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Dietary Leucine Requirement of Juvenile Japanese Seabass (*Lateolabrax Japonicus*)

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Abstract A 56-day feeding trial was conducted to examine the dietary leucine requirement of juvenile Japanese seabass in seawater floating net cages (1.5 m × 1.5 m × 2.0 m). Six isonitrogenous (crude protein 40%) and isoenergetic (gross energy 20 kJ g^{-1}) diets were formulated to contain different concentrations of leucine (0.9%, 1.49%, 2.07%, 2.70%, 3.30% and 3.88% of dry matter). Crystalline L-amino acids were supplemented to simulate the whole body amino acid pattern of Japanese seabass except for leucine. Three groups (30 fish individuals each, 8.0 g ± 0.20 g in initial weight) were fed to apparent satiation at 5:00 and 17:30 every day. During the experimental period, the water temperature ranged from 26 to 32℃ and salinity from 26 to 30, and the dissolved oxygen was maintained at 7 mg L^{-1}. The results showed that weight gain (*WG*), nitrogen retention (*NR*), feed efficiency (*FE*) and protein efficiency ratio (*PER*) were significantly increased when dietary leucine was increased from 0.90% to 2.70% of dry matter, and then declined. *WG* was the highest when fish were fed D4 containing 2.70% of leucine. No significant differences were observed in body composition among dietary treatments ($P > 0.05$). Considering the change of *WG*, the optimum dietary leucine requirement of juvenile Japanese seabass was either 2.39% of dry matter or 5.68% of dietary protein.

Key words growth; leucine; requirement; Japanese seabass

1 Introduction

Leucine is believed to be an indispensable amino acid in most aquatic species because its carbon skeleton cannot be synthesized *in vivo*. It functions as a major regulator in body metabolism at multiple levels (Balage *et al*., 2011). Usually, leucine can activate protein synthesis and decrease proteolysis, thus favoring a positive nitrogen balance (Dardevet *et al*., 2002; Crozier *et al*., 2005; Donato *et al*., 2007). Dietary leucine supplementation has been shown to reduce diet-induced obesity (Zhang *et al*., 2007; López *et al*., 2010; Vianna *et al*., 2012; Freudenberg *et al*., 2012; Eller *et al*., 2013) and inflammation in adipose tissue (Macotela *et al*., 2011; Toneto *et al*., 2012). In addition, it is also important to produce hemoglobin, maintain plasma glucose level and increase growth hormone production. However, leucine deficiency may reduce growth and diet conversion (Wilson and Halver, 1986), and serve as an antagonist of valine and isoleucine when the proportion of these three amino acids in diets is mal-balanced (D'Mello, 2003). The antagonism has been found to depress the growth of chinook salmon (Chance *et al*., 1964), lake trout (Hughes *et al*., 1984) and rainbow trout (Yamamoto *et al*., 2004). Therefore, it is necessary to determine the appropriate dietary leucine content.

The amino acid requirement of cultured species can be decided with a well-developed method. Dietary protein level was fixed at the optimal crude protein, which was satisfied for the maximal growth of cultured species. The composition of amino acids in the diet was simulated by either whole chicken egg protein (Luo *et al.*, 2005; Khan and Abidi, 2007) or the whole body protein of cultured species (Alliot *et al.*, 1974; Tibaldi and Tulli, 1999; Mai *et al.*, 2006) excluding the target amino acid. Diets were made isonitrogenous and isoenergetic by adjusting the non-essential amino acids and carbohydrate (Millamena *et al.*, 1997; Tibaldi and Tulli, 1999). Graded levels of a tested amino acid were supplemented to the basal diet, and the range of content covered the level of reference amino acids. Optimal requirement of target amino acid was estimated according to the growth performance of fish species (Tibaldi and Tulli, 1999; Khan and Abidi, 2007).

Japanese seabass is an economically important marine fish species and has been widely cultured in China. A few studies on its nutrient requirements have been conducted (Ai *et al.*, 2004a, 2004b; Zhang *et al.*, 2005). Mai *et al.* (2006) estimated that the dietary lysine requirement of juvenile Japanese seabass was 2.49% of dry diet (or 5.80% dietary protein) by broken-line analysis on the

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basis of *SGR* (specific growth rate). In order to gain the maximal growth of the species, knowledge of the other indispensable amino acid requirements is very important. The purpose of the present study was to quantify the dietary leucine requirement of this species.

