Yu et al., 2012; Zhao et al., 2008). However, the EAA requirements of large yellow croaker larvae have not been reported except for lysine (Xie et al., 2012) and methionine (Yu, 2006). On the basis of the previous study, the present study was designed to evaluate the optimal reference amino acid patterns of this fish larva by examining growth, survival, digestion and protein metabolism in relation to gene expression.

2. Materials and methods

2.1. Feed ingredients and diet formulation

Low-temperature-processed white fish meal, krill meal, squid meal, and yeast extract were used as the main protein sources. Cod liver oil and soybean lecithin were used as the main lipid sources (Ai et al., 2008; Xie et al., 2011). The mixture of betaine, glycine and alanine were used as the chemical stimulants. Before formulation, α-starch was precooked in order to improve its digestibility and stickiness. To improve water stability of the diets, sodium alginate was added to the mixture. The live copepod (the dominant species was *Calanus sinicus*) and five other isonitrogenous (55% crude protein, DM) and isolipidic (15% crude lipid, DM) semi-purified diets were designed as experimental diets, in which the control diet was produced using intact protein as the only protein source and four other diets were formulated with crystalline-AA replacing approximately 40% fish meal protein-bound nitrogen (Table 1). The AA patterns of these diets were adjusted according to the overall AA pattern (Table 2) of large yellow croaker egg protein (LEP), large yellow croaker larvae whole-body protein (LLP), large yellow croaker muscle protein (LMP) and white fishmeal protein (WFP) by supplementing crystalline-AA (Table 3). The amino acid compositions of experimental diets were shown in Table 4.

Micro-diet (MD) was manufactured by micro-bonding technology. The particle size of the formulated diets ranged from 150 to 250 μm for larvae between 15 and 22 days after hatch (DAH), and 250 to 425 μm diets for larvae between 23 and 45 DAH. The pellets were dried at 50 °C in a constant temperature oven for 5 h. The dry pellets were ground into two sizes above and stored at −20 °C until used in order to avoid lipid peroxidation.

2.2. Experimental procedure

Large yellow croaker (*L. crocea*) larvae used in this study were obtained and reared at the hatchery of the Aquatic Technology Extension Station of Ningde (Fujian, China). All larvae in the hatchery were fed with rotifers, *Brachionus plicatilis* ($0.5–1.5 \cdot 10^4$ ind L^{-1}) from 3 to 8 days after hatch (DAH), *Artemia nauplii* ($1.0–1.5 \cdot 10^4$ind L^{-1}) from 6 to 11 DAH,

Table 1
Formulation and proximate analysis of the experimental diets (% dry weight).

Ingredient(% dry weight)	Control	CAA
L T[a] — white fish meal[b]	50	30
L T[a] — shrimp meal[b]	10	7
L T[a] — squid meal[b]	15	10
Mixed amino acids[c]	0	20
Sodium alginate	1.5	1.5
Yeast	3	3
α-starch	6.0	12.5
Glycine	1.1	1.1
Glutamic	0.9	0.9
Vitamin premix[d]	1.7	1.7
Mineral premix[e]	1.5	1.5
Sodium benzoate	0.05	0.05
Antioxidant	0.05	0.05
Choline choride	0.2	0.2
Fish oil	4.5	6
Soybeans lecithin	4.5	4.5
Proximate composition (%, of dry matter)		
Crude protein	56.73	53.90—54.30
Crude lipid	16.16	14.45—15.16
Ash	15.42	10.14—10.99

[a] Low temperature.
[b] White fish meal, obtained from Cishan Fisheries (Shandong, china), crude protein, 71.5% dry matter, crude lipid, 6.89% dry matter; krill meal and squid meal, crude protein, 53% and 73% dry matter respectively, crude lipid, 3.5% and 3.7% dry matter respectively.
[c] Mixed amino acids: See the Table 3.
[d] Composition of Vitamin premix (IU or g kg^{-1} premix): retinal palmitate, 3,000,000 IU; cholecalciferol, 1,200,000 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}; ascorbyl polyphosphate (contained 25% ascorbic acid), 100.0 g kg^{-1}.
[e] Composition of Mineral premix (g kg^{-1} premix): $Ca(H_2PO_4)_2 \cdot H_2O$, 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; $ZnSO_4 \cdot 7H_2O$, 20.0.

Table 2
Amino acid composition (g/55 g protein) of different reference proteins.

