www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 14: 853-862 (2014) DOI: 10.4194/1303-2712-v14_4_02

Dietary Isoleucine Influences Non-Specific Immune Response in Juvenile Olive Flounder (*Paralichthys olivaceus*)

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Received 18 June 2014
Accepted 16 October 2014

Abstract

A 9-week feeding trial was conducted in low temperature season to evaluate the effects of varying levels of dietary isoleucine on hematology and innate immune response of juvenile olive flounder. Six isonitrogenous (45% crude protein) and isocaloric (4.45 kcal g^{-1} gross energy) diets containing graded levels of isoleucine (0.48, 0.87, 1.43, 1.94, 2.37 and 2.78% dry diet) were formulated. Triplicate groups of fish (8.59 ± 0.13 g) were fed the six test diets to apparent satiation twice daily. Fish growth performance was affected by dietary isoleucine levels and the highest growth was obtained at 1.43% isoleucine. The results showed the significant ($P<0.05$) increase of plasma total protein and cholesterol concentrations at isoleucine levels of 1.43 - 2.37% compared to those offered 0.48% isoleucine. Also, significant reductions in plasma alanine aminotransferase and aspartate aminotransferase activities were detected by increment of dietary isoleucine up to 1.94%. Fish innate immunity was significantly affected by isoleucine levels. Significantly higher lysozyme activity was found at 0.87 - 2.37% isoleucine and superoxide dismutase activity increased at 1.94-2.37% isoleucine levels. Respiratory burst activity and total immunoglobulin level were significantly enhanced at isoleucine levels of 1.43-1.94 and 1.94%, respectively. The findings in this study showed that inclusion of approximately 2% isoleucine in diets for olive flounder can enhance innate immunity. To estimate isoleucine requirement of the species further studies are required.

Keywords: Dietary isoleucine, olive flounder, *Paralichthys olivaceus*, growth, hematology, innate immunity.

Introduction

Protein is the most important component of fish feeds providing essential amino acids for tissue repair and growth (Luo *et al*., 2006). Proteins and their building blocks, amino acids, play very important roles in the structure and metabolism of living organisms (Meijer, 2003). Fishes cannot synthesize all the amino acids hence they require an exogenous source to meet the requirements (NRC, 2011). A consistent amount of proteins and amino acids needs to be incorporated in fish diets as they are continually used by the fish to build new proteins, peptides, free amino acids, enzymes, hormones, neurotransmitters and cofactors. Branched-chain amino acids (BCAA), isoleucine, leucine and valine, exert very important roles in certain biochemical reactions and growth. Isoleucine participates in production of certain biochemical compounds which are involved in energy production, and together with the other two BCAA promotes tissue building. It is the first limiting BCAA in meat and bone meal (Wang *et al*., 1997). Isoleucine deficiency results in biochemical malfunction including growth retardation (Ahmed and Khan, 2006; Khan and Abidi, 2007).

It has long been demonstrated that deficiency of dietary proteins and amino acids leads to impairment of immune function and increased susceptibility to infectious disease (Li *et al*., 2007). Results from both oral and parenteral feeding studies showed that protein intake and availability of certain dietary amino acids are vital for optimal immune function of the intestine and the proximal resident immune cells (Ruth and Field, 2013). A large body of studies has been conducted to assess the role of amino acids in immune function of animals including fish (Roch, 1999; Calder, 2006; Grimble, 2006; Kim *et al*., 2007). Early reports suggested that amino acids are used as important energy substrates for immune cells (Wu *et al*., 1991a, 1991b, 1991c; Field *et al*., 1994) and antioxidant defense mechanisms (Xue and Field, 2011). Several amino acids have been recognized to play significant roles in modulating various immune responses including the activation of lymphocytes, natural killer cells, and macrophages; proliferation of lymphocytes; regulation of intracellular redox states;

