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RESEARCH PAPER

Evaluation of the Dietary Protein Requirement of a Selectively Bred (F-5 Generation) Strain of Olive Flounder, *Paralichthys olivaceus*

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Abstract

We determined the optimum dietary protein level required for a family selected (F-5 generation) strain of olive flounder *Paralichthysolivaceus.* Six isocaloric diets (average 20.7 kJ/g diet gross energy) were formulated to contain crude protein levels (CP) as 30 (CP30), 40 (CP40), 45 (CP45), 50 (CP50), 55 (CP55) and 60% (CP60). Triplicate groups of fish averaging 58.4 ± 0.28g (mean ± SD) were randomly distributed into the tanks as groups of 25 fish and fed one of the 6 diets at apparent satiation twice a day. At the end of 8-weeks feeding trial, weight gain (WG) and specific growth rate of fish fed 50 to 60% CP diets were significantly higher than those of fish fed 30 to 45% CP diets. Feed efficiency and protein efficiency ratio were inversely related to the dietary protein level. Broken-line model analysis indicated that the optimum dietary protein level was 50.1% for maximum WG in selected strain of olive flounder. The second-order polynomial regression analysis revealed that the maximum WG occurred at 59.2%. Based on the study, the optimum dietary protein level for maximum growth of family selected olive flounder could be greater than 50.1%, but less than 59.2% CP with 20.7 kJ/g gross energy.

Keywords: Olive flounder, family selection, protein requirement, broken-line model, second-order polynomial regression

Introduction

The selective breeding in aquaculture is consistently growing around the world. However, it is still comparatively small practiced compared to farmed terrestrial animals (Neely *et al*. 2008). According to Gjedrem and Thodesen (2005) breeding programs aim to improve attributes of aquaculture such as increase of fish production with rapid growth and product quality. Several studies have been conducted in family selected strain of fish in terms of growth performance (Murata *et al*. 1996; Handeland *et al*. 2003), feed intake (Silverstein *et al*. 2001; Ogata *et al*. 2002), protein and digestible energy (Li *et al*. 1998), reproduction (Gjerde1986).

Olive flounder, *Paralichthys olivaceus* is one of the most commercially important marine cultured fish species in Asian markets especially Korea, Japan and China. The Korean aquaculture production of olive flounder has reached 45,739 tons in 2015 (Ministry of Oceans and Fisheries 2016). Because of its good flavor and excellent growth as well as high survival, researchers are now paying great attention for improvement of its quality. Research on selected strain of olive flounder was initiated in the 2004 by NIFS (National Institute of Fisheries Science, Korea), to increase production and improve quality of cultured fish (Min *et al*. 2010). The strain of olive flounder used in several types of research on genetic evaluation (Kim *et al*. 2008), broodstock (Kang *et al*. 2006) and hatching rate (Min *et al*. 2009). According to result of internal rate of return and benefit-cost ratio, whole economical value of "selected breeding program" is 1,121,060.40 dollars (Hwang and Myeong, 2010). However, no studies have ever tried to estimate the optimum dietary protein level in selected strains of olive flounder, which have been carried out on protein requirement of juvenile olive flounder (Kikuchi *et al*. 1992; Lee *et al*. 2002; Kim *et al*. 2002).

Protein is an essential component of fish diet needed for growth, reproduction and survival of fish (Wilson and Halver 1986). In fish feed, protein provides the essential and non-essential amino acids for body protein synthesis and in part supplies energy for maintenance (Kaushik and Médale 1994; NRC, 2011). When protein levels are inadequate in fish diet, a reduction of growth is observed. Dietary protein content is the most important factor affecting fish growth and feed cost (Lovell 1989). Aquafeeds constitutes about 70% of the total investment in aquaculture system (Pillay 1995). Besides, protein is the most expensive component in the supplementary

