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Short Communication

Optimum Dietary Threonine Level for Juvenile Japanese Flounder *Paralichthys olivaceus*

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Abstract

A 6-week feeding trial was conducted to estimate the optimum threonine level in the diet for growth of juvenile Japanese flounder, *Paralichthys olivaceus* with six test diets (50% crude protein) containing intact proteins (casein and gelatin) and a mixture of L- crystalline amino acids (CAA) as nitrogen sources. CAA were supplemented to correspond to the amino acid pattern found in the whole body protein of the Japanese flounder except for threonine. Test diets containing precoated CAA with six graded levels of threonine (0.80 to 2.72% of diet or 1.60 to 5.44% of protein) were fed to triplicate groups of the juveniles (2.00 ± 0.05 g) twice a day for six weeks at 5% body weight. Results showed that the weight gain, feed intake, feed conversion efficiency (FCE), specific growth rate, percent survival and nitrogen retention in the whole body were significantly ($P<0.05$) affected by dietary threonine levels. The optimum threonine level in the diet of Japanese flounder based on broken-line analyses of percent weight gain was 1.57% of diet or 3.14% of protein. Broken-line analysis of FCE and nitrogen retention indicated that the optimum dietary threonine level was 1.60 and 1.61% of diet (3.20 and 3.22% of protein) respectively, close to the value estimated by the weight gain data.

Introduction

The culture of Japanese flounder, *Paralichthys olivaceus* is increasing in some Asian countries like Japan and Korea due to the high demand and popularity of this fish. Culture of this fish mainly depends on fishmeal or minced whole fish in the feeds as protein sources. The nutritive value of protein is influenced by its amino acid composition along with digestibility (Wilson and Poe 1985). As protein is the most expensive component in diets, the optimum dietary levels of the amino acids should be established to formulate cost-effective diets for flatfish like Japanese flounder. The necessity of essential amino acid requirements for diet preparation in semi-intensive aquaculture has been alluded to by De Silva and Anderson (1995).

Threonine is one of the ten essential amino acids (EAA) required for normal growth of various fishes (Wilson 1989). In pigs and chicks, threonine after methionine and lysine, is usually the most limiting amino acid in practical diets (Saldana et al. 1994; Kidd et al. 1997). Threonine is assumed to be the second or third limiting amino acid in some practical diets for the striped bass or its hybrids (Small and Soars 1999). Tibaldi and Tulli (1999) have shown that threonine could be potentially marginal or limiting in practical diet formulations, especially when plant protein sources such as maize or wheat gluten are used to replace substantial amounts of dietary fishmeal protein for European sea bass.

Quantitative dietary requirements of ten EAA have been established for only a limited number of cultured fish species including common carp (Nose 1979), rainbow trout (Ogino 1980), Nile tilapia (Santiago and Lovell 1988), *Catla catla* (Ravi and Devaraj 1991), Japanese eel, channel catfish, chinook salmon (NRC 1993), chum salmon (Akiyama and Arai 1993), coho salmon (Arai and Ogata 1993), milk fish (Borlongan and Coloso 1993) and white sturgeon *Acipenser transmontanus* (Ng and Hung 1995). Recently, we have successfully quantified the optimum dietary level of methionine and arginine for Japanese flounder by dose-response analysis (Alam et al. 2002a; 2001; 2000) using precoated crystalline amino acid based diets. The purpose of the present study was to quantify the optimum dietary threonine level for maximum growth of the juvenile Japanese flounder.