2 Materials and Methods

2.1 Experimental Diets

Six isonitrogenous (crude protein 40%) and isoenergetic (gross energy 20 kJ g^{-1}) diets were formulated with six different levels of leucine ranging from 0 to 3.0% of dry diet with an increment of 0.6% (Table 1). Dietary leucine was quantitatively increased at the expense of glutamic acid. Glutamic acid was used to be blank, because it is non-essential and has little effect on the results of the experiment. L-crystalline amino acids mixture was used, so that the levels of all amino acids, except for leucine, would simulate the whole body amino acid pattern of Japanese seabass (initial body weight 8.0 g ± 0.20 g). The amino acid (AA) content of experimental diets is shown in Table 2. All the dietary AA contents were maintained nearly the same as the corresponding AA contents in 43% of whole body protein except for leucine and glutamic acid. The final level of leucine was 0.90%, 1.49%, 2.07%, 2.70%, 3.30% and 3.88% of dry weight, respectively, by adding crystalline leucine, which was determined by auto amino acids analyzer (Biochrom Ltd®, England). The range of dietary leucine content covered the leucine level (3.02%) in 43% crude protein from the whole body tissue of this species. The diets were marked as D1, D2, D3, D4, D5 and D6, respectively.

The ingredients were ground into fine powder through a 320 μm mesh. The diets were prepared by thoroughly mixing the dry ingredients, blending with the oil and water, and then forcing the paste through a pelletizer (F-26 (II), South China University of Technology) to obtain pellets. The moist pellets were dried in an oven at 45℃ for 12 h. The dry pellets were crushed and sieved to obtain suitable pellet sizes (1.5 mm× 2.0 mm and 2.5 mm × 3.0 mm), then sealed in bags and stored at −15℃ until used.

2.2 Experimental Procedure

Experimental fish were obtained from a commercial farm in Ningbo, China. Prior to the feeding trial, the fish were reared in floating sea cages (3.0 m × 3.0 m × 3.0 m), and fed the control diet (D1) for two weeks to acclimate to the experimental diets and conditions. At the start of the experiment, the fish were fasted for 24 h and weighed after being anesthetized with eugenol (1:10000) (Shanghai Reagent Corp, China). Juvenile fish in similar sizes were randomly assigned to 18 cages (1.5 m × 1.5 m × 2.0 m), 30 each. Diets each were randomly assigned to three cages. To prevent the waste of dietary pellets, fish were slowly hand-fed in small batches at 05:00 and 17:30 every day till visual satiation of fish feeding behavior, *i.e.*, never coming up to water surface for feed. Feed consumption was recorded daily. Feeding trial lasted for 56 days. During the experimental period, the water temperature ranged from 26 to 32℃, and salinity from 26 to 30. Photoperiod was about 14 h^{-1} d^{-1} and dissolved oxygen was approximately 7 mg L^{-1}. At the end of the experiment,

Table 1 Formulation and composition of the test diets used for the leucine requirement of Japanese seabass (g per 100 g dry matter)

