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Original Research Article

A preliminary study of dietary protein requirement of juvenile marbled flounder (*Pseudopleuronectes yokohamae*)

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ABSTRACT

An 8-week feeding trial was conducted to determine the optimal dietary protein level for juvenile marbled flounder. Five semi-purified test diets were formulated to contain different protein levels (CP) including 42.7%, 47.4%, 53.3%, 58.8%, and 64.5% (dry matter), named as CP42.7, CP47.4, CP53.3, CP58.8, and CP64.5, respectively. Five hundred and twenty-five juveniles (6.0 ± 0.1 g) were randomly distributed into 15 tanks (300 L tanks), resulting in 35 fish per tank ($n = 3$ tanks). Fish were fed the test diets 5 times per day until satiation. The CP58.8 resulted in the highest gain in weight and the best efficiency in feed utilization among the tested protein levels ($P < 0.05$). Fish fed the CP58.8 diet showed significantly higher whole-body protein and lipid contents than the fish that were fed the other diets ($P < 0.05$). Fish fed the CP53.3, CP58.8, and CP64.5 diets showed a significantly higher dorsal-muscle lipid content than the fish that were fed the CP42.7 and CP47.4 diets ($P < 0.05$). The one-slope straight broken-line regression analysis on the results of the thermal growth coefficient and feed conversion ratio indicated that the estimated optimum dietary protein level was 58.8%. Taken together, it is suggested that the dietary protein level of 58.8% is optimal for better growth and high efficiency in feed utilization for the juvenile marbled flounder.

1. Introduction

Marbled flounder, *Pseudopleuronectes yokohamae* belonging to the order *Pleuronectiformes* and family *Pleuronectidae* inhabit all the coasts of Korea, southern Hokkaido in Japan, and East China Sea (Chyung, 1986; Kim and Youn, 1994; Ji et al., 2016). Due to the high market value which is attributed to their good taste, the marbled flounder is a commercially important species in both Korea and Japan. The capture production for this species solely in Korea has not yet been reported, but the production for righteye flounders in general was recorded as 12,291 metric tons in 2018 (KOSTAT, 2019). The capture production of the flounders reported in Japan was 41,400 metric tons in 2018 (MAFF, 2019). An official record for aquaculture production of this species has not yet been reported.

Aquaculture is the best option for providing sustainable seafood (Baluyut, 1989). There are various factors determining the success of aquaculture, including water quality, environmental aspects, genetics, reproduction and life cycles, nutrition, food and feeding, disease, post-harvest technology and processing, and economics and marketing (Lucas and Southgate, 2012). To date, most research conducted on this species is related to ecology, including the development of fertilized eggs, larvae, and juveniles (Yusa, 1960; Han et al., 2001), reproduction and population dynamics (Lee et al.,

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1985; Kang et al., 1985; Kume et al., 2006; Seo et al., 2010), feeding habits (Huh et al., 2012), age, and growth (Solomon et al., 1987; Kim et al., 1991, 2015; Lee et al., 2009; Joo and Gwak, 2014). Among the factors for successful aquaculture, foods are critical because they generally constitute half of the total production costs. Thus, it is necessary to develop cost-effective feeds that maximize efficiency in growth and feed utilization. Further, a highly desirable goal is to determine the optimal nutrient requirements for a targeted fish. Though it has been reported that culture of the marbled flounder is established in Japan (Tucker, 2012), almost no information on nutrient requirements of this species is available.

Proteins are critical molecules in fish tissue, constituting 65% to 75% of the total on a dry-weight basis because they play an important role in the structure and metabolism of fish (Wilson, 2002; NRC, 2011). Due to its importance, numerous protein requirement studies have been conducted on cultured fish: e.g., channel catfish (*Ictalurus punctatus*, Garling and Wilson, 1976), chinook salmon (*Oncorhynchus tshawytscha*, DeLong et al., 1958), common carp (*Cyprinus carpio*, Ogino and Saito, 1970), Japanese eel (*Anguilla japonica*, Nose and Arai, 1972), gilthead bream (*Chrysophrys aurata*, Sabaut and Luquet, 1973), yellowtail (*Seriola quinqueradiata*, Takeda et al., 1975), Japanese seabass (*Lateolabrax japonicus*, Ai et al., 2004), starry flounder (*Platichthys stellatus*, Lee et al., 2006; Wang et al., 2017), tiger puffer (*Takifugu rubripes*, Kim and Lee, 2009), mangrove red snapper (*Lutjanus argentimaculatus*, Abbas and Siddiqui, 2013), turbot (*Scophthalmus maximu*, Liu et al., 2015), red spotted grouper (*Epinephelus akaara*, Wang et al., 2016), and zebra sea bream (*Diplodus cervinus*, Coutinho et al., 2016). These studies demonstrated that protein requirements vary among the fish, ranging from 32% to 57%. Therefore, an adequate protein composition in feed is required for maintaining optimal fish growth. Moreover, it is also known that feeds with excessive protein levels could not only increase feed costs but also nitrogen loss (Ullah-Khan et al., 2019; Teles et al., 2020).

Therefore, we conducted a feeding trial to evaluate the effects of graded protein levels on growth performance and biochemical compositions of the juvenile marbled flounder. To our knowledge, the current study may be the first one that tests the various nutrient levels in this species.

2. Materials and methods

All animal care and standard operating procedures in the present study were approved by the Institutional Animal Care and Use Committee of National Institute of Fisheries Science, Korea and conducted in accordance with the Guidelines for Experimental Animals (2018-NIFS-IACUC-09).

2.1. Experimental diets

Five semi-purified test diets were formulated to contain 5 different crude protein (CP) levels, including 42.7% (CP42.7), 47.4% (CP47.4), 53.3% (CP53.3), 58.8% (CP58.8), and 64.5% (CP64.5) as a dry-matter basis. Chilean anchovy meal and vitamin-free casein were used as ingredients for the main protein source. The gross energy of the test diets was designed to be isocaloric as ca. 20.0 kJ/g. The diet formulation and analyzed nutrient contents of the test diets are provided in Table 1, and amino acid content of the test diets are presented in Table 2. Water was added (the amount was 60% of the total volume of the diet) to the mixed ingredient, which was kneaded by a kneading machine (HYVM-1214; Hanyoung Food Machinery, Hanam, Korea) for 1 h. The dough was then pelleted through a meat chopper machine (SMC-32; SL Co., Incheon, Korea). The size of the pellet (ca. Ø 2 mm) was adjusted using sieves. The diets (sinking pellets) were air dried in an oven at 60 °C for 2 h, then were stored in a freezer at −30 °C until use.

Table 1
Formulation and proximate composition of the experimental diets (g/kg dry-matter).