fish feed (Fagbenro *et al*. 1992). Any reduction in dietary protein level without affecting fish growth can substantially reduce the cost of fish feed (Fiogbé 1996; Kim *et al*. 2003; Jamabo and Alfred-Ockiya 2008). For many fish species, there is an optimum requirement of dietary protein to supply adequate amino acids for maximizing growth (Siddiqui and Khan 2009). If too much protein is supplied in the diet, only part of it is used to make new protein for growth, and the remainder will be converted into energy, which results in increased feed cost and increased ammonia nitrogen excretion as an indicator of water pollution. Thus, from economical as well as environmental perspective, it is important that inclusion of the dietary protein should be optimized (Siddiqui and Khan 2009; Akpinar/*et al*. 2011). Therefore, the aim of the present study was to estimate the optimum dietary protein level on growth performance and proximate composition in family selected strains of olive flounder (F-5 generation).

Materials and Methods

Experimental Diets

The formulation and proximate composition of the experimental diets are shown in Table 1. Six experimental diets (gross energy, 20.7 kJ/g) were formulated to contain graded levels of protein 30%, 40%, 45%, 50%, 55% and 60% designated as CP30, CP40, CP45, CP50, CP55 and CP60, respectively, using brown fish meal and casein as the main protein source, and fish oil was used as lipid source. The estimated values for levels of protein were 30.1%, 39.3%, 44.5%, 50.1%, 54.5% and 59.3%. Diets were prepared by mixing the dry ingredients in an electric mixer, followed by the addition of oil and water. This mixture was formed into a dough, and dry pellets were made by passing the dough through a screw-type pelleting machine and then air dried the pellets for approximately 48 h. The pellets were crumbled and sieved through a sieve (180 mm) to obtain the appropriate and uniform pellet size. The formulated diets were stored at -20 ^{0}C until use.

Experimental Fish and Feeding Trial

The experiment was carried out for at the National Institute of Fisheries Science (NIFS), Pohang, Rep. of Korea. In this study, selectively bred offspring (F-5 generation) of olive flounder were obtained from Geoje Marine Hatchery (Geoje, Korea) of National Institute of Fisheries Science (NIFS). Prior to the start of the feeding trial, all fish were fed the basal diet for two weeks to become acclimatized to the experimental environment. At the start of the experiment, 25 sub-adult olive flounder with an initial average weight 58.4 ± 0.28 g (mean ± SD) were randomly distributed into each of the 18 tanks of 150-L supplied with flow-through sea water at 2-L/min. Each tank was then randomly assigned to one of three replicates of the six dietary treatments. Fish were fed twice daily (10.00 h and 17.00 h) at satiation for 8 weeks. Supplemental aeration was provided to maintain the dissolved oxygen (DO) near saturation, and water temperature during the experiment was

Table 1. Dietary composition of the experimental diets for family selected olive flounder (% dry matter basis)

	Protein levels (%)					
Ingredients[1]	CP30	CP40	CP45	CP50	CP55	CP60
Fishmeal[2]	25.7	39.7	46.7	53.7	60.7	67.8
Casein[2]	10.0	10.0	10.0	10.0	10.0	10.0
Alpha – starch[2]	31.9	21.2	15.9	10.6	5.50	0.00
Wheat flour[2]	11.0	11.0	11.0	11.0	11.0	11.0
Yeast[2]	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix[3]	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[3]	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin C	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin E	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
Cellulose	6.50	4.36	3.30	2.25	1.05	0.00
Fish oil	9.40	8.20	7.60	7.00	6.30	5.70
Proximate composition (%)						
Crude protein	30.1	39.3	44.5	50.1	54.5	59.3
Crude lipid	10.9	11.8	11.8	11.8	11.9	11.9
Crude ash	6.2	8.2	9.5	10.6	11.9	12.8
Estimated energy (kJ/g)[4]	20.1	20.4	20.7	21.1	21.1	20.8