gene expression; and production of cytokines (Yoneda *et al*., 2009). BCAA are involved in biosynthesis of glutamine, as part of the immune system, by providing α-amino group (Newsholme and Calder, 1997). Also, the carbon skeletons of BCAA are required by leucocytes for protein synthesis (Waithe *et al*., 1975). It has been reported that deficiency of BCAA results in immune impairment. Petro and Bhattacharjee (1981) reported that mice fed BCAA-deficient diet exhibited increased susceptibility to *Salmonella typhimurium*, impaired antibody production, reductions in serum concentrations of transferrin and complement C3, and increased numbers of bacteria in liver and spleen. Isoleucine has been reported to influence innate immunity including induction of the antimicrobial peptide beta-defensin from Madin–Darby bovine kidney (MDBK) epithelial cells (Fehlbaum *et al*., 2000). Also, it has been shown that isoleucine is incorporated into human leucocyte cellular proteins and lipids (Burns, 1975) and improves the serum complement component 3 (C3) level in mice (Petro and Bhattacharjee, 1980). However, there is only a single available study on immunomodulating effects of isoleucine in fishes. Zhao *et al*. (2013) showed that dietary isoleucine promotes the immune responses in Jian carp (*Cyprinus carpio* var. Jian).

Olive flounder has been the most important cultured marine fish species in Korea. Its total production reached ~37,000 tons in 2013 in Korea (Ministry of Maritime Affairs and Fisheries of Korea, 2013). Because of importance of olive flounder in Korean aquaculture industry, this study was conducted to examine the effects of different dietary isoleucine levels on growth, hematology and non-specific immune response of the species.

Materials and Methods

Experimental Diets

Six isonitrogenous (45% crud protein) and isocaloric (4.45 kcal g^{-1} gross energy) diets were formulated using fish meal and a mixture of crystalline amino acids to contain graded levels of isoleucine (0.48–2.78% dry diet). The mixture of crystalline amino acids without isoleucine was prepared according to Dabrowski *et al*. (2003) and used as the main protein source and fish meal was included to increase palatability of the semi-purified diets. Proximate and essential amino acid composition of fish meal used in this study is provided in Table 1. The basal diet contained a minimum level of isoleucine from fish meal and supplemented with incremental levels (0.5%) of L-isoleucine. Analyzed dietary isoleucine concentrations were 0.48, 0.87, 1.43, 1.94, 2.37 and 2.78% (Table 2). The experimental diets were kept isonitrogenous by decreasing the level of glycine as the isoleucine level was increased. Isoleucine concentration of the experimental diets was determined using an automated amino acid analyzer (Beckman 7300, Beckman Instruments, Palo Alto, CA). The energy value of diets was estimated on the basis of physiological fuel value, i.e., 3.99 kcal g^{-1} proteins or carbohydrates and 9.01 kcal g^{-1} lipids (Lee and Putnam, 1973). All dry ingredients were thoroughly mixed and after addition of squid liver oil and double distilled water pelleted through a meat chopper machine (SMC-12, Kuposlice, Busan, Korea) in 3 mm diameter. The diets were freeze-dried for 24 h, crushed into desirable particle sizes, sealed in bags and stored at -20°C until used.

Fish and Experimental Conditions

Juvenile olive flounder were transported from a private hatchery to the Marine and Environmental Research Institute of Jeju National University (Jeju, South Korea). All the fish were fed the basal diet for one week to be acclimated to the semi-purified diet and the experimental conditions. At the end of the acclimation period, 45 randomly selected fish (8.59 ± 0.13 g) were stocked into each polyvinyl circular tanks of 150 L capacity and supplied with filtered seawater at a flow rate of 3 L min^{-1} and aeration to maintain enough dissolved oxygen. Triplicate groups of fish were hand-fed the six test diets to apparent satiation (twice a day, 09:^{00} and 17:^{00} h) for 9 weeks. Uneaten food was siphoned out 30 min after feeding and weighed to determine the feed intake. Growth of fish was measured with three-week intervals. Feeding was stopped 24 h prior to weighing or blood sampling to minimize handling stress on fish. The water temperature during the feeding trial ranged from 13 to 17°C and the photoperiod was maintained on a 12:12 light:dark schedule.

