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Effects of Dietary Protein Levels on Growth Performance, Plasma Parameters, and Digestive Enzyme Activities in Different Intestinal Segments of *Megalobrama amblycephala* at Two Growth Stages

Wuxiao Zhang [1,2], Silei Xia [1], Bo Liu [3], Hongyan Tian [1], Fei Liu [1], Wenping Yang [1], Yebing Yu [1], Caiyuan Zhao [4], Naresh Kumar Dewangan [4], Aimin Wang [1,*] and Tao Teng [3,5,*]

[1] College of Marine and Biology Engineering, Yancheng Institute of Technology, Yancheng 224051, China; zhangwx257258@163.com (W.Z.); susanxia1990323@126.com (S.X.); tianhyy@outlook.com (H.T.); liufei@ycit.edu.en (F.L.); yangwenping@ycit.edu.cn (W.Y.); yuyebing2005@126.com (Y.Y.)
[2] Key Laboratory of Agricultural Environmental Microbiology, Ministry of Agriculture and Rural Affairs, College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China
[3] Key Laboratory of Freshwater Fisheries and Germplasm Resources Utilization, Ministry of Agriculture, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi 214081, China; liub@ffrc.cn
[4] College of Animal Science and Technology, Henan University of Animal Husbandry and Economy, Zhengzhou 450046, China; zhaocaiyuan326@foxmail.com (C.Z.); nareshbiotech@yahoo.in (N.K.D.)
[5] Tongwei Agricultural Development Co., Ltd., Key Laboratory of Nutrition and Healthy Culture of Aquatic, Livestock and Poultry, Ministry of Agriculture and Rural Affairs, Healthy Aquaculture Key Laboratory of Sichuan Province, Chengdu 610093, China
* Correspondence: blueseawam@ycit.cn (A.W.); tengt@tongwei.com (T.T.)

Abstract: An 8-week rearing trial was designed to estimate the dietary protein requirement and evaluate the effects of dietary protein on growth performance, plasma parameters, and digestive enzyme activities of blunt snout bream at two growth stages. Six practical diets were prepared to feed two sizes of fish (larger fish: initial weight of 153.69 ± 0.85 g; smaller fish: initial weight of 40.89 ± 0.28 g) with graded protein levels (26%, 28%, 30%, 32%, 34%, and 36%). Our results show that the final weight, weight gain (WG), and specific growth rate (SGR) of the fish initially rose to peak values and then declined as the dietary protein levels increased. The higher WG and SGR were recorded in the larger fish fed diets containing 30%, 32%, and 34% protein, and in the smaller fish fed a 30% protein diet, all significantly higher than those in the control group ($p < 0.05$). No significant differences were observed in the feed conversion ratio (FCR), viscerosomatic ratio (VR), hepatosomatic index (HSI), condition factor (CF), or survival rate among the treatments at both growth stages ($p > 0.05$). The plasma total protein (TP) content was highest at both growth stages in fish fed a 30% protein diet ($p < 0.05$). As the dietary protein level increased, the plasma urea content of the larger fish increased, peaked in the 34% protein group ($p < 0.05$), and then remained stable. In contrast, no significant difference in the plasma urea content was seen among the treatment groups of the smaller fish ($p > 0.05$). Protease activity in the fish foregut at both growth stages peaked in the 32% protein group ($p < 0.05$). In the midgut of the larger fish, protease activity was higher in the control group, while in the smaller fish, it was higher in the 36% protein group ($p < 0.05$). In the larger fish, hindgut protease activity was higher in the 34% protein group ($p < 0.05$), while in the smaller fish, there was no significant difference in the hindgut protease activity among all groups ($p > 0.05$). The dietary protein levels had no significant effect on lipase activity in the foregut, midgut, or hindgut, or on amylase activity in the foregut or midgut of the fish at the two growth stages ($p > 0.05$). However, hindgut amylase activity was highest in the control group of the smaller fish ($p < 0.05$). Based on regression analysis, the optimal

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dietary protein levels for the larger and smaller fish were 30.45% and 29.95%, respectively. Overall, appropriate dietary protein levels (30%) could improve the growth performance, immune function, and health status of fish at two growth stages and promote the adaptive response of their digestive system, especially the spatial regulation of protease activity in different gastrointestinal regions.