Amino acids	LEP[c]	LLP[d]	LMP[e]	WFP[f]
EAA[a]				
Valine	3.26	2.17	2.33	2.28
Methionine	2.63	1.32	1.90	1.42
Isoleucine	3.06	1.80	2.11	1.96
Leucine	4.71	3.28	4.26	3.59
Phenylalanine	1.98	1.60	2.09	1.88
Histidine	1.53	0.99	0.99	1.35
Lysine	3.85	3.35	4.14	3.64
Arginine	3.5	2.72	3.66	2.99
Threonine	2.46	1.95	2.47	2.01
Tryptophan	1.07	1.20	0.70	0.53
NEAA[b]				
Tyrosine	1.81	1.34	1.71	1.62
Proline	1.88	1.67	2.44	1.72
Aspartic acid	3.97	4.33	5.65	4.38
Serine	2.58	2.02	2.19	2.18
Glutamic acid	6.76	6.12	9.09	6.60
Glycine	2.17	3.01	4.27	2.93
Alanine	3.55	2.66	4.09	2.74
Cystine	0	0.17	0.51	0.60

[a] EAA: essential amino acids.
[b] NEAA: nonessential amino acids.
[c] LEP: large yellow croaker egg protein.
[d] LLP: large yellow croaker larvae whole-body protein.
[e] LMP: large yellow croaker muscle protein.
[f] WFP: white fishmeal protein.

Table 3
Amino acid composition (g/100 g dry diet) of ingredients and supplementation of CAA in different diets to simulate amino acid pattern of reference proteins (g/55 g protein).

Amino acids	Fish meal (30%)	Krill meal (7%)	Squid meal (10%)	Yeast powder (3%)	CAA added[g]			
					LEP[c]	LLP[d]	LMP[e]	WFP[f]
EAA[a]								
Valine	0.888	0.206	0.190	0.064	1.692	1.116	0.671	1.079
Methionine	0.552	0.117	0.090	0.023	1.670	0.712	0.863	0.727
Isoleucine	0.765	0.185	0.187	0.055	1.661	0.848	0.636	0.899
Leucine	1.401	0.336	0.350	0.095	2.208	1.536	1.512	1.645
Phenylalanine	0.732	0.182	0.140	0.050	0.742	0.710	0.708	0.897
Histidine	0.528	0.102	0.101	0.025	0.671	0.369	0.103	0.687
Lysine	1.419	0.362	0.309	0.089	1.411	1.630	1.413	1.699
Arginine	1.167	0.347	0.363	0.064	1.322	1.146	1.230	1.249
Threonine	0.783	0.179	0.192	0.060	1.080	1.000	0.924	0.926
Tryptophan	0.207	0.055	0.058	0.006	0.671	1.036	0.280	0.239
NEAA[b]								
Tyrosine	0.630	0.149	0.130	0.038	0.741	0.577	0.539	0.775
Proline	0.672	0.269	0.166	0.069	0.577	0.725	0.938	0.661
Aspartic acid	1.707	0.459	0.439	0.119	0.976	2.190	2.170	1.940
Serine	0.849	0.154	0.197	0.062	1.143	1.032	0.637	1.058
Glutamic acid	2.574	0.681	1.090	0.191	1.766	2.409	3.347	2.498
Glycine	1.143	0.217	0.305	0.059	0.299	1.693	1.979	1.399
Alanine	1.068	0.266	0.230	0.080	1.665	1.375	1.900	1.274
Cystine	0.234	0.022	0.023	0.017	0.000	0.000	0.149	0.344

[a] EAA: essential amino acids.
[b] NEAA: nonessential amino acids.
[c] LEP: large yellow croaker egg protein.
[d] LLP: large yellow croaker larvae whole-body protein.
[e] LMP: large yellow croaker muscle protein.
[f] WFP: white fishmeal protein.
[g] CAA were added to simulate dietary amino acid patterns to those of LEP, LLP, LMP and WFP (Table 2).