Item	Treatment[1]				
	CP42.7	CP47.4	CP53.3	CP58.8	CP64.5
Ingredients					
Chilean anchovy meal[2]	370	420	465	515	560
Casein, vitamin free[3]	170	192	215	235	260
Fish oil[4]	43	39	35	30	26
Tapioca starch[5]	237	179	125	70	14
Wheat flour[6]	100	100	100	100	100
Mineral mixture[7]	10	10	10	10	10
Vitamin mixture[8]	10	10	10	10	10
Calcium phosphate dibasic[9]	5	5	5	5	5
Choline chloride (50%)[10]	5	5	5	5	5
Carboxylmethyl cellulose[11]	10	10	10	10	10
α-Cellulose[12]	40	30	20	10	0
Proximate composition[13]					
Moisture	9	21	14	17	30
Crude protein	427	474	533	588	645
Crude lipid	80	81	80	78	79
Crude ash	76	84	91	99	107
Gross energy, MJ/kg	19.8	19.7	20.0	20.2	20.2

[1] Five different crude protein (CP) levels, including 42.7% (CP42.7), 47.4% (CP47.4), 53.3% (CP53.3), 58.8% (CP58.8), and 64.5% (CP64.5) as dry-matter basis.
[2] Chilean anchovy meal (CP: 66.1%, CL: 9.1%), Blumar Seafoods, Santiago, Chile.
[3] Casein, vitamin free (CP: 83.1%, CL: 0.1%), Sigma-aldrich Co., St Louis MO, USA.
[4] Ewha fats and oils industry, Busan, Rep. of Korea.
[5] Sonish starch technology Co., Ltd., Chachoengsao, Thailand.
[6] Kyongwon feed, Co., Ltd., Dangjin, Rep. of Korea (CP: 13.3%, CL: 2.9%).
[7] Mineral premix contained the following ingredients (g/kg premix): NaCl, 43.3; $MgSO_4 \cdot 7H_2O$, 136.5; $NaH_2PO_4 \cdot 2H_2O$, 86.9; KH_2PO_4, 239; $CaHPO_4$, 135.3; Ferric citrate, 29.6; $ZnSO_4 \cdot 7H_2O$, 21.9; Ca-lactate, 304; $CuCl_2$, 0.2; $AlCl_3 \cdot 6H_2O$, 0.15; KI, 0.15; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0.
[8] Vitamin premix contained the following amount which were diluted in cellulose (g/kg premix): 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-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003.
[9] Daejung chemicals & metals Co., Ltd., Siheung, Rep. of Korea.
[10] Solton Biochem, Corp, Cheonan, Rep. of Korea.
[11] Sigma-aldrich Co., St Louis MO, USA.
[12] Sigma-aldrich Co., St Louis MO, USA.
[13] Values of proximate composition are presented as means of duplication.

2.2. Experimental fish and feeding trial

Three thousand juveniles produced in the Fisheries Resources Institute, Gyeongsangbuk-do located near our research facility (about 45 km) were stocked in 8,000-L polyethylene circular tanks (3.5-m diameter and 0.8-m height) and were raised by feeding a commercial diet (Otohime number B1, crude protein 51%, crude lipid 11%, crude ash 15%, pellet size: 250 to 360 μm; Otohime number B2, crude protein 51%, crude lipid 11%, crude ash 15%, pellet size: 360 to 650 μm; Otohime number C1, crude protein 51%, crude lipid 11%, crude ash 15%, pellet size: 580 to 840 μm; Marubeni Nisshin Feed Co, Ltd., Tokyo, Japan) until reaching a targeted size. Prior to the start of the feeding trial, fish were fed a pelleted diet (crude protein 51% and crude lipid 11%) prepared using the same ingredients of the test diet for 2 weeks of acclimation. Five hundred and twenty-five juveniles (6.0 ± 0.1 g; mean ± standard deviation) were randomly distributed into 15 tanks (300-L polyethylene circular tank; 0.8-m diameter and 0.5 m height) resulting in 35 fish per tank ($n = 3$ tanks). Fish were hand-fed the test diet 5 times a day (09:00, 11:00, 13:00, 15:00, and 17:00) to apparent satiation for 8 weeks. Water temperature, pH, and dissolved oxygen levels were maintained at 17.8 ± 2.0 °C, 8.1 ± 0.2, and 9.0 ± 3.6 mg/L, respectively, throughout the trial.

Table 2
Constitutional amino acid content of the experimental diets (g/100 g, dry matter basis).

Item	Treatment[1]				
	CP42.7	CP47.4	CP53.3	CP58.8	CP64.5
Non-essential amino acids[2]					
Alanine	2.1 (5.0)	2.3 (4.9)	2.7 (5.1)	2.9 (5.0)	3.1 (4.8)
Aspartic acid	3.8 (8.9)	4.3 (9.0)	4.7 (8.8)	5.1 (8.7)	5.6 (8.7)
Cysteine	0.3 (0.8)	0.3 (0.7)	0.4 (0.8)	0.4 (0.7)	0.4 (0.7)
Glutamic acid	8.0 (18.6)	9.0 (19.1)	9.6 (17.9)	10.6 (18.0)	11.5 (17.8)
Glycine	1.9 (4.4)	2.1 (4.3)	2.4 (4.5)	2.6 (4.5)	2.8 (4.4)
Proline	3.0 (6.9)	3.4 (7.1)	3.6 (6.7)	3.9 (6.7)	4.1 (6.4)
Serine	2.1 (5.0)	2.3 (4.9)	2.5 (4.6)	2.8 (4.7)	3.0 (4.7)
Tyrosine	1.5 (3.6)	1.6 (3.4)	2.2 (4.2)	2.3 (3.9)	2.3 (3.6)
Total	22.7 (53.1)	25.3 (53.5)	28.0 (52.6)	30.7 (52.2)	32.9 (51.0)
Essential amino acids[2]					
Arginine	1.9 (4.4)	2.1 (4.4)	2.5 (4.7)	2.8 (4.7)	3.0 (4.7)
Histidine	1.1 (2.5)	1.2 (2.6)	1.4 (2.6)	1.6 (2.7)	1.7 (2.7)
Isoleucine	1.9 (4.5)	2.2 (4.7)	2.5 (4.7)	2.8 (4.8)	2.9 (4.5)
Leucine	3.6 (8.5)	4.0 (8.5)	4.5 (8.4)	4.9 (8.4)	5.2 (8.1)
Lysine	3.3 (7.8)	3.9 (8.2)	4.2 (7.8)	4.6 (7.8)	5.0 (7.7)
Methionine	0.8 (2.0)	1.1 (2.4)	1.5 (2.7)	1.6 (2.6)	1.6 (2.5)
Phenylalanine	2.0 (4.8)	2.2 (4.7)	2.5 (4.7)	2.7 (4.5)	2.9 (4.4)
Threonine	1.9 (4.4)	2.1 (4.4)	2.3 (4.3)	2.5 (4.2)	2.7 (4.3)
Valine	2.4 (5.7)	2.7 (5.6)	3.1 (5.9)	3.4 (5.8)	3.5 (5.4)
Total	19.0 (44.4)	21.5 (45.4)	24.5 (45.9)	26.8 (45.6)	28.6 (44.3)
Total (non-essential + essential)	41.7 (97.6)	46.9 (98.8)	52.5 (98.4)	57.5 (97.7)	61.5 (95.3)

[1] Five different crude protein (CP) levels, including 42.7% (CP42.7), 47.4% (CP47.4), 53.3% (CP53.3), 58.8% (CP58.8), and 64.5% (CP64.5) as dry-matter basis.
[2] The values in parentheses are expressed as grams per 100 g of protein.