Keywords: protein requirement; *Megalobrama amblycephala*; growth performance; plasma parameters; digestive enzyme activities; different growth stages

Key Contribution: The optimal protein requirements for two sizes of *Megalobrama amblycephala* were determined, and the effects of dietary protein levels on plasma protein metabolism and digestive enzyme activities in different parts of the intestinal tract were investigated.

1. Introduction

Protein is regarded as the main nutrient for fish, providing energy and essential amino acids [1,2]. It is an important macronutrient in nutritional studies, as it forms the costly part of commercial fish feed and is crucial for growth and development, also key for maintaining fish optimal growth and reproduction [3–5]. Insufficient dietary protein can lead to stunted growth or even cessation of growth, as the body must divert protein from less critical tissues to sustain the function of more vital organs and systems [6]. An excessive intake of dietary protein can be equally detrimental. The surplus protein is metabolized into energy, which not only increases feed costs but also leads to higher levels of ammonia nitrogen in the water, potentially harming the aquatic environment [7]. Therefore, determining the optimal protein requirements for fish is crucial for promoting their best growth and efficient diet utilization.

Megalobrama amblycephala, also referred to as the blunt snout bream, is a herbivorous freshwater fish that is indigenous to China. In recent years, the aquaculture industry has experienced rapid growth in the farming of this species, primarily due to its rapid growth rate, high feed efficiency, tender flesh, and strong disease resistance [8]. As a result, the total production of this fish in China reached approximately 0.74 million tons in 2023 [9]. The natural diet of the blunt snout bream consists mainly of aquatic plants, such as zooplankton. Consequently, its nutritional requirements are relatively simple. However, the blunt snout bream can adapt to formulated feeds, and understanding its nutritional requirements is essential for developing appropriate feed formulations that support optimal growth and health. In the context of fish nutrition, the demand for dietary protein is a critical factor that can significantly influence the overall health and productivity of the fish. The blunt snout bream, with its herbivorous tendencies, is no exception to this rule. In China, diets for blunt snout bream fingerlings were recommended to have at least 30% protein [10]. A previous study found that blunt snout bream requires 27–30% protein for optimal growth at about 20 °C water temperature, and the optimum protein requirement ranges from 25.6% to 41.4% when the water temperature is from 25 °C to 30 °C [11]. The specific protein requirements for this species can vary depending on its size, life stage, and environmental conditions [12]. Therefore, research into the most effective protein levels for different sizes of *Megalobrama amblycephala* is essential to optimize aquaculture practices.

The plasma biochemical parameters of fish provide valuable insights into their metabolic status and overall health [13]. For instance, levels of enzymes, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), can indicate liver function, while urea and creatinine levels can reflect kidney function [6,14]. Monitoring these param-

eters in relation to dietary protein levels can help in assessing whether the protein intake is appropriate or excessive, potentially leading to stress or metabolic disorders [15].

The activity of digestive enzymes in fish depends on their diet, and different diets can affect it [16]. It was reported that the utilization of feed protein is affected by the activity of digestive enzymes [17]. Also, protease activity changes in response to different levels of protein in the diet [18]. The activity of digestive enzymes in the intestine is a critical factor in determining the efficiency with which dietary protein is utilized. Enzymes such as proteases, amylases, and lipases play a vital role in breaking down complex nutrients into simpler forms that can be absorbed by the fish [19]. Understanding how different protein levels affect these enzymes can help in designing feeds that maximize nutrient absorption and minimize waste.