Materials and Methods

Experimental diets

Test diets (Table 1) were formulated from semipurified ingredients to contain 50% crude protein with a mixture of intact proteins and crystalline amino acids (1:1, w/w) as nitrogen sources. CAA were added to the diets to simulate the amino acid pattern found in 50% whole body protein of Japanese flounder (Alam et al. 2000) except for threonine. Diet 1 contained the minimum level of threonine (0.82% of diet, or 1.64% of protein) from intact protein sources. Five additional test diets were prepared by adding incremental levels (0.4%) of L-threonine to diet 1 to reach (calculated values) 1.22, 1.62, 2.02, 2.42 and 2.82% of diet or 2.44, 3.24, 4.04, 4.84 and 5.64% of dietary protein. The analyzed values for threonine in the diets were 0.80, 1.18, 1.57, 2.00, 2.35, and 2.72% of diets. These levels were selected to cover the known range of threonine required values for other fish species such as common carp, Japanese eel, channel catfish, chinook salmon, Nile tilapia (NRC 1993), red drum (Boren and Gatlin 1995), hybrid striped bass (Keembiyehetty and Gatlin 1997) and European sea bass (Tibaldi and Tulli 1999). Diets were kept isonitrogenous by decreasing L-glutamic acid while increasing the threonine levels. Levels of other ingredients were kept constant for all diets. To prevent leaching losses in water, precoated CAA were prepared as described in the previous study (Alam et al. 2000). During preparation, all of the diets were adjusted to pH 7.0 to 7.5

by adding 4 N NaOH. The dry pellets were ground, sieved and stored at –30 °C prior to feeding.

Experimental fish and feeding experiment

Juvenile Japanese flounder obtained from Matsumoto Suisan, Miyazaki, Japan, were transported to Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University and maintained on a commercially formulated flatfish diet (Higashimaru Feeds, Kagoshima, Japan). Prior to the feeding experiment, all fish underwent a 2-week conditioning period during which they adjusted to the commercial diet and standardized environmental conditions. The feeding trial was conducted in eighteen 54 l capacity rectangular (60 x 30 x 30cm, W x H x L) vinyl chloride tanks and 15 juveniles with a mean weight of 2.0 g ± 0.05 were stocked randomly in each tank. Each test diet was fed to three replicate groups of juveniles. The juveniles were fed the respective diets by hand at a rate equal to 5% of body weight per day divided into two equal feedings at 0830 and 1600 h. The juveniles were weighed every two weeks and the amount of feed was adjusted accordingly. Uneaten feed was removed one hour after feeding and dried using a freeze drier. Fecal matter was removed by siphoning the water from the bottom of each tank one hour before giving the diet. The water flow of the tank was 1.2 $l \cdot min^{-1}$ and a 12:12 h light-dark photoperiod was maintained. The water quality was checked regularly. The water

Table 1. Composition of the test diets ($g \cdot 100^{-1}$ dry diet) with graded levels of threonine

Ingredient	Test diets					
	1	2	3	4	5	6
Casein	17.00	17.00	17.00	17.00	17.00	17.00
Gelatin	8.00	8.00	8.00	8.00	8.00	8.00
Amino acid mixture[1]	24.86	24.46	24.06	23.66	23.26	22.86
Squid liver oil	5.00	5.00	5.00	5.00	5.00	5.00
Soybean lecithin	5.00	5.00	5.00	5.00	5.00	5.00
Vitamin mixture[2]	6.00	6.00	6.00	6.00	6.00	6.00
Mineral mixture[2]	5.00	5.00	5.00	5.00	5.00	5.00
a-Starch	12.00	12.00	12.00	12.00	12.00	12.00
Carboxymethyl cellulose (CMC)	4.40	4.40	4.40	4.40	4.40	4.40
k-Carrageenan	2.50	2.50	2.50	2.50	2.50	2.50
a-Cellulose	9.24	9.24	9.24	9.24	9.24	9.24
Attractants [3]	1.00	1.00	1.00	1.00	1.00	1.00
L-threonine	0	0.40	0.80	1.20	1.60	2.00
Total	100	100	100	100	100	100
Total threonine						
% of diet (calculated)	0.82	1.22	1.62	2.02	2.42	2.82
% of diet (analyzed)	0.80	1.18	1.57	2.00	2.35	2.72
% of protein	1.60	2.36	3.14	4.00	4.70	5.44
Crude protein (% diet)	49.3	49.3	50.0	50.7	51.8	50.2
Total lipid	9.7	9.5	9.4	9.4	9.6	9.3
Ash	5.8	6.1	6.1	5.7	5.7	5.8