2.3. Measurement

At the end of the feeding trial, all fish stocked in each tank were weighed to calculate indices of growth performance such as weight gain (WG) and thermal growth coefficient (TGC) as well as indices of feed utilization efficiency, including daily feed intake (DFI), feed conversion ratio (FCR), protein efficiency ratio (PER), protein retention efficiency (PRE), and energy retention efficiency (ERE). Twenty fish were euthanized with an overdose of 2-phenoxyethanol (150 parts per million; Sigma–Aldrich, St. Louis, MO, USA) to measure individual weight and the total length for calculating condition factor. Then, the fish were dissected to measure the weight of viscera, liver, and digestive tract from esophagus to rectum for calculating viscerosomatic index (VSI), hepatosomatic index (HSI), and digestive tract index (DTI). The length of the digestive tract was also measured to calculate the relative length of gut (RLG). These were measured as a biological index. Equations of each measurement are as follows:

$$WG\ (\%) = \{[\text{Final wet weight (g)} - \text{Initial wet weight (g)}]/\text{Initial wet weight (g)}\} \times 100;$$

$$TGC = [\text{Final wet weight (g)}^{1/3} - \text{Initial wet weight (g)}^{1/3}] \times (\text{Sum of days} \times \text{Average temperature } (^{\circ}\text{C}))^{-1} \times 1{,}000;$$

$$\text{Survival } (\%) = \text{Number of fish at harvest/Number of fish stocked} \times 100;$$

$$DFI\ (\%) = \text{Feed consumption (g)}/\{[\text{Initial wet weight (g)} + \text{Final wet weight (g)}]/2 \times \text{Days}\} \times 100;$$

$$FCR = \text{Dry feed intake (g)/Wet weight gain (g)};$$

$$PER = \text{Wet weight gain (g)/ Protein intake (g)};$$

$$PRE\ (\%) = (\text{Final wet weight} \times \text{Protein content in final fish body} - \text{Initial wet weight} \times \text{Protein content in initial fish body})/(\text{Dry feed intake} \times \text{Protein content in diet}) \times 100;$$

$$ERE\ (\%) = (\text{Final wet weight} \times \text{Energy content in final fish body} - \text{Initial wet weight} \times \text{Energy content in initial fish body})/(\text{Dry feed intake} \times \text{Energy content in diet}) \times 100;$$

$$CF = [\text{Wet weight (g)}/\text{Total length}^3\ (\text{cm})] \times 100;$$

$$VSI\ (\%) = [\text{Wet weight of viscera (g)}/\text{Wet weight (g)}] \times 100;$$

$$HSI\ (\%) = [\text{Wet weight of liver (g)}/\text{Wet weight (g)}] \times 100;$$

$$DTI\ (\%) = [\text{Wet weight of digestive tract (g)}/\text{Wet weight (g)}] \times 100;$$

$$RLG = \text{Digestive tract length (cm)/ Total length (cm)}.$$

Five fish from each tank were euthanized with the overdose of 2-phenoxyethanol (150 parts per million; Sigma–Aldrich Co., Ltd., St. Louis, MO, USA) and pooled for analysis of whole-body proximate composition analysis. Another 5 fish from each tank were anesthetized with 2-phenoxyethanol (50 parts per million; Sigma–Aldrich Co., Ltd., St. Louis, MO, USA) for collecting blood by puncturing the caudal vein using a heparinized syringe. The collected blood was chilled in ice for 3 min, then centrifuged at 5,000 rpm (12,225 × *g*; CF-10; Daihan Scientific Co., Ltd, Daegu, Korea) to separate out plasma. The plasma samples were kept at −80 °C for later analysis of growth hormone (GH), insulin-like growth factor-I (IGF-I), and hepatocyte growth factor (HGF). The 5 fish were subjected to sample collection for analysis of nutrient contents in dorsal muscle, viscera (without liver, spleen, and gall balder), and liver.

Plasma GH, IGF-I, and HGF levels were measured through enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Cat. #E12121Fh for GH, E12122Fh for IGF-I, and E16527Fh for HGF, CUSABIO, Wuhan, China). The assay procedure followed the manufacturer's protocol. The nutrient contents, including moisture, crude protein, crude lipid, and crude ash were analyzed through standard methods (AOAC, 1997). In brief, moisture was determined by drying a sample in an oven (OF–W155, Daihan Scientific Co., Ltd, Daegu, Korea) at 135 °C for 2 h. Crude protein

was measured by the Kjeldahl method (Gerhardt VAP 50 OT/TT125, KG, Germany). Nitrogen conversion factor of 6.25 was used to convert the amount of nitrogen detected into protein content. Crude lipid was determined by the extraction of the lipid with ethyl ether (Soxtec, 2043; Foss, Hillerød, Denmark). Crude ash was determined in a muffle furnace (FHPX-14, Daihan Scientific Co., Ltd, Daegu, Korea) at 600 °C for 6 h. All biochemical analyses were performed in triplicate.

2.4. Regression analysis

A one-slope straight broken-line model and a second-order polynomial model, which are widely accepted by fish nutritionists to determine an optimal nutrient requirement (Zeitoun et al., 1976; Robbins et al., 1979), were tested to determine the optimal protein level for the juvenile marbled flounder. Results of TGC and FCR obtained from conducting the current trial were subjected to each model analysis using a nlsLM (modified nonlinear least-squares by the Levenberg–Marquardt algorithm; Elzhov et al., 2016) function for the one-slope broken-line model and a lm (linear model) function for the second-order polynomial model, which both are provided in the standard library of R (R Core Team, 2015). Model descriptions and R codes relevant to the tested model can be found in Lee et al. (2014). As the calculated coefficients of the second-order polynomial model tested for each of the results were not statistically different from zero, outcomes of the one-slope straight broken-line model analysis were presented.

2.5. Statistical analysis

Results were analyzed using IBM SPSS 19 software package for Windows (SPSS Inc., Chicago, IL, USA). Data were evaluated for assumptions, including normality and homogeneity of variance, using the Shapiro–Wilk and Levene's tests, respectively, and no violation was detected ($P > 0.05$). Statistical analyses of data were conducted using ANOVA with a 95% significance level ($P < 0.05$). When a significant treatment effect was detected, the Tukey's HSD test was used to assess significant differences among means.

3. Results

3.1. Growth performance and feed utilization efficiency

Growth performance and feed utilization efficiency were significantly influenced ($P < 0.05$) by the protein levels ranging from 42.7% to 64.5% tested in the current study on the juvenile marbled flounder (Table 3). Final body weight, WG, and TGC significantly increased by increasing protein levels up to 58.8% (i.e., CP58.8), then the value of these measurements was relatively reduced when the marbled flounder were fed the CP64.5 diet in comparison to those fed the CP58.8 diet. There was no significant effect of the different protein levels on the survival rate of the marbled flounder ($P > 0.05$). The pattern of changes in the efficiency of feed utilization followed the pattern exhibited by the indices of growth performance. Feed conversion ratio significantly decreased when protein levels were increased up to 58.8%. The value of the FCR in the marbled flounder fed the CP64.5 diet was relatively elevated in comparison to those fed the CP58.8 diet ($P < 0.05$). The marbled flounder fed the CP58.8 diet showed the highest value of PER, PRE, and ERE. Biological indices, including CF, VSI, HSI, DTI, and RLG were not significantly influenced by the protein levels ($P > 0.05$).

3.2. Chemical composition

The nutrient composition of whole body and dorsal muscle of the marbled flounder was significantly affected by the various protein level (Table 4) ($P < 0.05$). Whole-body moisture content of the marbled flounder fed the CP58.8 and CP64.5 diets was significantly lower than that of fish fed the other diets ($P < 0.05$). Whole-body crude protein and lipid contents of the marbled flounder fed the CP58.8 and CP64.5 diets were significantly

Table 3
Growth performance, feed utilization efficiency and biological indices of juvenile marbled flounder fed diets containing graded protein levels.