Table 4
Amino acid profiles of the experimental diets and live copepod (%, dry matter) .[a]

AA/∑AA	LEP[b]	LLP[c]	LMP[d]	WFP[e]	Control[f]	Live copepod[g]
EAA[h]						
Valine	6.04	5.02	4.30	4.97	4.60	10.36
Methionine	4.67	2.95	3.18	2.89	2.77	2.93
Isoleucine	6.00	4.40	3.95	4.50	4.21	6.08
Leucine	9.30	7.88	8.00	7.79	7.66	7.92
Phenylalanine	4.48	4.89	4.75	5.44	4.17	4.29
Histidine	2.54	2.03	1.85	2.44	1.97	2.10
Lysine	6.63	6.95	6.74	6.92	7.53	6.89
Arginine	7.37	6.99	7.20	7.11	7.68	6.01
Threonine	4.22	4.18	3.97	3.92	3.88	4.26
Tryptophan	ND[i]	ND[i]	ND[i]	ND[i]	ND[i]	ND[i]
∑EAA	51.25	45.29	43.95	45.96	44.47	50.84
NEAA[j]						
Tyrosine	7.68	2.81	2.64	3.10	2.98	5.76
Proline	3.88	4.38	4.60	4.22	4.45	3.20
Aspartic acid	14.47	10.21	10.08	9.53	9.71	9.21
Serine	3.89	3.75	3.40	3.68	3.61	3.99
Glutamic acid	14.32	15.27	16.71	15.41	16.79	14.01
Glycine	8.15	11.32	11.01	10.94	10.63	4.86
Alanine	7.30	6.86	7.46	6.66	6.18	8.13
Cystine	0.11	0.16	0.17	0.49	0.29	ND[g]
∑NEAA	48.69	54.76	56.07	54.04	54.65	49.16

[a] Values are the mean of triplicate samples.
[b] LEP: large yellow croaker egg protein.
[c] LLP: large yellow croaker larvae whole-body protein.
[d] LMP: large yellow croaker muscle protein.
[e] WFP: white fishmeal protein.
[f] Control: fishmeal containing intact protein.
[g] Live copepod: crude protein, 57.8% dry matter; crude lipid, 10.6% dry matter.
[h] EAA: essential amino acids.
[i] ND: not determined.
[j] NEAA: non-essential amino acids.

and live copepod and the control diet from 10 to 14 DAH, and then the larvae were weaned to experimental diets. The feeding experiment was carried out in 18 fiberglass cylindrical tanks (water volume 500 L) at a stocking density of 5000 larvae (mean body weight 3.15 ± 0.15 mg) per tank. They were supplied with seawater that had been filtered through two-grade sand filter. During the rearing period, water temperature ranged from 22.8 to 24.5 °C, pH from 7.8 to 8.2 and salinity from 22 to 26‰. About 200–300% of the water volume was renewed daily. All rearing tanks were provided with continuous aeration and maintained under a 14:10 h, light/dark regime. The maximum light intensity was 8.5 W m^{-2} during daytime at the water surface. The water surface was skimmed with a polyvinylchloride pipe regularly to remove the suspended waste. Also, accumulations of feed and feces at the tank bottoms were siphoned twice daily. From 15 to 45 DAH, the fish were manually fed to satiation with the experimental diets or live copepod five times (6:00, 8:30, 12:30, 14:30, and 17:00) daily.

2.3. Sampling and dissection

At the beginning of the experiment, three hundred larvae at 15 DAH were randomly sampled to measure wet body weight. At the end of the experiment, survival was determined by counting the individuals remaining in each tank. All fish were deprived of food for 1 day before sampling to empty their guts. Fifty individuals were randomly sampled from each tank to measure wet body weight. Fifty individuals were randomly collected from each tank and immediately frozen in liquid nitrogen and then stored at −80 °C for enzymatic and gene expression assays. The remaining fish from each tank were collected and stored at −20 °C for body composition analysis.

Pancreatic and intestinal segments of 45 DAH larvae were separated under a dissecting microscope as described by Cahu and Zambonino-Infante (1994) and Ma et al. (2005) on a glass plate maintained at 0 °C.

2.4. Analytical methods

Chemical analysis of the experimental diets and whole fish were performed following the standard procedures (AOAC, 1995). Dry matter after drying in an oven at 105 °C until constant weight; crude protein (N × 6.25) by the Kjeldahl method after acid digestion using a Kjeltec system (Kjeltec-2300,Hoganas, Sweden); crude lipid by petroleum ether extraction in a Soxtec System HT apparatus (B-801, Flawil, Switzerland); ash by incineration in a muffle furnace at 550 °C for 16 h.

Samples of feed ingredients and experimental diets were freeze-dried for AA analysis. The samples (0.02 g) were hydrolyzed with 15 mL of 6 N HCl at 110 °C under an atmosphere of nitrogen for 24 h, then filtered and added to ultrapure water (from Milli-Q system, Milli-pore, Billerica, MA, USA) in a 50 mL volumetric flask. A 2 mL solution was then transferred to a glass bottle and dried in a vacuum drying chamber (VD23, Tuttlingen, Germany). Thereafter, 2 mL of ultrapure water was added to adjust pH and dried in the vacuum drying chamber twice, and then 2 mL of ultrapure water was added to the bottle and was freeze-dried. And 2 mL of loading buffer was added to dissolve the remains. The supernatant was analyzed by the ninhydrin method with an automatic AA analyzer (Biochrom 30, GE, Biochrom Ltd, Cambridge, UK).