[1]Supplied as L-form. Histidine, 0.69; isoleucine, 1.28; leucine, 2.14; methionine, 0.89; phenylalanine, 2.10; tryptophan 0.36; valine, 1.37; arginine, 1.88, lysine 3.29; aspartic acid, 3.22; serine, 1.02; glycine, 1.14; alanine, 2.04; tyrosine, 0.97; and glutamic acid, variable.
[2]according to Alam et al. (2000).
[3]Taurine 0.5, betaine 0.4 and inosine-5´monophosphate 0.1.

temperature ranged from 18.1 to 20.5°C during the feeding period and dissolved oxygen was maintained near saturation with continuous aeration. Ranges of other parameters were pH 7.9 to 8.2 and salinity was 33.2 to 34.5 ppt. Growth studies were conducted for a period of six weeks. A pooled sample of ten juveniles at the beginning and a pool of five fish per tank at the end of the feeding trial were sacrificed and used to determine the whole body proximate composition, nitrogen retention and amino acid composition.

Chemical and statistical analysis

Amino acid analysis was performed following the high performance liquid chromatography (HPLC) as described by Teshima et al. (1986). Crude protein (total-N x 6.25) and total lipid contents of the test diets and whole bodies were determined using the Kjeldahl and Bligh and Dyer (1959) methods, respectively. Ash and moisture contents were analyzed following the Association of Official Analytical Chemists (AOAC 1990) method. All data were tested using one way analysis of variance (super-ANOVA Abacus concept, California). Significant differences between means were evaluated using the Tukey Kramer test (Kramer 1956). Probabilities of $P<0.05$ were considered significant. The optimum dietary threonine level was determined according to the broken-line method (Zeitoun et al. 1976; Robbins et al. 1979) using the software package StatMost ver 3, DataMost Corporation, South Sandy, USA).

Results

The results of the present study indicated that the mean weight gain, specific growth rate (SGR), feed intake (FI), feed conversion efficiency (FCE), percent survival and nitrogen (N) retention of the Japanese flounder juveniles were significantly affected by the dietary threonine level (Table 2). The maximum weight gain and SGR were recorded in fish fed the diet containing 1.57% of threonine whereas the minimum weight gain and SGR were obtained in the group fed the diet without supplemental threonine. Weight gain and SGR significantly increased as the threonine level increased up to 1.57% of diet. Increasing threonine level from 1.57 to 2.72% of diet resulted in significantly decreased weight gain and SGR. Feed intake was lowest for the groups fed the 0.80% of threonine and highest for the group fed 1.57% of threonine. Significantly lower feed intake was observed for the groups fed more than 1.57% of dietary threonine. The best FCE was observed for the juveniles fed the diet containing 1.57% of threonine and increasing the threonine level from 1.57 to 2.00% of diet slightly decreased FCE. FCE significantly decreased further increment of threonine up to 2.72% of diet; however, no statistical differences were found between the juveniles fed threonine levels 2.35 and 2.72% of diet. Survival of juvenile Japanese flounder was more than 80% for all dietary treatments with no significant differences. Except for reduced growth, no other nutritional deficiency signs were observed in Japanese flounder fed threonine-deficient diets. The lowest N retention (5.08%) was found in the juveniles fed

the threonine-deficient diet (0.80% of diet). Increasing the threonine level up to 1.57% of diet increased N retention, but no statistical difference occurred between 1.57 and 2.00% threonine. However, further increase in the dietary threonine level, significantly ($P<0.05$) decreased the N retention of the juveniles.