Item	Treatment[1]					*P*-value
	CP42.7	CP47.4	CP53.3	CP58.8	CP64.5	
Growth performance						
IBW, g	6.1 ± 0.5	6.0 ± 0.3	6.0 ± 0.2	5.9 ± 0.6	5.9 ± 0.3	0.99
FBW, g	16.2 ± 1.0^{c}	18.4 ± 0.8^{bc}	19.6 ± 1.0^{b}	23.5 ± 0.6^{a}	20.2 ± 0.8^{b}	0.002
WG, %	167.9 ± 15.7^{b}	209.7 ± 6.7^{b}	228.9 ± 25.4^{ab}	305.9 ± 32.2^{a}	243.5 ± 30.0^{ab}	0.025
TGC	0.72 ± 0.04^{c}	0.84 ± 0.02^{c}	0.90 ± 0.07^{ab}	1.08 ± 0.05^{a}	0.93 ± 0.06^{ab}	0.006
Survival, %	88.2 ± 7.5	86.3 ± 10.9	94.0 ± 4.7	97.1 ± 2.9	97.0 ± 1.7	0.67
Feed utilization efficiency						
DFI, %	2.73 ± 0.05	2.62 ± 0.28	2.62 ± 0.06	2.05 ± 0.18	2.24 ± 0.08	0.05
FCR	1.92 ± 0.18^{c}	1.59 ± 0.08^{bc}	1.49 ± 0.12^{b}	0.98 ± 0.07^{a}	1.22 ± 0.11^{ab}	0.002
PER	1.39 ± 0.09^{b}	1.44 ± 0.10^{b}	1.36 ± 0.08^{b}	1.80 ± 0.11^{a}	1.35 ± 0.10^{b}	0.045
PRE, %	21.3 ± 1.2^{b}	23.6 ± 2.7^{b}	22.0 ± 0.9^{b}	33.6 ± 1.4^{a}	22.5 ± 1.1^{b}	<0.001
ERE, %	40.7 ± 2.9^{c}	53.6 ± 7.2^{bc}	52.8 ± 2.0^{bc}	89.3 ± 7.2^{a}	68.5 ± 4.5^{ab}	<0.001
Biological indices						
CF	1.51 ± 0.03	1.50 ± 0.03	1.61 ± 0.09	1.59 ± 0.06	1.55 ± 0.04	0.58
VSI, %	4.58 ± 0.02	4.46 ± 0.15	4.76 ± 0.17	4.84 ± 0.18	4.41 ± 0.18	0.27
HSI, %	1.62 ± 0.04	1.51 ± 0.01	1.63 ± 0.10	1.67 ± 0.05	1.51 ± 0.07	0.31
DTI, %	2.78 ± 0.04	2.77 ± 0.10	3.00 ± 0.04	3.04 ± 0.13	2.73 ± 0.12	0.13
RLG	1.33 ± 0.01	1.33 ± 0.03	1.37 ± 0.06	1.35 ± 0.03	1.27 ± 0.02	0.34

IBW = initial body weight; FBW = final body weight; WG = weight gain; TGC = thermal growth coefficient; DFI = daily feed intake; FCR = feed conversion ratio; PER = protein efficiency ratio; PRE = protein retention efficiency; ERE = energy retention efficiency; CF = condition factor; VSI = viscerosomatic index; HSI = hepatosomatic index; DTI = digestive tract index; RLG = relative length of the gut.

[a, b, c] Values (means ± standard errors of triplication) in a same row with different superscript letters are significantly different (Tukey's test, $P < 0.05$), and with no superscript letter indicates no sigificant difference ($P \geq 0.05$).

[1] Five different crude protein (CP) levels, including 42.7% (CP42.7), 47.4% (CP47.4), 53.3% (CP53.3), 58.8% (CP58.8), and 64.5% (CP64.5) as dry-matter basis.

Table 4
Whole-body, dorsal muscle, viscera and liver composition (g/kg, wet-matter basis) of juvenile marbled flounder fed diets containing graded protein levels.

Item	Treatment[1]					*P*-value
	CP42.7	CP47.4	CP53.3	CP58.8	CP64.5	
Whole body, g/kg						
Moisture	765.1 ± 1.7^{a}	760.7 ± 3.5^{a}	758.0 ± 4.8^{a}	742.0 ± 1.8^{b}	743.6 ± 3.3^{b}	<0.001
Crude protein	162.1 ± 1.9^{b}	161.0 ± 3.2^{b}	166.0 ± 4.7^{b}	182.3 ± 4.0^{a}	168.4 ± 1.6^{b}	0.007
Crude lipid	31.2 ± 3.3^{b}	37.7 ± 1.1^{b}	36.3 ± 2.6^{b}	45.4 ± 2.0^{a}	37.0 ± 2.3^{b}	0.024
Crude ash	33.2 ± 1.1	31.9 ± 1.1	33.4 ± 1.0	29.8 ± 1.2	31.4 ± 1.4	0.26
Dorsal muscle, g/kg						
Moisture	777.3 ± 1.1	776.7 ± 1.0	775.7 ± 3.6	775.6 ± 2.8	771.3 ± 2.2	0.42
Crude protein	201.7 ± 0.4	202.3 ± 1.2	201.9 ± 1.4	200.8 ± 3.2	209.0 ± 1.2	0.05
Crude lipid	1.2 ± 0.1^{c}	2.5 ± 0.8^{b}	4.8 ± 0.2^{a}	4.2 ± 0.2^{a}	4.0 ± 0.1^{a}	<0.001
Crude ash	15.5 ± 0.2^{ab}	15.8 ± 0.2^{ab}	16.1 ± 0.2^{a}	15.6 ± 0.2^{ab}	14.9 ± 0.2^{b}	0.044
Viscera, g/kg						
Moisture	781.8 ± 1.2	788.5 ± 3.7	784.7 ± 1.0	788.3 ± 2.8	787.5 ± 2.2	0.36
Crude protein	163.4 ± 1.6	158.2 ± 0.8	158.0 ± 0.5	157.0 ± 4.5	153.6 ± 1.0	0.06
Crude lipid	19.4 ± 0.3	19.7 ± 0.5	17.0 ± 0.6	17.1 ± 0.8	19.5 ± 0.6	0.06
Crude ash	14.4 ± 0.1	14.4 ± 0.3	14.5 ± 0.1	14.3 ± 0.2	13.9 ± 0.2	0.30
Liver, g/kg						
Moisture	610.7 ± 4.6	627.9 ± 5.1	618.5 ± 2.4	620.0 ± 11.1	608.8 ± 5.5	0.29
Crude protein	98.3 ± 2.9	102.1 ± 2.2	104.5 ± 2.0	104.0 ± 1.9	101.2 ± 1.2	0.30
Crude lipid	174.9 ± 6.1	170.5 ± 6.5	177.0 ± 4.6	185.5 ± 1.48	183.0 ± 7.7	0.74
Crude ash	11.3 ± 0.4	11.8 ± 0.1	10.9 ± 0.2	11.4 ± 0.1	10.8 ± 0.2	0.06

[a, b, c] Values (means ± standard errors of triplication) in the same row with different superscript letters are significantly different (Tukey's test, $P < 0.05$), and with no letter superscript mean no significant difference ($P > 0.05$).
[1] Five different crude protein (CP) levels, including 42.7% (CP42.7), 47.4% (CP47.4), 53.3% (CP53.3), 58.8% (CP58.8), and 64.5% (CP64.5) as dry-matter basis.

Table 5
Plasma insulin-like growth factor I (IGF-I), hepatocyte growth factor (HGF), and growth hormone (GH) levels in juvenile marbled flounder fed diets containing graded protein levels (pg/mL).