Trypsin activity was assayed according to Holm et al. (1988), using Na-Benzoyl-DL-arginine-p-nitroanilide (BAPNA, B-4875, Sigma) as the zymolyte; Leucine-Aminopeptidase (LA) and alkaline phosphatase (AP) were assayed both in intestinal segments and BBM according to Maroux et al. (1973) and Bessey et al. (1946), using Leucine-p-nitroanolide (L-9125,Sigma) and p-Nitrophenylphosphate (PNPP, 106850, Merck)as the zymolyte, respectively. Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to the method described by Reitman and Frankel (1957). For fish at 45 DAH, the dissected samples, 0.2–0.3 g

pancreatic segments, were homogenized in 2 ml cold (0 °C) ultrapure water (from Milli-Q system), were centrifuged at 3300 ×*g* for 3 min, and the supernatant was collected for further assaying. In addition, 0.2–0.3 g intestinal segments were homogenized to purify brush border membranes (BBMs) according to a method developed for intestinal scraping (Crane et al., 1979) and adapted to intestinal segments (Cahu and Zambonino-Infante, 1994). Before $CaCl_2$ solution addition, 1 ml 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.

All enzymes activities are expressed as U per mg of protein. Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA, A-2153, Sigma) as a standard. One unit of enzyme activity is defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of substrate per min under the specified conditions.

2.5. RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA from larvae body was extracted using Trizol Reagent (Invitrogen, USA) and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. The RNA was treated with RNA-Free DNase (Takara, Shiga, Japan) to remove DNA contaminant and reverse transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara, Shiga, Japan) following the manufacturer's instructions. Real-time RT-PCR was carried out in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany). The primer sequence for β-actin and TOR were designed following the published sequences from large yellow croaker (Yao et al., 2009; Genbank accession no. KC510145) and listed in Table 5. The amplification was performed in a total volume of 25 μl, containing 1 μl of each primer (10 μM), 1 μl of the diluted first strand cDNA product, 12.5 μl of 2× SYBR® Premix Ex Taq™II (Takara, Shiga, Japan) and 9.5 μl of sterilized double-distilled water. The real-time PCR program was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 20 s. At the end of each PCR reaction, melting curve analysis was performed to confirm that only one PCR product was present in these reactions. Standard curves were made with five different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analyzed according to the following equation $E = 10^{(-1/\text{Slope})} - 1$. The primer amplification efficiency was 0.84 for TOR and 0.83 for β-actin. The absolute ΔC_T value (TOR–β-actin) of the slope is 0.033, which are close to zero and indicate that $\Delta\Delta C_T$ calculation for the relative quantification of target genes can be used. To calculate the expression of TOR, the comparative CT method ($2^{-\Delta\Delta Ct}$ method) was used as described by Yao et al. (2009).

2.6. Calculations and statistical analysis

The following calculations were performed:

$$\text{Specific growth rate}\left(\text{SGR},\%\text{day}^{-1}\right) = ((\text{LnW}_f - \text{LnW}_i)/\text{d}) \times 100$$

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.

$$\text{Survival rate}\,(\text{SG},\%) = N_f/N_i \times 100$$

Where N_f is the number of fish larvae in each tank at the end of the experiment and N_i is the number of fish larvae in each tank at the beginning of the experiment.

Results are given as mean ± S.E. Data from each treatment were subjected to a one-way analysis of variance (ANOVA) using the software program SPSS 16.0. Tukey's honest significant difference test (Tukey HSD test) was chosen as a multiple comparison test and the significance level of 5% was used.

3. Results

3.1. Survival and growth

Survival rate was significantly higher in larvae fed the LLP diet (19.2%) than that of fish fed the LMP diet (15.5%). However, there was no significant difference in survival rate among larvae fed LEP, LLP, WFP and control diet, which were all significantly lower compared to larvae fed live copepod ($P < 0.05$, Table 6).

Growth of larvae, expressed as specific growth rate (SGR), was significantly higher in fish fed the WFP diet than that of fish fed LEP or LLP diet ($P < 0.05$). There was no significant difference in SGR among larvae fed WFP, LMP, control diet and live copepod ($P > 0.05$, Table 6).