The lowest protein content was observed in juveniles fed the diet containing the lowest threonine level; whereas, the highest value for protein was obtained from the group fed the diet containing 1.57% threonine (Table 3). Table 4 shows the amino acid composition of the whole body after six weeks of feeding. Although variation in levels of certain amino acids, such as arginine and valine in the whole body were significant ($P<0.05$) among the dietary groups, the actual differences were small.

Weight gain data subjected to broken-line analysis indicated optimum dietary threonine (R) level for the juvenile Japanese flounder was of 1.57% of

Table 2. Weight gain, feed intake (FI), feed conversion efficiency (FCE), percent survival, specific growth rate (SGR), N-retention of juvenile Japanese flounder fed diets graded levels of threonine for 42 days. Values are means ± SE for three replications. Means with different letters in the same column differ significantly ($P<0.05$)

Threonine level % of diet (% protein)	Weight gain[1] (%)	FI[2] (g/40days)	FCE[3] (%)	Survival (%)	SGR[4] (%)	N retention[5] (%)
0.80 (1.60)	43.5 ± 3.2a	2.93 ± 0.23a	24.7 ± 3.56a	86.7 ± 3.85a	0.96 ± 0.07a	5.08 ± 0.59a
1.18 (2.36)	127.1 ± 9.64b	3.36 ± 0.30ab	59.5 ± 4.18b	91.1 ± 2.22a	2.03 ± 0.11b	15.05 ± 1.10b
1.57 (3.14)	323.5 ± 12.79e	4.32 ± 0.26c	111.3 ± 8.20d	84.4 ± 5.88a	3.54 ± 0.08e	38.15 ± 0.89e
2.00 (4.00)	235.6 ± 5.46d	3.78 ± 0.15b	103.7 ± 9.79cd	87.8 ± 2.23a	2.97 ± 0.02d	31.59 ± 2.60de
2.35 (4.70)	191.7 ± 12.07cd	3.73 ± 0.22b	81.2 ± 2.26bc	84.4 ± 3.58a	2.66 ± 0.13cd	25.95 ± 2.22cd
2.72 (5.44)	168.9 ± 18.56bc	3.47 ± 0.20ab	70.0 ± 3.31b	93.3 ± 3.84a	2.47 ± 0.14bc	17.50 ± 2.43bc

[1]Weight gain = (Final body weight – initial body weight)/ initial body weight x100.
[2]FI = Feed intake.
[3]Feed conversion efficiency = weight gain (g) x 100/total feed intake in dry weight basis (g).
[4]Specific growth rate = [ln (mean final weight) - ln (mean initial weight) /42 day] x 100.
[5]Nitrogen retention = N gain x 100/ N intake.

Table 3. Effects of dietary levels of threonine on body composition (% wet basis) of Japanese flounder. Values are means ± SEM of triplicate groups. Initial body composition was 80.3% moisture, 13.5% of protein, 1.64% lipid and 2.80% ash. Means with different letters in the same column differ significantly ($P< 0.05$)

Threonine level % diet (% protein)	Moisture	Crude protein	Total lipid	Ash
0.80 (1.60)	81.3 ± 0.21b	12.4 ± 0.09a	1.87 ± 0.05a	3.55 ± 0.04d
1.18 (2.36)	78.5 ± 0.59a	14.4 ± 0.16b	2.29 ± 0.12ab	2.88 ± 0.05c
1.57 (3.14)	77.9 ± 0.46a	15.0 ± 0.09bc	2.60 ± 0.15b	2.46 ± 0.01a
2.00 (4.00)	77.6 ± 0.24a	15.2 ± 0.16c	2.43 ± 0.03ab	2.59 ± 0.06ab
2.35 (4.70)	78.2 ± 0.32a	4.8 ± 0.01bc	2.04 ± 0.24ab	2.77 ± 0.01bc
2.72 (5.44)	78.2 ± 0.41a	14.7 ± 0.13b	2.08 ± 0.06ab	2.63 ± 0.01ab

diet. The relationship between % weight gain (Y) and dietary methionine level (X) is:

$$Y = -266.2 + 364.2\ X \text{ if } X \leq R, \text{ and } Y = 521.2 - 134.9X \text{ if } X > R.$$

This level is equivalent to 3.14 g/100 g protein. Broken-line analysis of FCE and N retention indicated that the optimum dietary threonine level was 1.60 and 1.61% of diet (3.20 and 3.22% of protein), respectively. These values were close to the value by weight gain data.