[1]Feed stuffs not mentioned here are the same feed stuffs as the domestic aquaculture feed companies are using currently.
[2] Suhyup Feed Co., Kyong-Nam, Korea.
[3] Contents (mg/kg) : KI 250, $MnSO_4$ H_2O 2800, $ZnSO_4$ H_2O 2,350, vitamin K 225, biotin (2%) 3,500, niacin 4,850, calcium pantothenate 11,000, folic acid 2,000, vitamin B_1 1,500, vitamin B_2 2,000, vitamin B_6 2,000 and vitamin C 50,000.
[4] Calculated based on carbohydrates, proteins and lipids are 17.2, 23.6, and 39.5 kJ/g, respectively (Blaxter, 1989).

maintained at 22.1 ± 3.2 °C. A photoperiod of 12h light: 12h dark was used throughout the experimental period.

Sample Collection and Analysis

At the end of the feeding trial, the total number and weight of fish in each tank were determined for the calculation of weight gain (WG), feed efficiency (FE), specific growth rate (SGR), protein efficiency ratio (PER) and survival rate. Three fish per tank were randomly selected, individually weighed and total length determined, then dissected to obtain liver and viscera for the determination of condition factor (CF), hepatosomatic index (HSI) and viscerosomatic index (VSI), respectively.

Five fish per tank were randomly captured at the end of feeding trial, anesthetized with ethylene glycol phenyl ether, plasma was separated by centrifugation at 5000 ×g for 10 min and stored at −70°C for the determination of blood biochemical parameters including total cholesterol (TCHO), glucose, alkaline phosphatase (ALP), BUN (blood urea nitrogen), total protein (TP), triglyceride (TG), and alanine aminotransferase (ALT), aspartate aminotransferase (AST). Another set of blood samples from the same fish were taken without heparin and allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation at 5000 ×g for 10 min and stored at −70°C for the analysis of lysozyme activity as a non-specific immune parameter.

Three additional fish from each tank were used to analyze whole-body proximate composition. The proximate composition analyses of the experimental diets and whole-body of fish were performed by the standard methods of AOAC (1995). Samples of diets and fish were dried at 105^0C to a constant weight to determine their moisture contents. The ash content was determined by incineration at 550 ^{0}C. Protein was determined using the Kjeldahl method (N × 6.25) after acid digestion, and crude lipid was measured by ether extraction using the Soxhlet apparatus 1046 (Tacator AB, Hoganas, Sweden).

The plasma levels of total cholesterol, glucose, ALT, BUN, total protein, triglyceride, and activities of ALT and AST were measured using a chemical analyzer (Fuji DRI-CHEM 3500i; Fuji Photo Film, Tokyo, Japan). A turbidimetric assay was used for determination of serum lysozyme level using the method described by a turbimetric assay (Ellis, 1990) with slight modifications. Briefly, *Micrococcus lysodeikticus* (Sigma, 0.75 mg mL^{-1}) was suspended in sodium phosphate buffer (0.05 M, pH 6.2), 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 450 nm after incubation at room temperature for 0 and 30 min in a microplate reader (GENios Pro; TECAN, AUSTRIA). A reduction in absorbance of 0.001 min^{-1} was regarded as one unit of lysozyme activity.

Statistical Analysis

All data were analyzed by one-way ANOVA (Statistics 3.1; Analytical Software, St. Paul, MN, USA) to test for the dietary treatments. When a significant treatment effect was observed, Tukey's HSD *post-hoc* test was used to compare means. Treatment effects were considered significant at the $P<0.05$ level. An estimation of the dietary protein requirement, based on percentage weight gain of fish was conducted by a two-slope, broken-line analysis model (Robbins *et al*. 1979) and second order polynomial regression analysis.