Therefore, this study aimed to carry out a comprehensive investigation into the effects of dietary protein levels on the growth performance, plasma protein metabolism, and intestinal digestive enzyme activity of *Megalobrama amblycephala* at two sizes. Through the determination of optimal protein levels tailored to various sizes of this fish, researchers can make contributions to the advancement of more efficient and ecologically sustainable aquaculture feed formulations.

2. Materials and Methods

2.1. Experimental Diets

Based on previous research on the protein requirements of blunt snout bream [10,11], six isonitrogenous and isoenergetic diets were formulated. Using fish meal, casein, and gelatin as protein sources and soybean oil as a lipid source, they contained graded levels of protein (26%, 28%, 30%, 32%, 34%, and 36%) (Table 1). All the ingredients were ground into a powder and thoroughly mixed with the soybean oil and water. Then, they were forced through a pelletizer (Made in South China University of Technology, Guangzhou, China) and air-dried at 20 °C for 24 h, and then packaged in airtight plastic bags and stored at −20 °C.

Table 1. Formulation and chemical composition of experimental diets (% dry diet).

Ingredients	Group					
	26	**28**	**30**	**32**	**34**	**36**
Casein [a]	16.86	18.86	19.5	20.8	22	23.5
Gelatin [a]	4.25	4.25	4.85	5.5	5.71	5.86
White fish meal [a]	8.95	9.95	10.7	11.5	12.26	13
Corn starch	33.75	30.75	28.5	25.2	22.85	20
Dextrin	10	10	10	10	10	10
Microcrystalline cellulose	5.84	5.84	6.2	6.9	7.23	7.84
Carboxylmethyl cellulose	10	10	10	10	10	10
Soybean Oil	5.5	5.5	5.45	5.4	5.35	5.3
Vitamin mix and mineral mix [b]	1	1	1	1	1	1
Soybean lecithin	1	1	1	1	1	1
Calcium dihydrogen phosphate	2.65	2.65	2.6	2.5	2.4	2.3
Chlorinated choline	0.15	0.15	0.15	0.15	0.15	0.15
Ethoxyquin	0.05	0.05	0.05	0.05	0.05	0.05
Methionine + cystine	0.47	0.51	0.53	0.56	0.59	0.63
Lysine	2.09	2.16	2.17	2.20	2.24	2.28
Analyzed nutrient compositions (% of dry diet)						
Crude protein	25.89	28.46	30.07	32.37	34.18	36.20
Crude lipid	6.56	6.62	6.61	6.60	6.60	6.59
Gross energy (KJ/g)	19.48	19.61	19.60	19.56	19.59	19.56

[a] Casein, obtained from Hua'an Biological Products Lit. (Linxia, China), 90.2% crude protein; gelatin, obtained from Zhanyun Chemical Lit. (Shanghai, China), 91.3% crude protein; white fish meal, obtained from Copeinca (Lima, Peru), 67.4% crude protein and 9.3% crude lipid. [b] Vitamin premix (IU or mg/kg of diet): vitamin A, 25,000 IU; vitamin D3, 20,000 IU; vitamin E, 200 mg; vitamin K3, 20 mg; thiamin, 40 mg; riboflavin, 50 mg; calcium pantothenate, 100 mg; pyridoxine HCl, 40 mg; cyanocobalamin, 0.2 mg; biotin, 6 mg; folic acid, 20 mg; niacin, 200 mg; inositol, 1000 mg; vitamin C, 2000 mg; choline, 2000 mg. Cellulose was used as a carrier. Mineral premix (g/kg of diet): calcium biphosphate, 20 g; sodium chloride, 2.6; potassium chloride, 5 g; magnesium sulphate, 2 g; ferrous sulphate, 0.9 g; zinc sulphate, 0.06 g; cupric sulphate, 0.02; manganese sulphate, 0.03 g; sodium selenate, 0.02 g; cobalt chloride, 0.05 g; potassium iodide, 0.004. Zeolite was used as a carrier.