Discussion

The estimated optimum dietary threonine level for juvenile Japanese flounder from weight gain data was found to be 1.57% of diet, which corresponds to

Table 4. Amino acid composition ($g \cdot 100^{-1}$ dry sample) of the whole body after feeding trial. Means with different letters in the same column differ significantly ($P < 0.05$)

Amino acids	Threonine level in diets					
	0.80	1.18	1.57	2.00	2.35	2.72
Arginine	2.79 ± 0.17a	3.00 ± 0.15ab	3.07 ± 0.24ab	3.67 ± 0.04b	2.93 ± 0.06ab	2.92 ± 0.05ab
Histidine	1.37 ± 0.01	1.39 ± 0.09	1.29 ± 0.12	1.30 ± 0.06	1.11 ± 0.02	1.26 ± 0.04
Isoleucine	2.07 ± 0.06	2.04 ± 0.10	2.03 ± 0.12	2.07 ± 0.03	2.12 ± 0.04	2.03 ± 0.05
Leucine	3.94 ± 0.07	3.89 ± 0.26	3.80 ± 0.25	3.85 ± 0.04	4.27 ± 0.86	3.93 ± 0.9
Lysine	4.73 ± 0.21	4.78 ± 0.20	4.64 ± 0.23	4.69 ± 0.19	3.98 ± 0.09	4.03 ± 0.4
Methionine	1.25 ± 0.02	1.23 ± 0.10	1.19 ± 0.01	1.24 ± 0.10	1.28 ± 0.03	1.24 ± 0.01
Phenylalanine	2.00 ± 0.02	1.94 ± 0.08	1.89 ± 0.16	1.87 ± 0.03	1.73 ± 0.09	2.00 ± 0.01
Threonine	2.18 ± 0.17	2.26 ± 0.01	2.19 ± 0.30	2.10 ± 0.04	1.89 ± 0.17	2.17 ± 0.17
Valine	2.16 ± 0.01ab	1.93 ± 0.09a	2.14 ± 0.10ab	2.34 ± 0.06b	2.26 ± 0.03ab	2.10 ± 0.09ab
Aspartic acid	4.86 ± 0.42	4.68 ± 0.41	4.57 ± 0.8	3.99 ± 0.08	3.79 ± 0.55	4.29 ± 0.21
Glutamic acid	9.50 ± 0.29	9.53 ± 0.20	9.07 ± 0.98	8.84 ± 0.15	7.83 ± 0.74	7.84 ± 0.25
Serine	2.21 ± 0.12	2.20 ± 0.09	2.04 ± 0.25	1.95 ± 0.02	1.75 ± 0.21	1.85 ± 0.06
Proline	2.59 ± 0.23	2.69 ± 0.17	2.57 ± 0.11	2.51 ± 0.18	2.25 ± 0.18	2.53 ± 0.30
Glycine	3.68 ± 0.52	3.85 ± 0.38	3.45 ± 0.36	3.26 ± 0.41	2.57 ± 0.04	3.14 ± 0.29
Alanine	3.53 ± 0.21	3.72 ± 0.09	3.69 ± 0.25	3.58 ± 0.26	2.89 ± 0.03	3.23 ± 0.22
Tyrosine	1.70 ± 0.08	1.59 ± 0.11	1.57 ± 0.12	1.59 ± 0.03	1.75 ± 0.10	1.63 ± 0.08
Taurine	1.09 ± 0.02	0.98 ± 0.14	0.83 ± 0.09	0.73 ± 0.01	0.69 ± 0.10	0.79 ± 0.07