Item	Treatment[1]					*P*-value
	CP42.7	CP47.4	CP53.3	CP58.8	CP64.5	
IGF-I	1,306 ± 139	1,506 ± 183	1,544 ± 132	1,558 ± 39	1,543 ± 138	0.20
HGF	1,255 ± 139	1,455 ± 183	1,513 ± 91	1,527 ± 70	1,570 ± 111	0.08
GH	2,773 ± 485	2,905 ± 427	2,949 ± 35	2,966 ± 358	2,936 ± 248	0.96

Values (means ± standard errors of triplication) in the same row with no letter superscript mean no significant difference (Tukey's test, $P > 0.05$).
[1] Five different protein (CP) levels, including 42.7% (CP42.7), 47.4% (CP47.4), 53.3% (CP53.3), 58.8% (CP58.8), and 64.5% (CP64.5) as dry-matter basis.

higher than those of fish fed the other diets ($P < 0.05$). Dorsal-muscle crude lipid content of the marbled flounder fed CP53.3, CP58.8, and CP64.5 diets was significantly higher than that of fish fed the other diets ($P < 0.05$). Viscera and liver nutrient contents were not significantly influenced by the protein level ($P > 0.05$).

3.3. Plasma growth hormone

There was no significant effect of the different protein levels on the plasma IGF-I, HGF, and GH levels in the marbled flounder (Table 5).

3.4. One-slope straight broken-line regression analysis

The one-slope straight broken-line regression analysis on the TGC and FCR results revealed that the estimated optimal protein level was almost the same as 58.8% (standard error = ±5.8%, $P < 0.05$, $R^2 = 0.5959$ for the TGC result; standard error = ±4.8%, $P < 0.01$, $R^2 = 0.7065$ for the FCR result) (Figs. 1 and 2).

4. Discussion

As there are few existing nutrient requirement studies on flounder, the comparison of the growth performance results from the current study with previous nutrient requirement studies on some flatfishes is given as follows: a previous study conducted on winter flounder (*Pleuronectes americanus*; initial body weight: 0.8 g) reported that those fed a diet containing protein and lipids levels of 50% and 10%, respectively, for 10 weeks resulted in a 445% WG (Hebb et al., 2003). Liu et al. (2015) reported that turbot (*S. maximu*; initial body weight: 38.2 g) fed a diet formulated at 63.6% protein for 8 weeks resulted in a WG of 140%. Starry flounder (*P. stellatus*; initial body weight: 29.8 g) exhibited a 226% WG when fed a diet containing protein and lipid levels of 45 and 14%, respectively, for 8 weeks (Wang et al., 2017). In the current study, the flounder (initial body weight: 6 g) fed a diet containing protein and lipid levels of 58.8 and 7.8%, respectively, for 8 weeks showed a 306% WG. These comparative results indicate that the current feeding trial using the test diets was appropriately conducted.

Protein requirements vary among fish species, ranging from 29% to 55% (Wilson, 2002). As carnivores are known to require higher dietary protein levels in comparison to herbivores (e.g., common

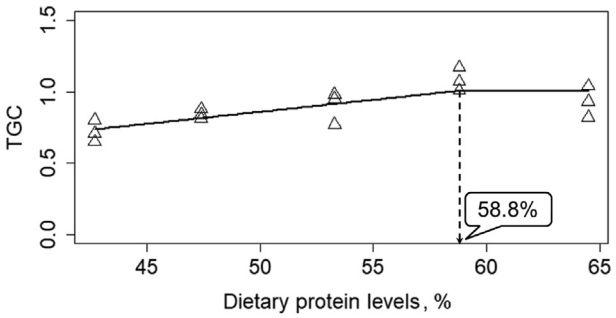

Fig. 1. A one-slope straight broken-line model fitted to the dataset of thermal growth coefficient (TGC) in juvenile marbled flounder in response to graded dietary protein levels. The value in the box indicates the estimated optimum protein level (58.8 ± 5.8)%. The model equation was: TGC = 1.0063 − 0.0168 (58.8 − x) (R^2 = 0.5959; P < 0.05) where (58.8 − x) is defined as zero when $x \geq$ 58.8. The x represents dietary protein levels (%).

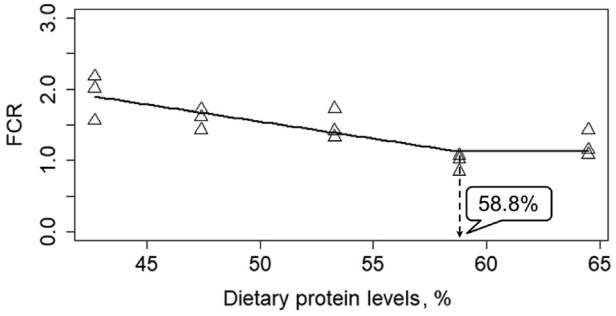

Fig. 2. A one-slope straight broken-line model fitted to the dataset of feed conversion ratio (FCR) in juvenile marbled flounder in response to graded dietary protein levels. The value in the box indicates the estimated optimum protein level (58.8 ± 4.8)%. The model equation was: FCR = 1.1234 + 0.0479 (58.8 − x) (R^2 = 0.7065; P < 0.01) where (58.8 − x) is defined as zero when $x \geq$ 58.8. The x represents dietary protein levels (%).

carp) and omnivores (e.g., salmonids) that possess better capability to utilize carbohydrate sources (i.e., protein sparing effect; Wilson and Halver, 1986; Wilson, 1994), it is reasonable to suggest that the flounder, a carnivore, may require a high level of protein such as 58.8%, resulting in the best growth performance. This is consistent with previous studies conducted on other flatfish, including turbot (protein requirement: 57% to 60%; Caceres-Martinez et al., 1984; Liu et al., 2015), sole (*Solea vulgaris*, 57% to 58%; Cadena-Roa, 1983), and plaice (*Pleuronectes platessa*, 57% to 70%; Cowey et al., 1970). In contrast to this, some studies conducted on other flatfish showed relatively lower protein requirements, showing that starry flounder (Lee et al., 2006; Wang et al., 2017) required as little as 45% to 50% protein, dependent on dietary lipid level. Further, Japanese flounder (*Paralichthys olivaceus*; Lee et al., 2002) showed the best growth in response to a dietary protein level of 45%. The reduction in growth (FBW, WG, and TGC) of the fish fed the diet at 64.5% protein compared with those fed the diet at 58.8% protein can be related to a reduction in available energy for growth as deamination and excretion of excessively absorbed amino acids may have required increased energy expenditure (Jauncey, 1982; Cho et al., 1985; Vergara et al., 1996). Insufficient energy sources like carbohydrates, resulting from high protein levels in fish diets, may negatively influence energy utilization by fishes. This is in line with a previous study reporting that plaice fed the 40%-protein diet containing the lipids and carbohydrates as an energy source gained more weight than those fed the 40%-protein diet containing the lipids only (Cowey et al., 1975). Pellet water stability and nutrient leaching in relation to the lower carbohydrate levels in the diet might have been associated the reduced growth. This property may be a crucial factor for grazing aquatic animals such as crustaceans. It is assumed, however, that the correlation between the water stability and growth performance was negligible in the current study because the pellet was mostly consumed by the flounder within a few minutes (i.e., instant feeders).