2.2. Experimental Fish and Feeding Trial

Experimental fish at two sizes were obtained from the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences (Wuxi, China). Prior to the feeding trial, these two sizes fish were reared in 200 L and 750 L tanks, respectively, for 2 weeks to acclimate to the experimental conditions while fed a commercial diet containing 32% protein and 5% lipid (1.5 mm in diameter, Wuxi Tongwei Feedstuffs, Wuxi, China). After fasting for 24 h, the healthy, similarly sized fish with a mean body weight of 40.89 ± 0.28 g were randomly sorted into eighteen 200 L tanks with 20 fish per tank (3 replicates per group). The same was carried out for another size of blunt snout bream, where the healthy, similarly sized fish with a mean body weight of 153.69 ± 0.85 g were randomly sorted into eighteen 750 L tanks with 25 fish per tank (3 replicates per group).

During the eight-week feeding trial, the fish were hand-fed the experimental diets to satiation three times daily (8:00, 12:00, and 16:00 h). Feed consumption and the number and weight of dead fish were recorded daily. The water temperature was controlled at 26–28 °C, pH 7.2–7.8. The dissolved oxygen concentration was higher than 5 mg/L, and the ammonia nitrogen concentration was <0.1 mg/L. The concentrations of ammonia-N were determined using the Nesslerization method, as described by Zhang et al. [20]. The temperature, pH, and dissolved oxygen were measured using a water quality instrument (YSI Inc., Yellow Springs, OH, USA).

The calculation formulas used for growth performance were as follows:

Specific growth rate, (SGR) = 100 × (Ln finial individual weight − Ln initial individual weight)/number of days;

Weight gain, (WG) = 100 × (final weight − initial weight)/initial weight;

Feed conversation ratio, (FCR) = (wet weight gain, g)/(dry feed weight, g);

Viscerosomatic ratio, (VR) = 100 × (viscera weight, g)/(body weight, g);

Hepatosomatic index, (HSI) = 100 × (liver weight, g)/(body weight, g);

Condition factor, (CF) = 100 × W/L^3, where W is the weight (g), and L is the length (cm);

Survival rate (%) = 100 × (final number of fish)/(initial number of fish).

2.3. Sample Collection

At the end of the experiment, the fish were fasted for 24 h and individually counted and weighed from each tank. Three fish were chosen from each tank and anesthetized with MS-222 (100 mg/L, Sigma Chemical Company, St. Louis, MO, USA), and then blood samples were collected immediately from the caudal vein using heparinized syringes. Following centrifugation at 3000 g for 10 min at 4 °C, the plasma was separated and stored at −80 °C. After the blood samples were collected, the intestine was excised and stored at −80 °C.

2.4. Chemical Analysis

The crude protein and crude lipid contents in the diets were determined according to the established methods of (AOAC, 2003) [21]: the crude protein content (N × 6.25) was determined by the Kjeldahl method using the semi-automatic Kjeldahl system (1030 Auto-analyzer, Tecator, Hoganos, Sweden) after acid digestion; the crude lipid content was measured by the ether extraction method using the Soxhlet system HT6 (Soxtec System, Tecator, Sweden); and the gross energy content was measured using the IKA C2000 basic bomb calorimeter (IKA Works Inc., Wilmington, NC, USA).

The plasma levels of alkaline phosphatase (ALP), total protein (TP), albumin (ALB), urea, and creatinine (Crea) were measured with an automatic biochemical analyzer (Mindray BS-400, Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China) using the AMP buffer method, biuret, bromocresol green, UV method, and sarcosine oxidase method, respectively. Determination of the low-density lipoprotein (LDL) and high-density lipopro-

tein (HDL) was carried out following the methods described by Mozanzadeh et al. [22]. The total globulin (GLB) content was estimated by subtracting the albumin from the total protein [22].