Ingredient	Diet no./Supplementation level					
	Diet 1 (0.0)	Diet 2 (0.6)	Diet 3 (1.2)	Diet 4 (1.8)	Diet 5 (2.4)	Diet 6 (3.0)
Fish meal	12.0	12.0	12.0	12.0	12.0	12.0
Soybean meal	16.0	16.0	16.0	16.0	16.0	16.0
Brower's yeast	3.0	3.0	3.0	3.0	3.0	3.0
Amino acid mixture	18.79	18.79	18.79	18.79	18.79	18.79
Fish oil	4.0	4.0	4.0	4.0	4.0	4.0
Soybean oil	3.0	3.0	3.0	3.0	3.0	3.0
Mineral mixture	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin mixture	2.0	2.0	2.0	2.0	2.0	2.0
Attractant	0.3	0.3	0.3	0.3	0.3	0.3
Mold inhibitor	0.1	0.1	0.1	0.1	0.1	0.1
Antioxidant	0.05	0.05	0.05	0.05	0.05	0.05
Lecithin	4.0	4.0	4.0	4.0	4.0	4.0
Wheat flour	28.0	28.0	28.0	28.0	28.0	28.0
Microcrystalline cellulose	3.76	3.76	3.76	3.76	3.76	3.76
Leucine	0.0	0.6	1.2	1.8	2.4	3.0
Glutamic acid	3.0	2.4	1.8	1.2	0.6	0.0
Proximate composition analysis (g per 100 g dry matter)						
Crude protein	40.4	40.1	39.3	39.3	41.9	39.9
Crude lipid	13.1	13.8	13.4	13.2	12.9	13.0
Gross energy (kJ g^{-1})	20.4	20.4	20.5	20.4	20.7	20.5
Ash	6.4	6.7	7.0	7.2	6.5	6.4
Moisture	5.8	6.8	6.4	6.0	5.2	6.2
Leucine	0.90	1.49	2.07	2.70	3.30	3.88

Notes: Fish meal (white fish meal): obtained from Hangzhou Wensli Biology Science and Technology Corporation (Zhejiang, China), crude protein 67.5% dry matter, crude lipid 7.8% dry matter; Soybean meal (de-hulled soybean meal): obtained from commercial market, crude protein 46.2% dry matter, crude lipid 1.7% dry matter; Beer yeast, crude protein 57.1% dry matter, crude lipid 3.5% dry matter; Wheat flour, crude protein 13.9% dry matter, crude lipid 1.9% dry matter. Amino acid mixture (g kg^{-1} diet): arginine, 24.3; histidine, 4.8; isoleucine, 9.8; lysine, 23.5; methionine, 9.7; phenylalanine, 9.6; valine, 10.6; aspartic acid, 25.9; serine, 11.5; glycine, 16.6; alanine, 21.7; tyrosine, 8.1; glutamic acid, 40.7; threonine, 12.0. Mineral premix (mg or g kg^{-1} diet): NaF, 2 mg; KI, 0.8 mg; $CoCl_2{\cdot}6H_2O$ (1%), 50 mg; $CuSO_4{\cdot}5H_2O$, 10 mg; $FeSO_4{\cdot}H_2O$, 80 mg; $ZnSO_4{\cdot}H_2O$, 50 mg; $MnSO_4{\cdot}H_2O$, 60 mg; $MgSO_4{\cdot}7H_2O$, 1200 mg; $Ca(H_2PO_4)_2{\cdot}H_2O$, 3000 mg; NaCl, 100 mg; Zoelite, 15.448 g. Vitamin premix (mg or g kg^{-1} diet): thiamin, 25 mg; riboflavin, 45 mg; pyridoxine-HCl, 20 mg; vitamin B_{12}, 0.1 mg; vitamin K_3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin, 1.20 mg; retinol acetate, 32 mg; cholecalciferol, 5 mg; alpha-tocopherol, 120 mg; ascorbic acid, 2000 mg; choline chloride, 2000 mg; ethoxyquin, 150 mg; wheat middling, 14.52 g. Attractant, glycine and betaine; Mold inhibitor, p-Aminobenzoic acid; Antioxidant: Ethoxyquin.

Table 2 Amino acid composition of ingredients and free amino acid supplementation (g per 100 g dry matter)

Amino acid	FM	SBM	WF	BY	Total	Addition	43% whole body protein
Indispensable amino acids							
Arginine	0.51	0.51	0.18	0.08	1.27	1.31	2.58
Histidine	0.16	0.18	0.08	0.03	0.46	0.09	0.55
Isoleucine	0.35	0.33	0.14	0.07	0.89	0.89	1.78
Lysine	0.62	0.44	0.10	0.12	1.28	2.08	3.36
Methionine	0.23	0.08	0.06	0.02	0.39	0.83	1.22
Phenylalanine	0.32	0.35	0.18	0.07	0.91	0.60	1.51
Valine	0.43	0.35	0.17	0.09	1.03	0.98	2.01
Threonine	0.34	0.28	0.10	0.07	0.80	0.80	1.60
Leucine	0.05	0.52	0.25	0.11	0.92	Varied	3.02
Non indispensable amino acids							
Cystine	0.52	0.31	0.00	0.07	0.90	1.97	2.87
Tyrosine	0.27	0.25	0.12	0.05	0.70	0.51	1.21
Aspartic acid	0.77	0.81	0.00	0.15	1.73	2.28	4.01
Serine	0.36	0.36	0.00	0.08	0.79	0.65	1.44
Glycine	0.05	0.05	0.08	0.01	0.18	–	0.24
Alanine	0.47	0.31	0.00	0.11	0.88	1.67	2.55
Glutamic acid	1.05	1.24	0.00	0.17	2.46	4.14	6.60
Total addition						18.79	