It is known that several factors affect protein requirements of fish, including dietary protein-to-energy balance, amino acid compositions and digestibility of proteins, amount of non-protein energy sources in test diets, fish size, and water temperature (Wilson and Halver, 1986; Wilson, 2002). Given the very limited information on nutrient requirement studies on flounder, it seems difficult to validate whether the estimated optimal protein level of 58.8% would have been an overestimation or not for the flounder in the given rearing conditions. Nonetheless, most of the ingredients used to make the test diets are conventionally accepted by fish nutritionists, and test diets were formulated to be isolipidic and isocaloric. Notably, the high-quality fish meal used in this study as the major protein source, originated from anchovy caught in Chile, indicating that its digestibility and palatability can be considered higher than other protein sources. In addition, the tapioca starch (also called cassava starch) used as the carbohydrate source has been shown to be fairly digestible (apparent digestibility of starch: 78% to 92%) in the studied fishes (Gominho-Rosa et al., 2015). Despite the fact that no information on amino acid requirements for the flounder is available, the essential amino acid profile was balanced among the test diets, and was generally comparable to requirements estimated for rainbow trout (NRC, 2011).

Indices of protein utilization, e.g., PERand PRE (i.e., net protein utilization), have been considered for determination of an optimal protein requirement, which is generally less than the requirement estimated by growth responses (Mambrini and Guillaume, 2001). This is, however, controversial among studies. Some studies conducted on hybrid tilapia (*Oreochromis niloticus* × *O. aureus*; Shiau and Huang, 1989), red spotted grouper (Wang et al., 2016), zebra sea bream (Coutinho et al., 2016), and tiger puffer (Kim and Lee, 2009) reported that the best efficiency in protein utilization was observed at the protein level below the level resulting in the maximum growth. On the other hand, some studies conducted on mangrove red snapper (Abbas and Siddiqui, 2013), starry flounder (Lee et al., 2006), turbot (Liu et al., 2015), and the current study demonstrated that the protein level resulting in the best outcome for PER was similar to that for growth responses. The highest PER value in response to the graded protein levels tested in each of the studies ranged from 1.58 to 3.11 in which the PER of 1.8 observed in the current study fell within the range.

Hepato- and viscero-somatic indices represent the proportion of liver and viscera weights to whole-body weight, respectively, and can be used as indices of the nutritional status of fish (Cui and Wootton, 1988) because liver and viscera are energy storage organs. In previous studies conducted on red spotted grouper (Wang et al., 2016) and mangrove red snapper (Abbas and Siddiqui, 2013), a continuous decrease in HSI and VSI values as the dietary protein levels increased was reported. This may not indicate that the lower values represent poor nutritional status of the tested fish, even when they were fed at the dietary protein level higher than the requirement, but may suggest that the rate of nutrient accumulation in the whole body outcompeted that indicated by liver tissues. In contrast to previous reports, few changes in these measurements in response to the tested protein levels were observed in the current study. The discrepancy in the response between the previous studies and current studies is unclear. This might be attributed to species-specificity in nutrient metabolism.

Changes in whole-body protein and lipid contents in response to graded protein levels generally follow the growth

responses that are exhibited, because excess dietary proteins result in protein deposition, which appears to be the main determinant of body WG in fish (Dumas et al., 2007). The results of whole-body nutrient contents shown in the current study agree with previous reports (Shiau and Huang, 1989; Lee et al., 2002; Coutinho et al., 2016; Wang et al., 2016). A few changes, responding to the graded protein levels, in the protein and lipid contents of viscera and liver, may suggest that the lowest dietary protein level of 43% was still sufficient to provide the essential nutrients required to support the balance between protein synthesis and degradation. When exogenous nutrient/energy input is not satisfactory, some amino acids and lipids are primarily mobilized to meet metabolic needs (Bar and Volkoff, 2012). Growth hormone (GH) and IGF-I play important roles in physiological processes of fish, including somatic growth, nutrient metabolism, reproduction, and osmoregulation (Johnsson and Björnsson, 1994; Péréz-Sánchez et al., 2002; Reinecke et al., 2005). It has been shown that the plasma GH concentration of gilthead sea bream was affected by dietary protein (Péréz-Sánchez et al., 1995). A significant correlation between dietary protein levels and hepatic IGF-I mRNA expression has also been reported in mirror carp (Huang et al., 2016) and Nile tilapia (*O. niloticus*, Qiang et al., 2012). In the current study, we did not observe this trend in plasma IGF-I, HGF, and GH in the marbled flounder in response to the protein levels. This may indicate that these are not sensitive measurements to dietary protein. A transcriptomic approach is of interest for further studies to investigate the relationship between protein-level dependent growth responses and mRNA expression of these hormones in the flounder.

When determining an optimum nutrient requirement, a regression model (e.g., broken-line and second-order polynomial model) has been preferentially used by fish nutritionists (Shiau and Huang, 1989; Abbas and Siddiqui, 2013; Wang et al., 2016) rather than ANOVA with multiple range tests because regression models consider the dose–response relationship between nutrient levels and growth continuous (Shearer, 2000). In the current study, commonly tested regression models, one-slope straight broken-line (e.g., Shiau and Huang, 1989) and second-order polynomial (e.g., Wang et al., 2016) models, were tested. The estimates obtained from testing the second-order polynomial were not adopted because the coefficient of the estimated model was not significantly different from zero (results were not included). The estimated optimal protein level found by the one-slope straight broken-line regression model analysis on the TGC and FCR results was almost the same. This was comparable to the protein level of 58.8% which showed the best growth performance of the flounder.

5. Conclusion

Taken together, the 58.8% protein diet containing 8% lipid with 20 gross energy (MJ/kg) is recommended for the optimum growth and efficient feed utilization of juvenile marbled flounder.

Author contributions

Jeong-Hyeon Cho: Conceptualization, Data Curation, Investigation, Writing - Original Draft, Visualization, Writing - Review & Editing. **Seunghyung Lee**: Conceptualization, Methodology, Data Curation, Investigation, Software, Writing - Original Draft, Visualization, Writing - Review & Editing. **Bong-Joo Lee**: Conceptualization, Supervision, Project administration, Funding acquisition. **Sang-Woo Hur**: Investigation, Validation, Resources. **Kang-Woong Kim**: Conceptualization, Funding acquisition, Project administration. **Maeng-Hyun Son**: Conceptualization, Funding acquisition, Resources. **Dong-Jae Yoo**: Investigation, Validation, Resources.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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References

Abbas G, Siddiqui PJA. The effects of varying dietary protein level on growth, feed conversion, body composition and apparent digestibility coefficient of juvenile mangrove red snapper, *Lutjanus argentimaculatus* (Forsskal 1775). Aquacult Res 2013;44:807–18. http://doi:10.1111/j.1365-2109.2012.03096.x.

Ai Q, Mai K, Li H, Zhang C, Zhang L, Duan Q, et al. Effect of dietary protein to energy ratios on growth and body composition of juvenile Japanese seabass, *Lateolabrax japonicas*. Aquaculture 2004;230:507–16. https://doi.org/10.1016/j.aquaculture.2003.09.040.

AOAC (Association of Official Analytical Chemists International). Official methods of analysis of AOAC international. 16th ed. Arlington, VA: AOAC International; 1997.

Bar N, Volkoff H. Adaptation of the physiological, endocrine, and digestive system functions to prolonged food deprivation in fish. In: McCue MD, editor. Comparative physiology of fasting, starvation, and food limitation. New York, USA: Springer; 2012. p. 69–90.

Baluyut EA. Aquaculture systems and practices: a selected review. Rome: UNDP/FAO Aquaculture Development and Coordination Programme; 1989. ADCP/REP/89/43.

Cadena-Roa M. Etude expérimentale de l'alimentation de la sole (*Solea vulgaris* Q.) en élevage intensif. Brest, France: Thése Doctorat de 3éme Cycle Univ. de Bretagne Occidentale; 1983.

Caceres-Martinez C, Cadena-Roa M, Métailler R. Nutritional requirements of turbot (*Scophthalmus maximus*). I- A preliminary study of protein and lipid utilization. J World Maric Soc 1984;15:191–202. https://doi.org/10.1111/j.1749-7345.1984.tb00153.x.