Intestinal digestive enzyme activity was determined according to the method described by Bowyer et al. [23]. The fish gut was divided into three sections (foregut, midgut, and hindgut). The intestines were homogenized in 10 volumes (w/v) of 4000× g for 20 min at 4 °C, and the supernatant was used as the enzyme source. The activities of the protein concentration, lipase, and amylase in the intestines of blunt snout bream were determined by spectrophotometry using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The activity of protease was assayed following the Forint phenol reagent method in 0.01 M Tris-HCL (pH 7.4) buffer using 2% casein as a substrate. The reaction was carried out for 10 min at 30 °C, stopped with 0.1 M trichloroacetic acid (TCA), and then centrifuged at 3000× g for 5 min at 4 °C. Then, 0.5 mL of the supernatant was added to 2.5 mL of 0.4 M $NaHCO_3$ and 0.5 mL of 50% Folin phenol reagent, and the optical density was read at 680 nm using tyrosine as a standard. The specific activity of protease was expressed as 1 μmol of hydrolyzed tyrosine per minute per milligram of protein (U/mg). The specific activity of amylase was expressed as 1 mol of reducing sugars per min per mg of protein (U/mg). The specific activity of lipase was expressed as 1 μmol of hydrolyzed substrate per minute per gram of protein (U/g).

2.5. Statistical Analysis

All data were presented as the means ± SEMs (standard errors of the mean). The data were subjected to one-way analysis of variance (ANOVA) using SPSS 27.0 software for Windows (International Business Machines Corporation, Armonk, NY, USA). Significant differences in the means among the dietary treatments were evaluated by Tukey's multiple range test. Mean differences were considered significant at a p value of less than 0.05.

3. Results

3.1. Growth Performance

As the dietary protein levels increased, the final weight, weight gain (WG), and specific growth rate (SGR) of the blunt snout bream at two sizes initially rose to their peak values, and then subsequently exhibited a downward trend (as shown in Table 2). A higher final weight was observed in the larger fish (initial weight of 153.69 g) fed 30% and 32% protein, significantly higher than that in the control group ($p < 0.05$). Moreover, higher WG and SGR were recorded in the larger fish fed diets with 30%, 32%, and 34% protein, also significantly higher than those in the control group ($p < 0.05$). For the smaller fish (with an initial weight of 40.89 g), the highest final weight, WG, and SGR were achieved with a 30% protein diet, which were also significantly higher than those in the 28% protein group and the control group ($p < 0.05$). Broken-line regression analysis showed, based on the WG, that the optimal dietary protein levels in the larger fish and smaller fish were estimated to be 30.45% and 29.95% (Figure 1), respectively. The feed conversion ratio (FCR), viscerosomatic ratio (VR), hepatosomatic index (HSI), condition factor (CF), and survival rate of the fish at two growth stages were not significantly different among the treatments ($p > 0.05$).

Table 2. Effects of dietary protein levels on growth performance in blunt snout bream at two growth stages.