Notes: FM, fish meal; SBM, soybean meal; WF, wheat flour; BY, brewer's yeast. Change varied to 0–3.0%.

the fish were fasted for 24 h and fish each cage were weighed and counted.

2.3 Measurement and Analysis

Ten fish per cage at the termination of the feeding trial were sampled and frozen (−20℃) for the analysis of proximate whole body composition. Proximate analysis on feedstuffs, diets and fish were performed according to the standard methods of AOAC (2003). Dry matter was achieved by drying in an oven set at 105℃ and the weight was constant (DHG-9140A, Shanghai). Crude protein (N ×6.25) was measured by Kjeldahl method after acid digestion (FOSS Kjeltec 2300, Sweden). Lipid was assayed by ether extraction using Soxhlet (BUCHI B-811, Swistzerland). Ash content was determined by incineration in a muffle furnace at 600℃ for 12 h (SK-4-10, Shenyang, China). For amino acids analysis (except for methionine and cystine), the whole fish tissue samples were freeze-dried, and then hydrolyzed with 6 mol L^{-1} HCl at 110℃ for 22 h, followed by analyzing with an amino acids analyzer (Biochrom Ltd®, England). For methionine and cystine, the samples were oxidized with performic acid at −10℃ for 3 h to obtain methionine sulfone and cysteic acid, and then freeze-dried twice with deionized water. The freeze-dried ingredients were hydrolyzed and analyzed by the reverse-phase high performance liquid chromatography (HPLC, HP1100, USA). The energy was measured with an adiabatic bomb calorimeter (PARR1281, USA).

2.4 Calculation and Statistical Analysis

The following variables were calculated:

$$\text{Weight gain } (WG)=100\times\frac{W_f-W_i}{W_i},$$

$$\text{Nitrogen retention } (NR)=\frac{\text{Nitrogen gained}}{\text{Nitrogen intake}}\times 100,$$

$$\text{Feed efficiency } (FE)=\frac{\text{Wet weight gain (g)}}{\text{Dry diet fed (g)}},$$

$$\text{Protein efficiency ratio } (PER)=\frac{\text{Wet weight gain (g)}}{\text{Protein fed (g)}},$$

$$\text{Feed intake } (\%\,d^{-1})=\frac{\text{Dry diet fed of each fish}}{\frac{(W_f+W_i)t}{2}}\times 100,$$

where W_f and W_i were final and initial fish weights and t is the experimental duration in days.

All data were subjected to variance and regression analysis if being appropriate using SPSS 16.0 for windows. Differences between the means were tested by Tukey's multiple range test. The level of significance was chosen at $P<0.05$. Comparing the coefficient of determination (R^2) of broken-line model

$$Y=L-U(R-X),$$

and second-order polynomial model

$$Y=a+bX+cX^2 \quad \text{(Zeitoun } et\ al.\text{, 1976)}.$$

The second-order polynomial model was chosen as the best fit model which gave maximum of R^2.

3 Results

3.1 Growth Performance

In the present study, the survival rate of Japanes seabass ranged from 92.0% to 98.9%, and no significant difference in survival was found among diets (Table 3). *WG* significantly increased with the increase of dietary leucine concentration from 0.90% to 2.70%, and then significantly decreased when dietary leucine concentration increased from 2.70% to 3.88%. *WG* was the highest when fish fed D4 with 2.70% leucine content. There were no significant differences in *NR* of fish fed diets containing leucine over 0.90% of dry matter. However, *NR*s of fish fed D3 (2.07% leucine) and D4 (2.70% leucine) were significantly higher than those of fish fed D1 (0.90% leucine). The changing trends of *FE* and *PER* were similar with that of *NR*, significantly increasing with the increase of dietary leucine concentration from 0.90% to 2.07%, and then decreased when dietary leucine concentration increased from 2.07% to 3.88%. *FI* of fish fed D1 was the highest, which was significantly higher than that of fish fed D3 and D5. Considering the change of *WG*, the optimum dietary leucine requirement of juvenile Japanese seabass was 2.39% of dry diet (5.68% of dietary protein)

(Fig.1), which was estimated through quadratic regression analysis.