Cho CY, Cowey CB, Watanabe T. Finfish nutrition in Asia. Methodological approaches to research and development. Otawa, Canada: IDRC; 1985.

Chyung MK. The fishes of Korea. Seoul, Korea: Iljisa Pub. Co.; 1986. p. 767.

Coutinho F, Peres H, Castro C, Pérez-Jiménez A, Magalhães R, Pousão-Ferreira P, et al. Dietary protein requirement of zebra sea bream (*Diplodus cervinus*, Lowe 1838) juveniles. Aquacult Nutr 2016;22:465–71. https://doi.org/10.1111/anu.12269.

Cowey CB, Adron JW, Blair A, Pope J. The growth of O-group plaice on artificial diets containing different levels of protein. Helgoländer Wiss Meersunters 1970;20:602–9. https://doi.org/10.1007/BF01609932.

Cowey CB, Adron JW, Brown OA. Studies on the nutrition of marine flatfish. The metabolism of glucose by plaice, (*Pleuronectes platessa*) and the effect of dietary energy source on protein utilization in plaice. Br J Nutr 1975;33:219–31. https://doi.org/10.1079/BJN19750026.

Cui Y, Wootton RJ. Effects of ration, temperature and body size on the body composition, energy content and condition of the minnow, *Phoxinus phoxinus* (L). J Fish Biol 1988;32:749–64. https://doi.org/10.1111/j.1095-8649.1988.tb05414.x.

DeLong DC, Halver JE, Mertz ET. Nutrition of salmonoid fishes. VI. Protein requirements of chinook salmon at two water temperatures. J Nutr 1958;65:589–99. https://doi.org/10.1093/jn/65.4.589.

Dumas A, de Lange CFM, France J, Bureau DP. Quantitative description of body composition and rates of nutrient deposition in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 2007;273:165–81. https://doi.org/10.1016/j.aquaculture.2007.09.026.

Elzhov TV, Mullen KM, Spiess A-N, Bolker B, Mullen MKM, Suggests M. R-Package 'minpack.lm'. 2016. Retrieved from, http://CRAN.R-project.org/package=minpack.lm.

Garling DLJr, Wilson RP. Optimum dietary protein to energy ratio for channel catfish fingerlings, *Ictalurus punctatus*. J Nutr 1976;106:1368–75. https://doi.org/10.1093/jn/106.9.1368.

Gominho-Rosa MC, Rodrigues APO, Mattioni B, Francisco A, Moraes G, Fracalossi DM. Comparison between the omnivorous jundia catfish (*Rhamdia quelen*) and Nile tilapia (*Oreochromis niloticus*) on the utilization of dietary