Item	Protein Level (% of Dry Diet)						
	26	28	30	32	34	36	*p* Value
Trial 1 (initial body weight of 153.69 g)							
Initial weight (g)	152.86 ± 0.29	153.76 ± 0.64	154.27 ± 0.27	153.72 ± 0.87	153.52 ± 0.36	153.67 ± 0.65	0.659
Final weight (g)	278.98 ± 1.41 ^{a}	285.80 ± 1.79 ^{ab}	297.24 ± 0.12 ^{b}	294.72 ± 5.05 ^{b}	294.36 ± 1.08 ^{ab}	289.48 ± 4.60 ^{ab}	0.018
SGR (% day^{-1})	1.07 ± 0.01 ^{a}	1.11 ± 0.01 ^{ab}	1.17 ± 0.00 ^{b}	1.16 ± 0.02 ^{b}	1.16 ± 0.01 ^{b}	1.13 ± 0.02 ^{ab}	0.009
WG	82.50 ± 0.61 ^{a}	85.88 ± 1.21 ^{ab}	93.02 ± 0.08 ^{b}	91.70 ± 2.21 ^{b}	91.74 ± 1.15 ^{b}	88.37 ± 2.49 ^{ab}	0.007
FCR	2.75 ± 0.02	2.45 ± 0.10	2.21 ± 0.20	1.89 ± 0.04	1.98 ± 0.02	2.17 ± 0.21	0.281
VR	13.91 ± 3.54	15.49 ± 1.38	11.16 ± 0.72	15.24 ± 1.16	13.43 ± 1.49	11.88 ± 1.66	0.38
HSI	1.68 ± 0.32	1.63 ± 0.13	1.56 ± 0.15	1.82 ± 0.10	1.58 ± 0.17	1.76 ± 0.09	0.873
CF	2.12 ± 0.11	2.15 ± 0.03	2.02 ± 0.02	2.14 ± 0.03	2.18 ± 0.02	2.08 ± 0.02	0.204
Survival (%)	92.00 ± 2.30	98.67 ± 1.33	96.00 ± 4.00	96.00 ± 2.31	98.67 ± 1.33	94.67 ± 1.33	0.227
Trial 2 (initial body weight of 40.89 g)							
Initial weight (g)	40.78 ± 0.04	40.81 ± 0.02	41.02 ± 0.09	40.92 ± 0.09	40.93 ± 0.04	40.87 ± 0.04	1.53
Final weight (g)	80.97 ± 1.05 ^{a}	81.81 ± 0.67 ^{a}	90.62 ± 1.85 ^{b}	85.32 ± 0.26 ^{ab}	84.34 ± 1.66 ^{ab}	84.27 ± 0.17 ^{ab}	0.004
SGR (% day^{-1})	1.23 ± 0.02 ^{a}	1.25 ± 0.02 ^{a}	1.41 ± 0.04 ^{b}	1.31 ± 0.01 ^{ab}	1.29 ± 0.03 ^{ab}	1.30 ± 0.01 ^{ab}	0.005
WG	98.54 ± 2.59 ^{a}	100.50 ± 1.65 ^{a}	120.92 ± 4.39 ^{b}	108.51 ± 0.71 ^{ab}	106.04 ± 3.91 ^{ab}	106.04 ± 0.67 ^{ab}	0.005
FCR	1.99 ± 0.10	2.12 ± 0.08	1.72 ± 0.08	1.85 ± 0.01	2.26 ± 0.33	2.13 ± 0.24	0.348
VR	16.17 ± 3.70	14.05 ± 0.60	15.71 ± 0.96	14.02 ± 2.01	18.70 ± 2.54	16.25 ± 0.66	0.637
HSI	1.72 ± 0.10	1.70 ± 0.17	1.28 ± 0.09	1.59 ± 0.20	1.54 ± 0.09	1.29 ± 0.11	0.143
CF	2.09 ± 0.14	1.97 ± 0.01	2.02 ± 0.11	1.97 ± 0.02	2.07 ± 0.05	1.92 ± 0.01	0.55
Survival (%)	73.33 ± 13.02	98.33 ± 1.67	100	98.33 ± 1.67	86.67 ± 8.33	91.39 ± 3.25	0.120

Note: Values are presented as means ± SEM (n = 3). Values with different superscripts in the same row are significantly ($p < 0.05$) different. SGR, specific growth rate; WG, weight gain; FCR, feed conversation ratio; VR, viscerosomatic ratio; HSI, hepatosomatic index; CF, condition factor.