Table 3 Effect of dietary leucine on the growth and survival of juvenile Japanese seabass (*L. japonicus*) fed experimental diets for 56 days†

Diet no. (level of Leu % diet)	*WG* (%)	*NR* (%)	*FE*	*PER*	*FI* (% d^{-1})	Survival (%)
D1(0.90)	319.0^{b}	25.26^{b}	0.63^{b}	1.56^{b}	3.3^{a}	94.4
D2(1.49)	353.0^{ab}	31.33^{ab}	0.73^{ab}	1.82^{ab}	3.08^{ab}	97.8
D3(2.07)	366.1^{ab}	36.06^{a}	0.88^{a}	2.23^{a}	2.62^{b}	98.9
D4(2.70)	390.4^{a}	33.95^{a}	0.80^{ab}	2.04^{a}	2.78^{ab}	94.4
D5(3.30)	345.9^{ab}	30.26^{ab}	0.76^{ab}	1.82^{ab}	2.74^{b}	92.0
D6(3.88)	317.2^{b}	30.55^{ab}	0.73^{ab}	1.83^{ab}	2.87^{ab}	95.6
Pooled S.E.M.	7.32	1.01	0.02	0.06	2.90	0.97
ANOVA						
F-value	6.226	4.460	4.990	5.997	4.917	1.073
P-value	0.005	0.016	0.011	0.005	0.011	0.422

Notes: †Value is the mean of three replicate groups ($n=3$). Means with different letter in the same column differ significantly ($P<0.05$); *WG*, weight gain; *SGR*, specific growth ratio; *FE*, feed efficiency; *PER*, protein efficiency ratio; *FI*, feed intake; S.E.M., standard error of means; ANOVA, one-way analysis of variance.

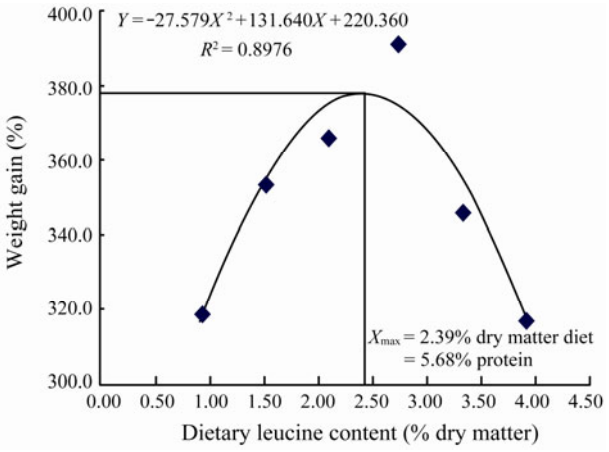

Fig.1 Effect of dietary leucine on the weight gain (*WG*) of juvenile Japanese seabass (*L. japonicus*) fed experimental diets for 56 days.

3.2 Whole Body Composition

No significant difference was observed in the contents of body protein (16.2%–17.2%), lipid (5.8%–7.5%), moisture (71.8%–73.6%) and ash (4.5%–5.2%) among diets ($P>0.05$) (Table 4).