3.2. Body composition

Dietary amino acid profiles significantly affected whole-body protein and lipid contents of larvae (Table 7, $P < 0.05$). However, whole-body moisture content was independent of dietary treatments. The whole-body protein content of larvae fed the control diet was significantly higher than the LEP, LLP and LMP treatments, which was significantly lower than live copepod treatment ($P < 0.05$). There were no significant differences in larvae whole-body lipid content among larvae fed artificial diets ($P > 0.05$), which was significantly lower than larvae fed live copepod ($P < 0.05$).

3.3. Specific activities of digestive and AA catabolism enzymes

Specific activities of trypsin (both in intestinal segment and pancreatic segment) in fish fed the WFP diet were significantly higher compared with LEP and LLP diet ($P < 0.05$). However, there were no significant differences among the WFP, control and live copepod treatment. The ratio "pancreatic enzyme in intestinal segment/pancreatic enzyme in pancreatic segment (trypsin-IS/ trypsin-PS)" was significantly higher in larvae fed the WFP diet compared with LEP and LLP diet ($P < 0.05$), but was not significantly different among larvae fed WFP, LMP, control diet and live copepod ($P > 0.05$). Activities of LA and AP in the purified BBM of larval intestine were significantly affected by dietary amino acid profiles ($P < 0.05$, Table 8). The specific activity of LA in larvae fed the WFP diet was significantly higher than LEP diet ($P < 0.05$), but was significantly lower than the control and live copepod treatments ($P < 0.05$). The specific activity of AP was significantly higher in larvae fed the WFP diet compared with LEP and LLP ($P < 0.05$), but was not significantly different from LMP and control diet ($P > 0.05$).

Specific activities of ALT and AST were not significantly different between larvae fed the WFP and the control diet ($P > 0.05$) though they were significantly higher than the other AA pattern diets

Table 5
Sequence of the primers used for qRT-PCR in this study.

Target gene	Reference	Forward (5′–3′)	Reverse (5′–3′)
TOR[a]	Genbank accession no. KC510145	GCTCTGGAAATGTAAAAGACCTGAC	GGACTGGATGCGGATGATGG
β-actin	Yao et al. (2009)	TTATGAAGGCTATGCCCTGCC	TGAAGGAGTAGCCACGCTCTGT

[a] TOR: target of rapamycin.

Table 6
The effects of different amino acid profiles on growth and survival of large yellow croaker larvae*.

index	LEP[2]	LLP[3]	LMP[4]	WFP[5]	Control[6]	Live copepod	*F*-value	*P*-value
Initial number	5000	5000	5000	5000	5000	5000		
Survival number	863 ± 45^{bc}	890 ± 69^{b}	777 ± 49^{c}	909 ± 16^{bc}	878 ± 13^{bc}	1131 ± 22^{a}		
Survival rate (%)	17.3 ± 0.90^{bc}	19.2 ± 0.13^{b}	15.5 ± 0.98^{c}	18.2 ± 0.31^{bc}	17.6 ± 0.27^{bc}	22.62 ± 0.43^{a}	14.621	<0.01
Initial weight (mg)	3.15 ± 0.15	3.15 ± 0.15	3.15 ± 0.15	3.15 ± 0.15	3.15 ± 0.15	3.15 ± 0.15		
Final weight (mg)	62.1 ± 2.85^{c}	68.6 ± 3.33^{c}	80.0 ± 7.00^{bc}	92.8 ± 4.41^{b}	90.2 ± 5.75^{b}	101.9 ± 1.38^{a}	12.04	<0.01
SGR[1] (%/day)	9.9 ± 0.15^{c}	10.3 ± 0.16^{c}	10.6 ± 0.03^{bc}	11.3 ± 0.16^{ab}	11.2 ± 0.22^{ab}	11.6 ± 0.04^{a}	15.675	<0.01

* Values in the same row with the same superscripts are not significantly different determined by the Tukey test ($P > 0.05$).
[1] SGR: specific growth rate.
[2] LEP: large yellow croaker egg protein.
[3] LLP: large yellow croaker larvae whole-body protein.
[4] LMP: large yellow croaker muscle protein.
[5] WFP: white fishmeal protein.
[6] Control: fishmeal containing intact protein.

($P < 0.05$). Specific activities of ALT and AST were significantly higher in larvae fed the live copepod than artificial diets ($P < 0.05$, Table 8).