Results and Discussion

After the 8-weeks feeding trial, growth performance and survival in family selected strains of olive flounder fed the experimental diets are presented in Table 2. Weight gain (WG) and specific growth rate (SGR) of fish fed CP50 diet was significantly higher than those of fish fed CP30, CP40 and CP45 diets ($F = 94.93$; $P=< .0001$ and $F= 87.52$; $P= < .0001$). However, there were no significant differences in WG and SGR among fish fed the CP 50, CP55 and CP60 diets. WG and SGR of fish increased with increasing dietary protein level from CP30 up to CP50 and then stable from CP50 up toCP60 diet. Similar trends have been demonstrated for other flat fish species (Caceres-Martinez *et al*. 1984; Devesa *et al*. 1994; Kim *et al*. 2002; Lee *et al*. 2003; Imsland *et al*. 2016). These unchanged growth performances of fish fed diets containing protein levels above the optimum probably due to decreasing available energy for growth and inadequate non-protein energy necessary to de-aminate and excretion of the excess amino acids absorbed (Jauncey 1982; Vergara *et al*. 1996; Kim *et al*. 2002). The dietary protein requirement of fish depends on various factors, including fish species, water temperature, feeding method and size of fish (NRC 1993).

According to a two-slope, broken-line model analysis, the optimum dietary protein level for maximum growth of fish was estimated to be 50.1% protein per kg diet (Figure 1). However, second order polynomial regression analysis showed that the maximum WG response point in the present study is 59.2% protein per kg diet ($y=-0.0681x^2 + 8.0686x - 77.146$; $R^2=0.97$). The above results indicated a higher protein requirement for the improved strain of olive flounder. However, this result is within the range of approximately 40-70% which is demonstrated for protein requirement of some carnivorous fish species (Caceres-Martinez *et al*. 1984; NRC 1993). In addition, it has been reported flatfish required higher protein compared with other fish species (Danielssen and Hjertnes 1993; Helland and Grisdale-Helland 1998). Thodesen *et al* (1999)

Table 2. Growth performance of family selected olive flounder fed the different protein level diets for 8 weeks[1]

	Experimental diets							
	CP30	CP40	CP45	CP50	CP55	CP60	F-value	p-value
IBW[2]	58.3±0.24^{a}	58.5±0.13^{a}	58.4±0.31^{a}	58.5±0.61^{a}	58.3±0.26^{a}	58.4±0.13^{a}	0.21	0.9500
FBW[3]	119±3.99^{c}	138±1.17^{b}	142±2.31^{b}	153±2.04^{a}	152±1.57^{a}	152±1.60^{a}	97.40	< .0001
WG[4]	104±3.64^{c}	136±1.05^{b}	143±2.76^{b}	162±4.28^{a}	160±3.55^{a}	160 ±1.36^{a}	94.93	< .0001
SGR[5]	1.35±0.03^{c}	1.62±0.01^{b}	1.67±0.02^{b}	1.82±0.03^{a}	1.80±0.03^{a}	1.80±0.01^{a}	87.52	< .0001
FE[6]	61.3±0.43^{f}	76.0±0.90^{e}	85.8±1.26^{d}	97.7±1.19^{c}	104±2.36^{b}	111±2.10^{a}	293.82	< .0001
PER[7]	2.04±0.01^{a}	1.93±0.02^{bc}	1.93±0.03^{bc}	1.95±0.02^{b}	1.91±0.04^{bc}	1.88±0.04^{c}	6.67	0.0034
HSI[8]	2.10±0.17^{a}	1.90±0.15^{ab}	1.75±0.20^{bc}	1.73±0.24^{bc}	1.62±0.07^{bc}	1.44±0.13^{c}	5.38	0.0080
CF[9]	0.95±0.01^{b}	0.99±0.03^{b}	1.05±0.03^{a}	1.05±0.01^{a}	1.04±0.02^{a}	1.03±0.03^{a}	9.85	0.0006
VSI[10]	3.29±0.04^{a}	3.16±0.04^{a}	2.97±0.07^{b}	2.96±0.12^{b}	2.90±0.09^{b}	2.85±0.09^{b}	13.91	0.0001
Survival[11]	97.3±2.04^{a}	96.0±0.00^{a}	97.3±4.07^{a}	97.3±2.04^{a}	98.7±0.77^{a}	98.7±2.04^{a}	0.42	0.8227