Sample Collection and Chemical Analyses

At the end of the feeding trial, all the fish in each tank were bulk-weighed and counted for calculation

Table 1. Proximate and essential amino acid composition of white fish meal (% dry matter)

	%
Essential amino acids	
Arg	3.65
His	2.60
Ile	2.59
Leu	4.97
Lys	5.46
Met	1.84
Phe	2.60
Thr	3.05
Val	3.04
Proximate composition	
Dry matter	90
Protein	67
Lipid	10
Ash	16

Table 2. Formulation and proximate composition of the experimental diets (% dry matter)

Ingredients	1	2	3	4	5	6
White fish meal	20.0	20.0	20.0	20.0	20.0	20.0
Free AA mix[1]	27.5	27.5	27.5	27.5	27.5	27.5
Isoleucine	0.0	0.5	1.0	1.5	2.0	2.5
Glycine	2.5	2.0	1.5	1.0	0.5	0.0
Dextrin	33.5	33.5	33.5	33.5	33.5	33.5
Taurine	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mix[2]	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin mix[3]	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride	1.0	1.0	1.0	1.0	1.0	1.0
Squid liver oil	12.5	12.5	12.5	12.5	12.5	12.5
Proximate composition						
Dry matter	93.1	93.0	94.0	93.4	94.0	94.0
Protein	44.9	45.2	45.0	45.4	45.6	45.1
Lipid	9.5	9.2	9.4	9.3	9.5	9.5
Ash	3.6	3.8	3.5	3.7	3.5	3.5
Isoleucine	0.48	0.87	1.43	1.94	2.37	2.78

[1] Free amino acid mixture composition: (g per 446 g dry weight mixture): arginine hydrochloride, 15; lysine hydrochloride, 18; methionine, 10; histidine, 7; valine, 12; leucine, 14; phenylalanine, 18; threonine, 8; tryptophan, 2; glutamic acid, 111; glycine, 231.

[2] Mineral premix (g kg^{-1}): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[3] Vitamin premix (g kg^{-1}): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

of growth parameters and survival. Five fish per tank (15 fish per treatment) were selected and kept at -20°C for whole-body composition analysis. Five fish per tank (15 fish per treatment) were randomly captured, anesthetized with 2-phenoxyethanol (200 mg L^{-1}), and blood samples were collected from the caudal vein with heparinized syringes for determination of hematocrit, hemoglobin and respiratory burst activity. After the above mentioned measurements with whole blood, plasma were separated by centrifugation at 5000 × g for 10 min and stored at -70°C for determination of total immunoglobulin (Ig) level and blood biochemical parameters including plasma total protein, glucose and cholesterol concentrations and activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Another set of blood samples (5 fish per tank, 15 fish per dietary treatment) were taken without heparin and allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation for 10 min at 5000 × g and stored at -70°C for the analysis of non-specific immune responses including lysozyme, myeloperoxidase (MPO) and superoxide dismutase (SOD) activities.

Analyses of moisture and ash contents of diet and fish whole-body samples were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using automatic Kjeltec Analyzer Unit 2300 (FossTecator, Höganäs, Sweden) and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Seoul, Korea). Hematocrit was determined by microhematocrit technique (Brown, 1980). Hemoglobin and plasma levels of total protein, glucose and cholesterol and activities of ALT and AST were determined by an automated blood analyzer (SLIM, SEAC Inc, Florence, Italy).

A turbidometric assay was used for determination of serum lysozyme level by the method described by Hultmark (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg ml^{-1}) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), then 200 µl of suspension was placed in each well of 96-well plates and 20 µl serum was added subsequently. The reduction in absorbance of the samples was recorded at 570 nm after incubation at room temperature for 0 and 30 min in a microplate reader (UVM 340, Biochrom, Cambridge, UK). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity.

Oxidative radical production by phagocytes during respiratory burst was measured through NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995). Briefly, blood and NBT (0.2%) (Sigma, USA) were mixed in equal proportion (1:1) and incubated for 30 min at room temperature, then 50 µl was taken out and dispensed into glass tubes. Then, 1 ml dimethylformamide (Sigma) was added and centrifuged at 2000 × *g* for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Genesys 10UV, Rochester, NY, USA). Dimethylformamide was used as blank.