3.4. The expression of TOR gene

At the end of the experiment, no significant differences were observed in larvae TOR gene expression among dietary treatments ($P > 0.05$, Fig. 1). Compared to the control (intact protein), the mRNA expression levels of TOR were decreased by about 2-fold, 0.41-fold, 1.73-fold, 1.15-fold and 1.43-fold in larvae fed LEP, LLP, LMP, WFP diets and live copepod, respectively.

4. Discussion

Compared to intact protein sources, incorporation of crystalline-AA in diets has been reported to lower growth performance and reduce efficiency of feed utilization in some fish species (Mambrini and Kaushik, 1994; Peres and Oliva-Teles, 2005; Rodehutscord et al., 1995). However, the present study showed that there were no significant difference on growth and survival of large yellow croaker larvae fed diets with white fishmeal amino acids(AA) profiles (supplementation of crystalline-AA) compared to the control (intact protein), which indicated that the large yellow croaker larvae can effectively utilize dietary crystalline-AA when the AA profile is suitable. The results were parallel with previous studies which also demonstrated that supplementation of crystalline-AA to AA-deficient diets can improve growth and feed utilization efficiency (Cheng et al., 2003; Robinson, 1991; Takagi et al., 2001).

The amino acid patterns of whole hen egg (Halver et al., 1959) and fish egg (Ketola, 1982) have been suggested to reflect the EAA requirement patterns of some fish. However, the poorest growth was observed in larvae fed the diet with amino acid profiles simulating large yellow croaker egg protein in the present study. This was in agreement with the findings of Japanese flounder, which also showed the lowest weight gain when fed the diet with supplementation of amino acids according to amino acid pattern of Japanese flounder egg protein (Alam et al., 2002). Due to the absence of accurate data on EAA requirements for a given species, it is generally accepted that the EAA profile of whole-body protein provides an acceptable approach to that of EAA requirements (Mambrini and Kaushik, 1995; Wilson and Poe, 1985). However, results of the present study showed that growth performance of fish fed the diet with the EAA profile similar to that of large yellow croaker larvae whole-body protein (LLP) diet was lower than that of fish fed the diet with white fishmeal protein (WFP) diet. Mambrini and Kaushik (1995) as well as Rollin et al. (2003), using different approaches, both have demonstrated that the use of whole-body protein EAA profile as a reference protein was not totally satisfactory as it could lead to underestimation or overestimation of some EAA.

In the present study, the EAA/NEAA ratio of all diets was around 50/50, but the histidine and phenylalanine in the WFP diet were about 20.1% and 11.2%, higher than that in the LLP diet, respectively. In addition, the cysteine in the WFP diet was almost triple of that in the LLP diet. Thus, the difference of the histidine, phenylalanine and cysteine between the WFP diet and the LLP diet could lead to the variation in SGR of the larvae fed the two diets. Furthermore, the AA composition could not be constant in larvae because of their high instantaneous growth. Hence, the LLP pattern, which referenced a certain period AA composition in larvae body, could not meet the requirement of AA over the entire larval stage. This was in agreement with some previous findings in rainbow trout (Green and Hardy, 2002), Japanese flounder (Alam et al., 2002), Atlantic salmon (Rollin et al., 2003) and European sea bass (Peres and Oliva-Teles, 2007), which found that AA profile of fishmeal protein was better to reflect the EAA requirement profile compared to AA profile of fish whole-body protein (Yúfera et al., 2011).

Since the larval digestive tract is not fully developed during the first weeks of life (Zambonino-Infante and Cahu, 2001), digestive processes, and particularly protein digestion, have been suggested as a major limitation to the utilization of artificial diets by fish larvae (Carvalho et al., 2004). Two main stages are considered crucial in the maturation process of the digestive function. The first is the achievement of pancreas secretion function and the second is the onset of brush border membrane (BBM) enzymes in intestine (Cahu and Zambonino-Infante, 1995; Ma et al., 2005). The ratio of trypsin-IS to trypsin-PS reflected the secretion level of pancreatic enzymes (Zambonino-Infante et al., 1996). In this experiment, the ratio was significantly influenced by the dietary AA patterns. The highest value of this ratio was found in larvae fed the WFP diet, which indicated the AA profile of WFP could strengthen the exocrine function of large yellow croaker larvae pancreas to some extent. Both AP and LA are mainly located in cell membranes, and the variation of their activities during larval development could reflect the maturation

Table 7
The effects of different amino acid profiles on body composition of large yellow croaker larvae (45 DAH, mean ± S.E., n = 3).[a]