[1] Value are mean ± SD from triplicate groups of fish where the values in each row with different superscripts are significantly different (P<0.05).
[2] IBW : initial body weight.
[3] FBW : final body weight.
[4] Weight gain (%): (final weight - initial weight) × 100 / initial weight.
[5] Specific growth rate (%/day): ($\log_e$ final weight – $\log_e$ initial weight) / days.
[6] Feed efficiency (%): (wet weight gain / dry feed intake) × 100.
[7] Protein efficiency ratio: (wet weight gain / protein intake).
[8] Hepatosomatic index (%) = liver weight × 100 / body weight.
[9] Condition factor: (fish weight / fish length^3) × 100.

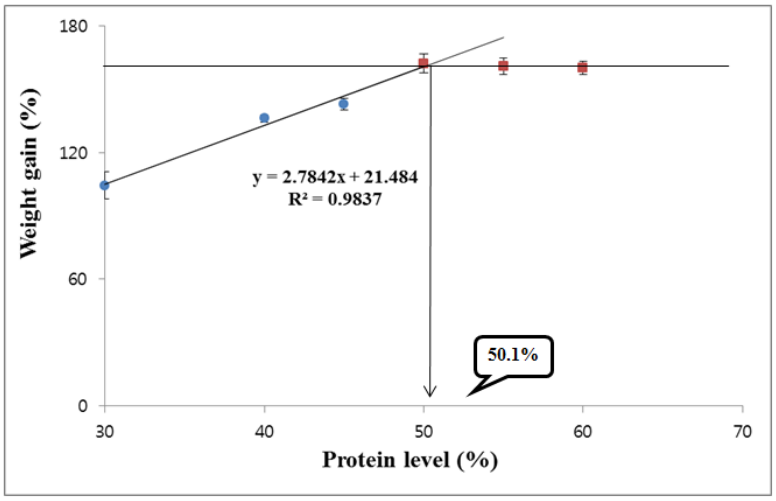

Figure 1. Broken line Second-order polynomial regression analysis of weight gain of olive flounder fed experimental diets at different protein levels.

reported that the family selection of fish increased growth rate which reduced the production cycle. Ogata *et al*. (2002) reported that selected strain of Japanese flounder fish required high protein level approximately 60% in the diet. In the present study, the protein requirement in the selected strain of olive flounder was determined higher than juvenile olive flounder probably due to selective breeding program. In most experiments, the WG is frequently used to determine nutrient requirements including protein. Numerous studies showed the protein requirement of fish based on WG using the broken-line analysis model and second order polynomial regression analysis (Anderson *et al*. 1981; Moore *et al*. 1988; Santiago and Reyes 1991). The broken line model corresponds to the minimum required nutrient level that will produce the maximum response. While, the polynomial regression analysis indicates the maximum response when the growth response is curvilinear (Tacon and Cowey 1985; Shearer 2000).

In this study, protein efficiency ratio (PER) tended to decrease with increase of dietary protein levels may be due to the excessive protein in the diet that was surpassed but used for the energy

(Dabrowski 1977; Santinha *et al*. 1996). Survival was not affected by the experimental diets. HSI decreased with increasing dietary protein levels and CF increased with increasing dietary protein levels (F = 5.38; p = 0.0080 and F = 9.85; p = 0.0006) which are consistent with Kim *et al*. (2016).

The whole-body and dorsal muscle proximate compositions are shown Table 3. Whole-body and dorsal muscle proximate compositions in terms of crude protein, crude lipid and crude ash was not affected significantly by protein levels. In the present study, the dorsal muscle moisture composition of olive flounder decreased with the increasing dietary protein levels (F = 9.81; p = 0.0009). Likewise, Lee *et al*. (2002) demonstrated that moisture of fish dorsal muscle was inversely correlated to the dietary protein levels in japanese flounder. In addition, proximate composition of fish showed a similar trend with Khan *et al*. (1993).