4 Discussion

In the present study, *WG* of Japanese seabass increased with the increase of dietary leucine content and then decreased when the content increased further. This result paralleled with that found in Indian major carp (Abidi and Khan, 2007). Our result indicated that leucine is essential for the growth of Japanese seabass; while dietary suboptimal or super optimal leucine can lead to a reduction in growth rate. The optimum dietary leucine requirement of juvenile Japanese seabass was 5.40%–5.98% of dietary protein within 95% confidence based on growth performance. The result was lower than that of blunt nose black bream (6.98%, Li, 1996), higher than those of other fishes, *e.g.*, 5.2% of Atlantic salmon (Rollin, 1999), 3.75%–3.92% of Indian major carp (Abidi and Khan, 2007), 4.30% of postlarval tiger shrimp (Millamena *et al.*, 1999) and white sturgeon (Ng and Hung, 1995), 4.40% rainbow trout (Kaushik, 1998), 3.90 of Japanese flounder (Forster and Ogata, 1998) and chinook salmon (Chance *et al.*, 1964), and 3.50% of channel catfish (Wilson *et al.*, 1980). In this experiment, soybean meal was used as intact protein. Japanese seabass is carnivorous and cannot utilize soybean meal very well, which leads to a low feed intake and low digestibility of amino acids. This could also lead to a high leucine requirement. It is well known that the crystalline amino acids can be absorbed faster by fish than those of intact protein. In this present study, the fast absorption of crystalline amino acids leads to a poor utilization for protein synthesis. This may be one of possible reasons that leucine requirement of juvenile Japanese seabass was higher than those of some other fish species.

Table 4 Effect of dietary leucine on the body composition of juvenile Japanese seabass (*L. japonicus*) fed experimental diets for 56 days†

Diet no. (level of Leu, %)	Body composition			
	Moisture (%)	Crude protein (% w.w.)	Crude lipid (% w.w.)	Ash (% w.w.)
D1(0.90)	72.5	16.2	6.5	5.2
D2(1.49)	71.8	17.2	6.9	4.7
D3(2.07)	71.8	16.2	7.5	4.9
D4(2.70)	73.6	16.7	5.8	4.7
D5(3.30)	72.5	16.6	6.6	4.7
D6(3.88)	72.7	16.7	6.2	4.5
Pooled S.E.M.c	0.30	0.14	0.22	0.07
ANOVA				
F-value	1.087	0.906	1.030	2.090
P-value	0.429	0.511	0.455	0.143

Notes: †Value is the mean of three replicate groups ($n=3$); w.w., wet weight; S.E.M., standard error; ANOVA, one-way analysis of variance.

Amino acid balance in diets is necessary for the optimal growth of animals. Leucine deficiency can cause low diet intake, severe biochemical malfunction and growth retardation (de la Higuera, 2001). However, excessive dietary leucine may depress the growth response of Japanese seabass as was documented in Indian major carp (Abidi and Khan, 2007). The reason might be that the excessive leucine leads to accumulation and oxidation of ketones and other toxic metabolites, which adversely affect the growth of fish (Abidi and Khan, 2007). In addition, the antagonism of BCAA (Branched-chain amino acid) in different proportions can also inhibit growth, and it was severe in a high excessive BCAA (Yamamoto *et al.*, 2004). In this experiment, when dietary leucine was not at the optimal level, an apparent antagonism among BCAA was observed, which depressed the growth of juvenile

Japanese seabass. The antagonism has been found also in chinook salmon (Chance *et al*., 1964), lake trout (Hughes *et al*., 1984) and rainbow trout (Yamamoto *et al*., 2006).

Leucine is the only amino acid being able to reproduce the effect of a mixture of amino acids on muscle protein synthesis. Many previous studies found the ingestion of leucine was able to acutely increase the protein synthesis rate (Garlick, 2005; Kimball and Jefferson, 2006; Rieu *et al.*, 2006). The body protein of Indian major carp was enhanced significantly with increasing dietary leucine concentrations up to 1.5%. However, beyond this level, a significant fall in body protein concentration was evident (Abidi and Khan, 2007). Choo *et al*. (1991) also proved that increasing dietary leucine did not increase the body protein of rainbow trout. In this study, there was no significant difference in protein, lipid and moisture contents. Probably, chronic supplementation of leucine or measurement of more sensitive parameters are necessary to detect the effect of supplemental leucine on nutrition status.

In a conclusion, leucine is essential for the growth of Japanese seabass, and fish can utilize crystalline leucine. On the basis of *WG*, the optimum dietary leucine requirement of juvenile Japanese seabass is 2.39% of dry diet (5.68% of dietary protein) as was estimated through second-order polynomial regression analysis.

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