Treatments	Protein (%)	Lipid (%)	Moisture (%)
LEP[b]	8.45 ± 0.11^{d}	1.91 ± 0.03^{b}	86.65 ± 0.53
LLP[c]	8.89 ± 0.04^{cd}	1.92 ± 0.01^{b}	85.82 ± 1.00
LMP[d]	8.60 ± 0.13^{d}	2.03 ± 0.13^{ab}	85.99 ± 1.49
WFP[e]	9.60 ± 0.22^{bc}	2.18 ± 0.01^{ab}	84.87 ± 0.89
Control[f]	9.77 ± 0.20^{b}	2.08 ± 0.04^{ab}	84.96 ± 0.97
Live copepod	11.30 ± 0.08^{a}	2.30 ± 0.03^{a}	83.99 ± 0.11
F-value	53.281	6.994	
P-value	<0.01	0.017	

[a] Values in the same column with the same superscripts are not significantly different determined by the Tukey test ($P > 0.05$).
[b] LEP: large yellow croaker egg protein.
[c] LLP: large yellow croaker larvae whole-body protein.
[d] LMP: large yellow croaker muscle protein.
[e] WFP: white fishmeal protein.
[f] Control: fishmeal containing intact protein.

Table 8
The effects of different amino acid profiles on specific activities of digestive enzymes and amino acid metabolic enzymes of large yellow croaker larvae (45DAH, mean ± S.E., n = 3)*.

Index		LEP[5]	LLP[6]	LMP[7]	WFP[8]	Control[9]	Live copepod	*F*-value	*P*-value
PS[3]	Trypsin[1]	37.98 ± 1.00^{b}	40.30 ± 4.94^{b}	82.53 ± 2.43^{a}	89.01 ± 4.28^{a}	98.67 ± 5.7^{a}	101.95 ± 4.91^{a}	46.524	<0.01
IS[3]	Trypsin[1]	26.16 ± 0.50^{c}	30.26 ± 0.66^{c}	73.24 ± 5.01^{b}	84.38 ± 2.03^{ab}	82.84 ± 1.23^{a}	90.63 ± 0.75^{a}	155.315	<0.01
Trypsin-IS/trypsin-PS[4]		0.70 ± 0.01^{c}	0.75 ± 0.02^{bc}	0.89 ± 0.06^{a}	0.95 ± 0.02^{a}	0.84 ± 0.01^{ab}	0.89 ± 0.01^{a}	10.962	<0.01
Specific activities of digestive enzymes in purified brush border membrane of intestine									
Leucine-Aminopeptidase[1]		57.03 ± 4.72^{c}	70.75 ± 4.93^{bc}	93.84 ± 6.02^{b}	96.09 ± 7.37^{b}	159.73 ± 3.84^{a}	184.33 ± 5.59^{a}	73.65	<0.01
Alkaline-phosphatase[2]		249.19 ± 14.83^{c}	313.34 ± 5.57^{c}	434.92 ± 28.61^{b}	469.33 ± 16.83^{b}	453.75 ± 33.65^{b}	607.39 ± 19.28^{a}	28.341	<0.01
Specific activities of amino acid metabolic enzymes in whole-body of larvae									
Aspartate aminotransferases[2]		16.79 ± 1.13^{d}	21.30 ± 1.06^{c}	26.79 ± 1.61^{c}	43.89 ± 1.60^{b}	47.55 ± 1.52^{b}	61.21 ± 1.79^{a}	137.698	<0.01
Alanine aminotransferase[2]		24.91 ± 3.5^{d}	34.00 ± 0.71^{c}	33.76 ± 1.32^{c}	59.59 ± 0.97^{b}	62.98 ± 0.86^{ab}	67.47 ± 1.03^{a}	264.835	<0.01

* Values in the same row with the same superscripts are not significantly different determined by the Tukey test ($P > 0.05$).
[1] The units of enzyme activity is mU mg^{-1} · protein.
[2] The units of enzyme activity is U mg^{-1} · protein.
[3] PS: pancreatic segments; IS: intestinal segments.
[4] Trypsin-IS: trypsin of intestinal segment; Trypsin-PS: trypsin of pancreatic segment.
[5] LEP: large yellow croaker egg protein.
[6] LLP: large yellow croaker larvae whole-body protein.
[7] LMP: large yellow croaker muscle protein.
[8] WFP: white fishmeal protein.
[9] Control: fishmeal containing intact protein.

process in cells of the intestinal membrane (Cahu et al., 1998). In this experiment, among AA pattern dietary treatments, activities of AP and LA were the highest in larvae fed the WFP diet. The result suggested that the WFP diet may have improved the development of intestinal membrane cells compared to other AA pattern diets.