Biochemical parameters have important roles for ascertain the health and physiological conditions of fish (Blaxhall 1972). Biochemical parameters of olive flounder fed different experimental diets including various protein levels are given in Table 4. Plasma ALT and TG contents of fish fed CP55 and CP60 diets were significantly higher than those of fish fed CP30 diet (F = 25.77; P= < .0001 and F=17.46; P= < .0001). However, there were no significant differences among fish fed CP30 to CP50 diets; whereas, fish fed CP30 diet had the highest plasma ALP, TCHO and TP levels. However, the ALP, TCHO and TP contents were gradually decreased with the increase of protein levels in the diets. BUN, Glucose and AST activities were not significantly changed by dietary protein levels indicating that the dietary treatment groups had no adverse effect on fish health status.

Lysozyme activity has an important role in protecting the host from the attack of bacteria in terms of non- specific immune response in fish (Ellis 1990). Lysozyme activity of fish fed CP30 to CP60 diets for 8 weeks was showed in Table 4. The results showed

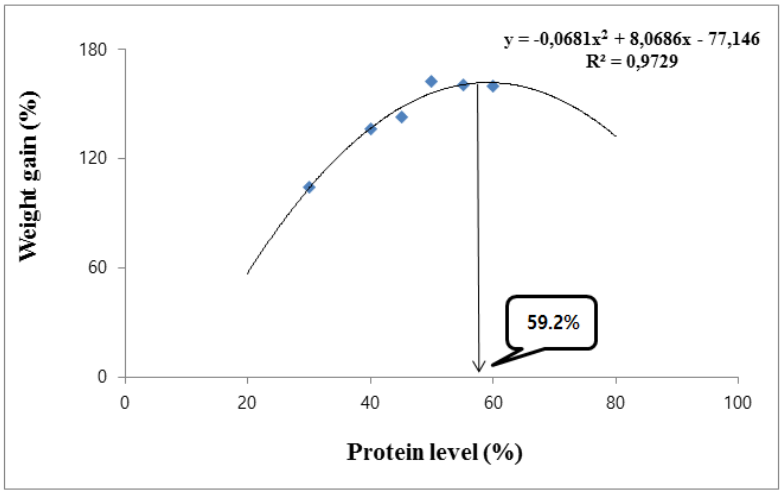

Figure 2. Second-order polynomial regression analysis of weight gain of olive flounder fed experimental diets at different protein levels.

Table 3. Whole-body and dorsal muscle proximate composition (%) of family selected olive flounder fed experimental diets[1]

	Experimental diets							
	CP30	CP40	CP45	CP50	CP55	CP60	F-value	p-value
Dorsal muscle								
Moisture	75.8±0.34^{a}	75.3±0.29^{ab}	75.4±0.10^{ab}	75.0±0.16^{b}	74.7±0.30^{b}	74.6±0.35^{b}	9.81	0.0009
Crude protein	21.9±0.39^{a}	22.6±0.82^{a}	22.8±0.33^{a}	22.9±0.62^{a}	22.8±0.23^{a}	22.8±0.90^{a}	1.29	0.3316
Crude lipid	0.45±0.06^{a}	0.41±0.08^{a}	0.47±0.08^{a}	0.36±0.08^{a}	0.46±0.06^{a}	0.34±0.05^{a}	1.80	0.1881
Crude ash	1.97±0.10^{a}	1.82±0.66^{a}	1.46±0.36^{a}	1.86±0.76^{a}	1.98±0.30^{a}	2.01±0.40^{a}	0.52	0.7534
Whole-body								
Moisture	71.9±0.55^{a}	70.8±0.67^{a}	71.8±1.24^{a}	71.2±0.49^{a}	71.1±0.50^{a}	71.8±1.17^{a}	0.98	0.4660
Crude protein	17.5±0.37^{a}	17.7±0.22^{a}	17.9±0.37^{a}	17.9±0.33^{a}	18.0±0.39^{a}	17.9±0.13^{a}	1.02	0.4457
Crude lipid	5.18±0.76^{a}	5.05±0.15^{a}	5.12±0.22^{a}	5.28±0.21^{a}	4.84±0.71^{a}	4.10±0.37^{a}	2.54	0.0860
Crude ash	3.57±0.35^{a}	3.45±0.11^{a}	3.55±0.29^{a}	3.69±0.29^{a}	3.48±0.48^{a}	3.36±0.22^{a}	0.40	0.8388