Serum myeloperoxidase (MPO) activity was measured according to Quade and Roth (1997). Briefly, twenty microliter of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma, USA) in 96-well plates. Then, 35 µl of 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma, USA) and H_2O_2 (5 mM) were added. The color change reaction was stopped after 2 min by

adding 35 µl of 4 M sulfuric acid. Finally, the optical density was read at 450 nm in the microplate reader.

Plasma Ig level was determined according to the method described by Siwicki and Anderson (1993). Briefly, plasma total protein concentration was measured using a micro protein determination method (C-690; Sigma), prior to and after precipitating down the Ig molecules using a 12% solution of polyethylene glycol (Sigma). The difference in protein concentration represents the Ig content.

Serum superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37°C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Statistical Analysis

All dietary treatments were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with Tukey's HSD multiple range test. Statistical significance was determined at $P<0.05$. Data are presented as mean ± SD. Percentage data were arcsine transformed before statistical analysis.

Results

The results showed the significant enhancement of growth performance and feed utilization at dietary isoleucine levels of ≥0.87% (Table 3). Feed conversion ratio decreased significantly at isoleucine levels of 0.87-1.94% and significantly higher protein efficiency ratio was found in fish fed 1.43-1.94% isoleucine compared to those offered 0.48% isoleucine. Fish survival was not affected by dietary treatments. Also, the results showed no significant effect of dietary isoleucine on whole-body composition (Table 4).

Higher hematocrit values were obtained at increased isoleucine levels, but the differences were not significant (Table 5). Significantly higher plasma total protein and cholesterol concentrations were found in groups fed 1.43-2.37% isoleucine. Also, the results revealed the significant reduction of plasma ALT and AST activities at dietary isoleucine levels of 1.43-1.94% and 0.87-1.94%, respectively.

Non-specific immune response parameters were affected by dietary isoleucine levels (Table 6). Significantly higher lysozyme and SOD activities were found in fish fed isoleucine levels of 0.87-2.37%

Table 3. Growth performance of olive flounder (initial body weight, 8.59±0.13) fed different levels of dietary isoleucine for 9 weeks

Ile level (% dry diet)	WG[1] (%)	SGR[2] (%)	FI[3] (g/fish)	FCR[4]	PER[5]	Survival (%)
0.48	81.54±4.68^{d}	0.95±0.04^{d}	18.71±3.47	2.68±0.61^{a}	0.84±0.22^{b}	80.00±13.5
0.87	119.12±4.07^{ab}	1.25±0.03^{ab}	19.89±1.14	1.92±0.06^{b}	1.14±0.04^{ab}	77.78±7.70
1.43	128.41±4.85^{a}	1.31±0.03^{a}	17.93±0.80	1.64±0.12^{b}	1.33±0.10^{a}	89.63±5.59
1.94	116.80±5.76^{ab}	1.23±0.04^{ab}	18.15±1.23	1.80±0.21^{b}	1.22±0.14^{a}	88.15±16.8
2.37	109.04±4.77^{bc}	1.17±0.04^{bc}	18.47±0.96	1.98±0.18^{ab}	1.10±0.10^{ab}	89.63±8.41
2.78	101.98±5.40^{c}	1.12±0.04^{c}	17.69±1.16	2.05±0.03^{ab}	1.05±0.01^{ab}	91.85±5.13

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1] Weight gain = [(final body weight – initial body weight) / initial body weight × 100];

[2] Specific growth rate = 100 × [(ln final body weight - ln initial body weight) / days];

[3] Feed intake (g/fish) = dry feed consumed (g) / fish;

[4] Feed conversion ratio = dry feed fed / wet weight gain;

[5] Protein efficiency ratio = wet weight gain / total protein given.