ALT and AST are the most important aminotransferases in fish livers (Cowey and Walton, 1989; Fynn-Aikins et al., 1995), and are considered valuable tools for evaluating the response of fish to diet modifications (Dean et al., 1986). In this study, the activities of AST and ALT were significantly higher in fish fed the WFP diet compared to the other AA patterns diets, which indicated that larvae fed the diet with amino acids referenced white fishmeal protein responded positively. The activity of AST or ALT is closely related to amino acid metabolism in fish, and the transaminase activity increased with the increase of amino acid metabolism (Cheng et al., 2010; Deng et al., 2009; Feng et al., 2012; Luo et al., 2012). In the present study, activities of AST and ALT in larvae were significantly higher than the other treatments except for the control. Dietary amino acid profiles simulating the white fishmeal can improve amino acid metabolism, therefore the larvae of this treatment expressed better growth performance. This can also explain why the whole-body protein content of larvae fed WFP is higher than the other treatments with different amino acids patterns. However, Peres and Oliva-Teles (2007) found that the activity of transaminase showed no clear response to the dietary AA pattern in European sea bass. In fish, the effect of dietary protein on hepatic activity of key enzymes involved in amino acid catabolism is relatively contradictory (Kim et al., 1992; Lupianez et al., 1989). Dietary protein-bound AA replacement by crystalline-AA or dietary AA imbalance was also reported to affect specific activities of AA catabolism enzymes (Gomez-Requeni et al., 2003; Moyano et al., 1991; Peres and Oliva-Teles, 2005, 2006).

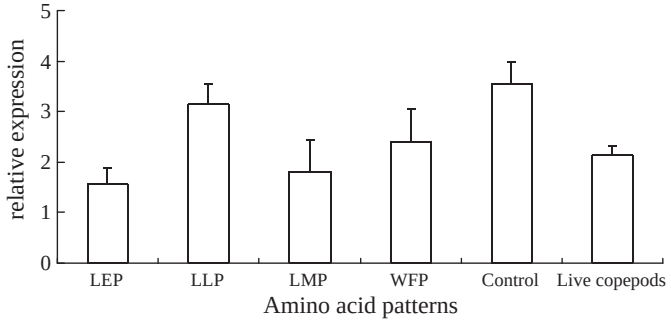

Fig. 1. Relative mRNA expression of TOR in large yellow croaker larvae (45DAH) fed diets containing different AA profiles. LEP: large yellow croaker egg protein; LLP: large yellow croaker larvae whole-body protein; LMP: large yellow croaker muscle protein; WFP: white fishmeal protein; Control: fishmeal containing intact protein; LC: live copepod. Values are means ± S.E. (n = 3).

TOR is part of a molecular complex, which is a key regulator of energy homeostasis, cell growth and protein translation, also is a nutrient sensor regulated by both amino acids and insulin (or other growth factors) (Wullschleger et al., 2006). In the present study, expression of TOR gene in larvae was not significantly affected by the dietary incorporation of crystalline-AA compared to intact protein. Also different AA patterns had no significant impact on in larval fish, which seems to indicate that the TOR gene expression could not be determined by dietary AA patterns. The TOR pathway could not be regulated by amino acids at the level of gene expression. In this study, nutritional status had no effect on the expression of TOR in fish. This result was similar to findings in cobia (Luo et al., 2012). However, some studies reported that supplementation of some amino acids impacts TOR gene expression in different aquatic animals (Chen et al., 2012; Sun et al., 2010; Wacyk et al., 2012; Zhao et al., 2012). These differences could be due to the different species, tissue, age, test methods (feeding or injection). The ways by which amino acids regulate TOR pathway are still not fully understood and need further investigation.

In conclusion, the growth performance, survival and activities of trypsin in the intestine of large yellow croaker larvae fed diets with white fishmeal protein AA profile were not significantly different from the control (intact protein), indicating that larvae could effectively utilize dietary crystalline-AA when the AA profile was suitable. Therefore, the optimal AA pattern in diets could improve larvae growth and protein metabolism to some extent, but had no significant effects on the TOR gene expression. Compared to the AA pattern tested of LEP, LLP, and LMP, the white fishmeal protein AA pattern appeared to be the most appropriate dietary AA pattern tested for large yellow croaker larvae.

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