[1] Values are mean ± SD from triplicate groups of fish where the means in each row with different superscript letters are significantly different (P<0.05).

Table 4. Blood parameters of family selected olive flounder fed experimental diets for 8 weeks[1]

	Experimental diets							
	CP30	CP40	CP45	CP50	CP55	CP60	F-value	p-value
AST[2] (U L^{-1})	12.7±1.53^{a}	14.0±1.00^{a}	14.3±2.52^{a}	14.7±1.53^{a}	15.0±2.00^{a}	15.7±2.52^{a}	0.84	0.5472
ALT[3] (U L^{-1})	1.33±0.58^{b}	1.33±0.58^{b}	2.67±0.58^{b}	2.33±0.58^{b}	4.67±0.58^{a}	5.33±0.58^{a}	25.77	< .0001
TCHO[4] (mg dL^{-1})	292±4.73^{a}	277±5.03^{ab}	233±10.4^{bc}	236±31.6^{bc}	227±13.7^{c}	211 ±18.8^{c}	10.51	0.0005
Glucose (mg dL^{-1})	3.33±0.58^{a}	4.00±1.00^{a}	5.33±0.58^{a}	5.00±1.00^{a}	5.33±0.58^{a}	6.33±2.25^{a}	2.20	0.1222
ALP[5] (U L^{-1})	284±5.13^{a}	265±7.02^{ab}	266±13.2^{ab}	261±6.24^{b}	263±7.57^{ab}	249±4.16^{b}	6.08	0.0050
TP[6] (g dL^{-1})	4.67±0.25^{a}	4.70±0.56^{ab}	3.87±0.81^{ab}	3.23±0.81^{b}	3.37±0.21^{ab}	2.87±0.15^{b}	6.03	0.0051
TG[7] (mg dL^{-1})	194±4.73^{c}	205±6.11^{bc}	208±9.17^{bc}	215±4.16^{ab}	226±2.31^{a}	228±2.52^{a}	17.46	< .0001
BUN[8] (mg dL^{-1})	3.47±0.35^{a}	3.10±0.17^{a}	4.00±0.96^{a}	4.17±0.86^{a}	3.33±0.12^{a}	4.47±0.75^{a}	2.14	0.1307
Non-specific immune response								
Lysozyme (U ml^{-1})	72.2±11.9^{a}	75.8±7.34^{a}	60.5±7.76^{a}	68.1±3.50^{a}	65.8±18.6^{a}	50.2±3.79^{a}	2.38	0.1016

[1] Values are mean ± SD from triplicate groups of fish where the means in each row with different superscript letters are significantly different (P<0.05).
[2] AST: aspartate aminotransferase
[3] ALT: alanine aminotransferase
[4] TCHO: total cholesterol
[5] ALP: alkaline phosphatase
[6] TP: total protein
[7] TG: triglycerides
[8] BUN: blood urea nitrogen

that lysozyme activity varied from 50 to 76 (U ml^{-1}) and no significant differences (F = 2.38; p = 0.1016) were observed among dietary treatments (P>0.05) which suggest that fish immunity was in intact condition. Moreover, we did not find any abnormalities in fish during the experimental period which might be reflected in survivability data of fish.

In conclusion, based on the broken-line analysis and second-order polynomial regression analysis of the WG the results of the present study revealed that the optimum dietary protein level of a selectively bred (F-5 generation) strain of olive flounder could be greater than 50.1% but less than 59.2% of diet.

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