Table 4. Whole-body composition (% wet basis) of olive flounder fed different levels of dietary isoleucine for 9 weeks

Ile level (% dry diet)	Protein	Lipid	Moisture	Ash
0.48	16.78±0.45	1.08±0.16	82.40±0.61	3.48±0.38
0.87	16.83±0.71	1.09±0.03	81.85±0.60	3.25±0.69
1.43	17.55±0.73	0.99±0.05	81.93±0.14	3.56±0.70
1.94	16.97±0.53	1.14±0.19	81.47±0.25	3.33±0.52
2.37	16.80±0.65	1.15±0.12	81.73±0.50	3.65±0.39
2.78	16.87±0.50	1.12±0.04	81.54±0.45	3.51±0.75

Values are mean of triplicate groups and presented as mean ± SD.

The lack of superscript letter indicates no significant differences among treatments.

Table 5. Hematological parameters of olive flounder fed different levels of dietary isoleucine for 9 weeks

Ile level (% dry diet)	Ht[1]	Hg[1]	Total protein[3]	ALT[4]	AST[5]	Glucose[6]	Cholesterol[7]
0.48	15.83±1.04	2.36±0.24	1.72±0.15^{c}	57.33±6.66^{a}	105.5±6.40^{a}	19.65±1.94	39.77±8.21^{c}
0.87	16.50±1.80	2.38±0.02	1.87±0.24^{bc}	56.20±4.20^{a}	83.74±5.24^{b}	24.02±4.19	49.12±9.42^{bc}
1.43	17.50±2.18	2.45±0.33	2.58±0.38^{ab}	35.79±2.44^{c}	64.94±7.58^{b}	24.31±3.58	77.55±5.80^{a}
1.94	17.67±1.76	2.59±0.25	2.96±0.28^{a}	43.26±4.95^{bc}	78.00±9.13^{b}	25.19±3.46	67.08±7.57^{ab}
2.37	17.33±0.76	2.30±0.13	2.88±0.21^{a}	55.37±5.37^{ab}	84.88±8.42^{ab}	24.60±2.40	64.85±7.86^{ab}
2.78	17.67±1.04	2.33±0.10	2.53±0.34^{ab}	62.81±2.66^{a}	85.03±7.97^{ab}	23.40±4.89	60.54±8.18^{abc}

Values are mean of triplicate groups and presented as mean ± SD. Values in the same column having different superscript letters are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.
[1]Hematocrit (%)
[2]Hemoglobin (g/dl)
[3]Total protein (g/dl)
[4] Alanine aminotransferase activity (U/L)
[5] Aspartate aminotransferase (U/L)
[6]Glucose (mg/dl)
[7]Total cholesterol (mg/dl)

Table 6. Innate immune response of olive flounder fed different levels of dietary isoleucine for 9 weeks

Ile level (% dry diet)	Lysozyme[1]	NBT[2]	MPO[3]	Ig[4]	SOD[5]
0.48	19.74±2.29^{c}	0.26±0.01^{b}	1.42±0.04	5.89±0.57^{b}	55.22±2.84^{c}
0.87	28.23±2.45^{ab}	0.34±0.03^{ab}	1.49±0.04	8.83±0.73^{ab}	59.06±3.16^{bc}
1.43	28.06±2.13^{ab}	0.41±0.04^{a}	1.51±0.15	10.36±0.21^{ab}	62.54±4.56^{abc}
1.94	34.21±2.48^{a}	0.42±0.08^{a}	1.65±0.09	12.92±3.18^{a}	68.41±1.51^{a}
2.37	33.27±2.59^{a}	0.35±0.03^{ab}	1.45±0.10	10.72±3.56^{ab}	64.58±2.10^{ab}
2.78	24.73±4.57^{bc}	0.37±0.05^{ab}	1.50±0.03	9.75±2.59^{ab}	58.67±3.57^{bc}

Values are mean of triplicate groups and presented as mean ± S.D. Values in the same column having different superscript letters are significantly different (P<0.05). The lack of superscript letter indicates no significant differences among treatments.
[1] Lysozyme (U/ml)
[2] Nitro blue tetrazolium activity (Absorbance)
[3] Myeloperoxidase (Absorbance)
[4] Total Immunoglobulin (mg/ml)
[5] Superoxide dismutase (% inhibition)

and 1.94-2.37%, respectively, compared to those fed the basal diet. Respiratory burst activity was significantly increased at the levels of 1.43-1.94% isoleucine. The highest Ig level was detected in fish fed 1.94% isoleucine and differed significantly from that of the fish fed 0.43% isoleucine. However, MPO activity did not significantly differ among experimental groups.