starch sources: diegestibility, enzyme activity and starch microstructure. Aquaculture 2015;435:92–9. https://doi.org/10.1016/j.aquaculture.2014.09.035.
Han KH, Park JT, Jin DS, Jang SI, Joung HH, Cho JK. Morphological development of larvae and juveniles of the marbled sole, *Limanda yokohamae*. Korean J Ichthyol 2001;13:161–5 [in Korean with English abstract].
Hebb CD, Castell JD, Anderson DM, Batt J. Growth and feed conversion of juvenile winter flounder (*Pleuronectes americanus*) in relation to different protein-to-lipid levels in isocaloric diets. Aquaculture 2003;221:439–49. https://doi.org/10.1016/S0044-8486(03)00064-4.
Huh SH, Nam KM, Park JM, Jeong JM, Baeck GW. Feeding habits of the marbled sole flounder, *Pleuronectes yokohamae* in the coastal waters off Tong-yeong, Korea. Korean J Ichthyol 2012;24:77–83. https://doi.org/10.3796/KSFT.2017.53.4.363 [in Korean with English abstract].
Huang JF, Xu QY, Chang YM. Effects of temperature and dietary protein on the growth performance and IGF-I mRNA expression of juvenile mirror carp (*Cyprinus carpio*). Aquacult Nutr 2016;22:283–92. https://doi.org/10.1111/anu.12254.
Jauncey K. The effects of varying dietary protein level on the growth, food conversion, protein utilization and body composition of juvenile tilapia, *Sarotherodon mossambicus*. Aquaculture 1982;27:43–54. https://doi.org/10.1016/0044-8486(82)90108-9.
Ji H-S, Kim J-K, Kim B-J. Molecular phylogeny of the families Pleuronectidae and Poecilopsettidae (PISCES, Pleuronectiformes) from Korea, with a proposal for a new classification. Ocean Sci J 2016;51:299–304. https://doi.org/10.1007/s12601-016-0026-8.
Johnsson JI, Björnsson BT. Growth hormone increases growth rate, appetite and dominance in juvenile rainbow trout, *Oncorhynchus mykiss*. Anim Behav 1994;48:177–86. https://doi.org/10.1006/anbe.1994.1224.
Joo HW, Gwak WS. Estimation of early growth and spawning period of marbled sole (*Pseudo Pleuronectes yokohamae*) in the water off Namhae of Korea as indicated from daily growth increments in otoliths. Sea J Korean Soc Oceanography 2014;19:35–40. https://doi.org/10.7850/jkso.2014.19.1.35 [in Korean with English abstract].
Kang YJ, Lee TY, Lee BD. Reproduction and population dynamics of marbled sole *Limanda yokohamae*. 2. Population dynamics. Korean J Fish Aqua Sci 1985;18:261–5 [in Korean with English abstract].
Kim YH, Kang YJ, Bae IJ. Age and growth of marbled sole *Limanda yokohamae* (Günther). Korean J Ichthyol 1991;3:130–9.
Kim H, Huh S, Kim S, Seo YI. Age and growth of marbled sole, *Pleuronectes yokohamae* (Günther) in the southern waters of Korea. J Korean Soc Fish Ocean Technol 2015;51:35–41. https://doi.org/10.3796/KSFT.2015.51.1.035 [in Korean with English abstract].
Kim IS, Youn CH. Taxonomic revision of the flounders (pisces: pleuronectiformens) from Korea. Korean J Ichthyol 1994;6:99–131 [in Korean with English abstract].
Kim S-S, Lee K-J. Dietary protein requirement of juvenile tiger puffer (*Takifugu rubripes*). Aquaculture 2009;287:219–22. https://doi.org/10.1016/j.aquaculture.2008.10.021.
KOSTAT (Statistics Korea). Fishery production survey, daejeon. Republic of Korea; 2019.
Kume G, Horiguchi T, Goto A, Shiraishi H, Shibata Y, Morita M, et al. Seasonal distribution, age, growth, and reproductive biology of marbled sole *Pleuronectes yokohamae* in Tokyo Bay, Japan. Fish Sci 2006;72:289–98. https://doi.org/10.1111/j.1444-2906.2006.01150.x.
Lee TY, Kang YJ, Lee BD. Reproduction and population dynamics of marbled sole *Limanda yokohamae*. 1. Reproduction. Korean J Fish Aqua Sci 1985;18:253–61 [in Korean with English abstract].
Lee S-M, Lee JH, Kim K-D, Cho SH. Optimum dietary protein for growth of juvenile starry flounder, *Platichthys stellatus*. J World Aquacult Soc 2006;37:200–3. https://doi.org/10.1111/j.1749-7345.2006.00027.x.
Lee S-M, Park CS, Bang IC. Dietary protein requirement of young Japanese flounder *Paralichthys olivaceus* fed isocaloric diets. Fish Sci 2002;68:158–64. https://doi.org/10.1046/j.1444-2906.2002.00402.x.
Lee JH, Kodama K, Oyama M, Kume G, Takao Y, Shiraishi H, et al. Changes in growth of marbled sole *Pseudopleuronectes yokohamae* between high and low stock-size periods in Tokyo Bay, Japan. Fish Sci 2009;75:929–35. https://doi.org/10.1007/s12562-009-0117-x.
Lee S, Wang Y, Hung SSO, Strathe AB, Fangue NA, Fadel JG. Development of optimum feeding rate model for white sturgeon (*Acipenser transmontanus*). Aquaculture 2014;433:411–20. https://doi.org/10.1016/j.aquaculture.2014.06.007.
Liu X, Mai K, Liufu Z, Ai Q. Dietary protein requirement of juvenile turbot (*Scophthalmus maximus* Linnaeus). J Ocean Univ China 2015;14:325–8. https://doi.org/10.1007/s11802-015-2360-3.
Lucas JS, Southgate PC. Aquaculture: farming aquatic animals and plants. 2nd ed. West-Sussex, UK: Wiley-Blackwell; 2012.
Mambrini M, Guillaume J. Protein nutrition. In: Guillaume J, Kaushik S, Bergot P, Métailler R, editors. Nutrition and feeding of fish and curstaceans. Chicester, UK: Springer-Praxis Publishing Ltd; 2001. p. 81–109.
MAFF (Ministry of Agriculture, Forestry and Fisheries). Annual report of Fisheries and aquaculture industry statistics. 2019. Japan.
NRC (Nutrient requirements of fish). Washington, DC: National Academy of Science of Washington; 2011.
Nose T, Arai S. Optimum level of protein in purified test diet for eel, *Anguilla japonica*. Bull Freshwater Fish Res Lab Tokyo 1972;22:145–55.
Ogino C, Saito K. Protein nutrition in fish. I. The utilization of dietary protein by young carp. Bull Japanese Soc Sci Fish 1970;36:250–4.
Péréz-Sánchez J, Martí-Palanca H, Kaushik SJ. Ration size and protein intake affect circulating growth hormone concentration, hepatic growth hormone binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, the Gilthead sea bream (*Sparus aurata*). J Nutr 1995;125:546–52. https://doi.org/10.1093/jn/125.3.546.
Péréz-Sánchez J, Calduch-Giner JA, Mingarro M, de Celis SVR, Gomez-Requeni P, Saera-Vila A, et al. Overview of fish growth hormone family. New insights in genomic organization and heterogeneity of growth hormone receptors. Fish Physiol Biochem 2002;27:243–58. https://doi.org/10.1023/B:FISH.0000032729.72746.c8.
Qiang J, Yang H, Wang H, Kpundeh MD, Xu P. Growth and IGF-I response of juvenile Nile tilapia (*Oreochromis niloticus*) to changes in water temperature and dietary protein level. J Therm Biol 2012;37:686–95. https://doi.org/10.1016/j.jtherbio.2012.07.009.
Reinecke M, Björnsson BT, Dickhoff WW, McCormick SD, Navarro I, Power DM, et al. Growth hormone and insulin-like growth factors in fish: where we are and where to go. Gen Comp Endocrinol 2005;142:20–4. https://doi.org/10.1016/j.ygcen.2005.01.016.
R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2015. http://www.R-project.org/.
Robbins KR, Saxton HW, Baker DH. Estimation of nutrient requirements from growth data. J Nutr 1979;109:1710–4. https://doi.org/10.1093/jn/109.10.1710.
Sabaut JJ, Luquet P. Nutritional requirements of the gilthead bream (*Chrysophrys aurata*), quantitative protein requirements. Mar Biol (N Y) 1973;18:50–4. https://doi.org/10.1007/BF00347920.
Seo YI, Joo H, Lee SK, Kim HY, Ko JC, Choi MS, et al. Maturity and spawning of marbled sole *Pleuronectes yokohamae* in the southern sea of Korea. Korean J Ichthyol 2010;22:83–9 [in Korean with English abstract].
Shearer K. Experimental design, statistical analysis and modelling of dietary nutrient requirement studies for fish: a critical review. Aquacult Nutr 2000;6:91–102. https://doi.org/10.1046/j.1365-2095.2000.00134.x.
Shiau SY, Huang SL. Optimum dietary protein level for hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) reared in seawater. Aquaculture 1989;81:119–27. https://doi.org/10.1016/0044-8486(89)90237-8.
Solomon G, Sano M, Simizu M, Nose Y. Age and growth of the pleuronectid flounder *Limanda yokohamae* in Tokyo bay, Japan. Nippon Suisan Gakkaishi 1987;53:711–6. https://doi.org/10.2331/suisan.53.711.
Takeda M, Shimeno S, Hosokawa H, Kajiyama H, Kaisyo T. The effect of dietary calorie-to-protein ratio on the growth, feed conversion and body composition of young yellow tail. Bull Japanese Soc Sci Fish 1975;41:443–7.
Teles AO, Couto A, Enes P, Peres H. Dietary protein requirements of fish – a meta-analysis. Rev Aquacult 2020:1–33. https://doi.org/10.1111/raq.12391.
Tucker J. Marine fish. In: Lucas JS, Southgate PC, editors. Aquaculture: farming aquatic animals and plants. 2nd ed. West-Sussex, UK: Wiley-Blackwell; 2012.
Ullah-Khan K, Tellechea-Rodrigues A, Menegasso-Mansano CF, Queiroz DMA, Kazue-Sakomura N, Romaneli RS, do-Nascomento TMT, Kochenborger-Fernandes JB. Dietary protein quality and proper protein to energy ratios: a bioeconomic approach in aquaculture feeding practices. Lat Am J Aquat Res 2019;47:232–9. https://doi.org/10.3856/vol47-issue2-fulltext-3.
Vergara JM, Fernandez-Palacios H, Robaina L, Jauncey K, De La Higuera M, Izquierdo M. The effects of varying dietary protein level on the growth, feed efficiency, protein utilization and body composition of gilthead sea bream fry. Fish Sci 1996;62:620–3. https://doi.org/10.2331/fishsci.62.620.
Wang J, Jiang Y, Li X, Han T, Yang Y, Hu S, et al. Dietary protein requirement of juvenile red spotted grouper. Aquaculture 2016;450:289–94. https://doi.org/10.1016/j.aquaculture.2015.08.007.
Wang J, Li B, Ma J, WangS, Huang B, Sun Y, et al. Optimum dietary protein to lipid ratio for Starry flounder (*Platichthys stellatus*). Aquacult Res 2017;48:189–201. http://doi:10.1111/are.12873.
Wilson RP, Halver JE. Protein and amino acid requirements of fishes. Annu Rev Nutr 1986;6:225–44.
Wilson RP. Amino acids and proteins. In: Halver JE, Hardy RW, editors. Fish nutrition. San Diego, CA, USA: Academic Press; 2002. p. 143–79.
Wilson RP. Utilization of dietary carbohydrate by fish. Aquaculture 1994;124:67–80. https://doi.org/10.1016/0044-8486(94)90363-8.
Yusa T. Difference of structures of eggs and larvae between *Limanda yokohamae* Gunther and *Limanda schrencki* Schmidt. Bull Ashamushi Marine Biol Station 1960;10:127–31.
Zeitoun IH, Ullrey DE, Magee WT, Gill JL, Bergen WG. Quantifying nutrient requirements of fish. J Fish Res Board Can 1976;33:167–72. https://doi.org/10.1139/f76-019.