3.14% of dietary protein. Estimates of optimum threonine level for the flounder derived from the other parameters such as FCE and N retention were slightly different with those based on weight gain. The optimum dietary level (% of protein) of the Japanese flounder determined in the present study is nearer to those of the commercially important finfish species namely, common carp (3.9%, Nose 1979), rainbow trout 3.4 (Ogino 1980) Japanese eel (4%, NRC 1993), Nile tilapia (3.75%, Santiago and Lovell 1988), juvenile striped bass (2.45%, Small and Soars 1999), hybrid striped bass (2.6%, Keembiyehetty and Gatlin 1997). However, the optimum dietary threonine level of the juvenile flounder is higher than the values reported for red drum (2.28%, Boren and Gatlin 1995) and European sea bass (2.3 to 2.6%, Tibaldi and Tulli 1999) and lower than the value reported for milkfish (4.5%, Borlongan 1991). Many factors have been identified that may affect optimum levels or amino acid requirements, including species and age, dietary protein sources, crystalline amino acids, environmental conditions and experimental design (Tacon and Cowey 1985; Moon and Gatlin 1991).

The reduction in growth of Japanese flounder fed high levels of threonine could be attributed to amino acid toxicity and amino acid catabolism. The accumulation of an amino acid or its degradative products in body pools may stress enzymatic systems and lead to further accumulation and possible toxicity. A significant depression in weight gain was observed in *C. catla* when fed diets supplying up to 50% excess of threonine with respect to the required value (Ravi and Devaraj 1991). Excess level of threonine also reduced growth of penaeid shrimp *Penaeus monodon* (Millamena et al. 1997) and Indian major carp, *Labeo rohita* (Murthy and Varghese 1996), which is similar to the findings of the present study for flounder. It has been reported that excessive levels of amino acids may become toxic and may have adverse effect on growth, because the disproportionate intake affects the absorption and utilization of other amino acids or decrease the diets' palatability (Harper et al. 1970; Borlongan and Coloso 1993; Murthy and Varghese 1996; 1998). For threonine, there is considerable evidence from studies with rats and chickens that a dietary imbalance or excess of certain amino acids increases dietary threonine requirement (Twes et al. 1980; Davies and Austic 1982; Kidd et al. 1997). Recently, we have tested the interaction of arginine and lysine levels on Japanese flounder (Alam et al. 2002). However, so far with fish such possible dietary antagonism has not been investigated in threonine requirement studies. Further study is needed to elucidate the actual mechanism of this adverse effect on flounder for high dose of threonine.

In the present study, low feed intake recorded for juveniles fed the diet deficient of threonine resulted to low growth as related to rainbow trout (Rodehustscord et al. 1995), carp (Nose 1979) and *C. catla* (Ravi and Devaraj 1991). On the other hand, lower feed intake was also recorded for Japanese flounder fed dietary threonine levels beyond the estimated optimum level. The poor growth of high dose-threonine groups may be due to loss of appetite, which resulted in low feed intake, hence depressed growth. Any other nutritional pathological signs except reduced growth were not observed in the Japanese flounder fed either threonine-deficient or excess diets.

The total amino acid composition of juveniles fed diets containing different levels of threonine did not show marked differences except some individual amino acids as related to other fishes (Mohanty and Kaushik 1991). Kaushik (1998) did not find any significant differences of total whole body amino acids detected from two different sizes of European sea bass, gilthead sea bream and turbot. The small differences of certain amino acids between the dietary treatments found in this study may be due to differences in the tissue levels of free amino acids. The threonine level found in the whole body was a little higher compared to the optimum level estimated by growth parameters.

Finally, this study indicates that the optimum level of dietary threonine at approximately 1.57% of diet or 3.14% of protein for maximum growth of juvenile Japanese flounder.

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