Discussion

In the present study the fish readily accepted the semi-purified test diets, however, relatively low growth rates were achieved due to low rearing water temperature. The results showed the significant enhancement of fish growth by increment of dietary isoleucine level up to 1.43% and thereafter a decreasing tendency was achieved. The increased fish growth in this study was primarily due to improved feed utilization efficiency as significantly lower FCR and higher PER were obtained by increment of dietary isoleucine level. It has long been demonstrated that a balanced amino acids profile is required for effective utilization of dietary protein for tissue synthesis (D'Mello, 1994; Yamamoto *et al*., 2000; Berge *et al*., 2002; Green and Hardy, 2002; Goemez-Requeni *et al*., 2003). In this study reduced growth was found in fish groups fed isoleucine deficient diets and those fed higher isoleucine levels than 1.43% probably indicating imbalances in dietary amino acids profile. Antagonistic interactions between BCAA including leucine, isoleucine and valine have been reported in chicks, pigs, rats and humans (D'Mello, 1994). In fish, such effects have not been fully investigated and the obtained results have been capricious (NRC, 2011). BCAA are known to produce antagonistic effects when the proportion of these three amino acids in diet is imbalanced. Reduced growth performance of olive flounder at over 1.43% isoleucine in this study can be attributed to antagonism between BCAA (Ahmed and Khan, 2006).

The results showed no significant influence of isoleucine on feed intake. Similarly, previous studies on catla (*Catla catla*) (Zehra and Khan, 2013) and Pacific white shrimp (*Litopenaeus vannamei*) (Liu *et al*., 2014) did not show any significant effect of dietary isoleucine on feed intake. However, Zhao *et al*. (2012) reported the significant increase of feed intake in Jian carp (*Cyprinus carpio* var. Jian) offered

incremental dietary isoleucine levels. Quantitative requirement for essential amino acids is generally estimated based on fish weight gain; however, regarding the secondary growth of fish in this study the data were not used for determination of optimal isoleucine requirement level and needs to be quantified in future studies. Fish whole-body composition was taken into account in the present study and the results revealed no significant changes with respect to variations in dietary isoleucine level.

Hematological parameters are being increasingly taken into account in amino acids requirement studies because of their sensitivity to dietary manipulations (Hrubec *et al*., 2000; Congleton and Wagner, 2006). Accordingly, in the current study the changes in hematological and blood biochemical parameters were examined and significant changes were detected. Zhao *et al*. (2013) reported the significant increase of red and white blood cells count in Jian carp fed increased dietary isoleucine levels. In the current study, numerically higher hematocrit values were observed at higher isoleucine levels, however the differences were not significant. Plasma total protein level has been measured frequently as an indicator of physiological condition in fish nutrition studies (Nakagawa *et al*., 2000; Farhangi and Carter, 2001; Watanabe *et al*., 2001; Harikrishnan *et al*., 2003). Total protein in plasma is the most stable component, and few dietary factors have been reported to affect the levels in fish. Plasma total protein is elevated when dietary protein intake increases (Leveille and Sauberlich, 1961) indicating improved physiological condition (Dawson and Bortolotti, 1997). Also, it has been suggested that increased blood protein level is associated with enhanced innate immune response in fish (Wiegertjes *et al*., 1996). In the current study significant enhancements in plasma total protein level was observed at increased isoleucine levels. There is no available study on the effect of dietary isoleucine on fish plasma total protein level, but in agreement to our study significant enhancements in fish serum/plasma protein levels have been reported following administration of optimum dietary lysine level in black sea bream (*Sparus macrocephalus*) (Zhou *et al*., 2010) and yellow catfish (*Pelteobagrus fulvidraco*) (Cao *et al*., 2012), and optimal dietary valine level in red sea bream (Rahimnejad and Lee, 2013). In general, ALT and AST are mainly distributed in the liver and spleen and play important roles in protein metabolism. Their concentrations in the blood increase when the liver and myocardial cells are damaged or their permeability increased. Both enzymes are used as valuable diagnostic means of stress responses in fish species (De Smet and Blust, 2001; Almeida *et al*., 2002; Choi *et al*., 2007) and their concentration in plasma increases in response to several factors such as pollution and ammonia and nitrite toxications (Das *et al*., 2004). In the current study significant reductions in plasma ALT and AST concentrations were achieved by increment of dietary isoleucine level up to 1.94% indicating an improvement in fish health status. It has been reported that dietary protein affects the plasma cholesterol level (Carroll and Hamilton, 1975; Kritchevsky, 1979; Terpstra *et al*., 1983) and that the main influencing factor is the amino acids composition of the protein source (Garlich *et al*., 1970; Olsen *et al*., 1970a,b; Coles and McDonald, 1972). In agreement to this notion, Sugiyama *et al*. (1996) reported a significant positive correlation between the plasma total cholesterol level and plasma concentration of valine in rats fed different dietary protein sources. Also, the results of our previous study on red sea bream showed the significant increase of plasma cholesterol concentration by increment of dietary valine level (Rahimnejad and Lee, 2013). Similarly, in the present study plasma cholesterol level elevated significantly when dietary isoleucine level increased.

It has been demonstrated that deficiency or excess of dietary protein (Glick *et al*., 1981, 1983; Payne *et al*., 1990) or amino acids (Bhargava *et al*., 1970, 1971; Tsiagbe *et al*., 1987a, 1987b) alters immune responses. BCAA have been shown to play very important roles in the immune organ development (Kidd, 2004). Konashi *et al*. (2000) reported that weights of lymphoid organs are modified by either the type of essential amino acids or the degree of deficiency; and that feeding the BCAA-deficient diet causes the most severe decrease in both thymus and bursa weights in chickens. Lysozyme as a bactericidal enzyme is well known as an important humoral indicator of innate immunity in fish. It is released by leucocytes and provides protection against both gram-positive and gram-negative bacteria by lysing the 1, 4-beta-linkages in the peptidoglycan layer found in bacteria cell walls (Ellis, 1999). Several factors affect the lysozyme activity in fish including nutritional status (Saurabh and Sahoo, 2008). In the present study, significantly higher lysozyme activities were found by increment of dietary isoleucine level up to 2.37% and thereafter a reduced activity was obtained. Similarly, Zhao *et al*. (2013) reported the significant enhancement in lysozyme activity of Jian carp fed increased dietary isoleucine levels. The respiratory burst is generated by macrophages/monocytes and granulocytes to attack invasive pathogens during phagocytosis and is widely used to evaluate the defense capabilities against pathogens (Dalmo *et al*., 1997). In the current study the respiratory burst activity was measured through NBT assay and significantly higher activities were achieved at increased isoleucine levels. There is no earlier report on the effect of isoleucine on fish respiratory burst activity; however our previous study on red sea bream (Rahimnejad and Lee, 2013) showed the enhanced respiratory burst activity at increased valine levels indicating the role of BCAA. Total immunoglobulins play important roles in innate and acquired immune response (Magnadóttir, 2006) and are regarded as a good indicator for the action of

immunonutrients. In this study higher Ig levels were found by increment of dietary isoleucine. In agreement to our results, Zhao *et al*. (2013) showed the significant elevation of IgM level in Jian carp fed increased dietary isoleucine level. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen including oxygen ions and peroxides. The over production of ROS may damage cell membranes (Liu *et al*., 2007). Radical scavenging enzymes such as SOD can provide protection against ROS damage. Earlier, Zhao *et al*. (2013) reported the enhancement of SOD activity in Jian carp when by increment of dietary isoleucine level, which is in agreement with the results of the present study.

In conclusion, under the rearing conditions in this study it was shown that supplementation of a proper level of isoleucine is necessary for maximal growth of olive flounder, however, the optimum requirement level remains to be determined in future studies. The most significant finding of this study was that dietary inclusion of approximately 2% isoleucine can enhance humoral innate immune response in olive flounder.

Acknowledgments

This research was supported by Basic Science Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (grant number NRF 2010-0009918).

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