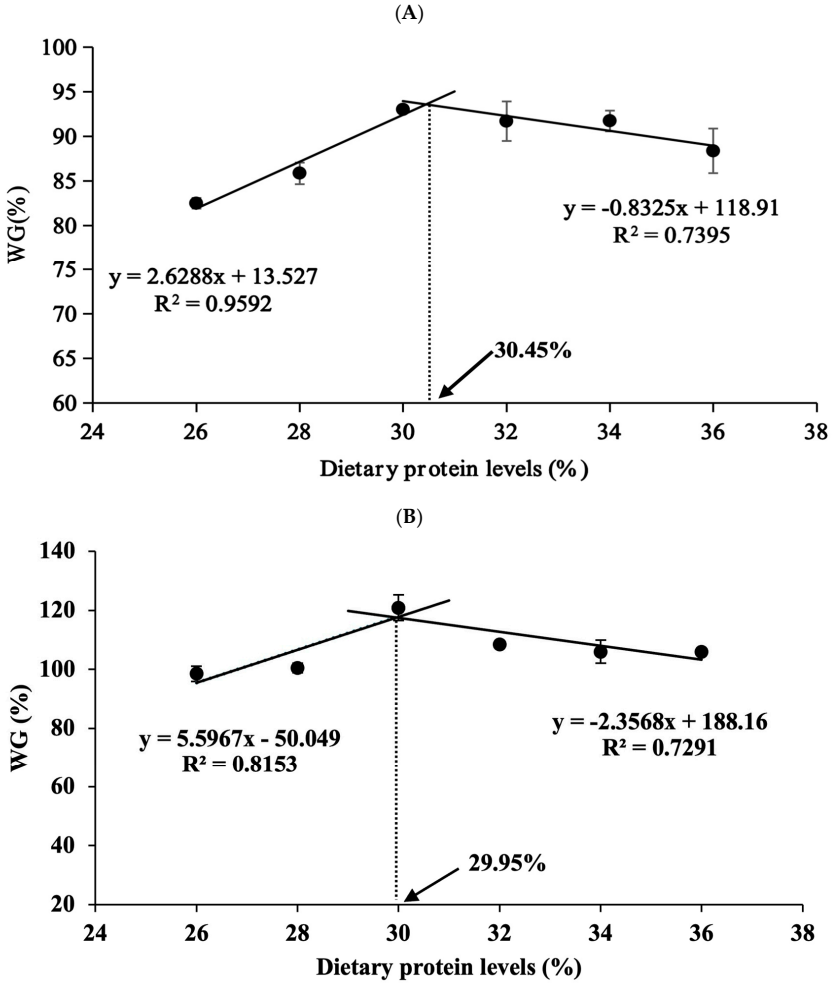

Figure 1. Regression analysis of weight gain (WG, %) against different graded levels of dietary protein. (**A**) Larger fish, initial body weight of 153.69 g; (**B**) smaller fish, initial body weight of 40.89 g.

3.2. Plasma Parameters

In all treatment groups, no significant differences were observed in the plasma levels of alkaline phosphatase (ALP), albumin (ALB), globulin (GLB), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or creatinine (Crea) at either fish growth stage ($p > 0.05$, Table 3). However, when fed a diet containing 30% protein, the plasma total protein (TP) content at both growth stages of blunt snout bream was highest compared to that of the other groups ($p < 0.05$). As the dietary protein level increased, the plasma urea content of the larger fish (with an initial weight of 153.69 g) increased accordingly, peaking in the 34% protein group. This value was significantly higher than that of the control group ($p < 0.05$), and subsequently remained stable. Conversely, no significant difference in the plasma urea content was observed among all treatment groups of the smaller fish (with an initial weight of 40.89 g) ($p > 0.05$). Furthermore, the plasma levels of urea and creatinine, particularly creatinine, were substantially higher in the larger fish compared to the smaller fish.

Table 3. Effects of dietary protein levels on plasma parameters in blunt snout bream at two growth stages.

Item	Protein Level (% of Dry Diet)						
	26	28	30	32	34	36	*p* Value
Trial 1 (initial body weight of 153.69 g)							
ALP	39.28 ± 8.46	44.93 ± 6.91	44.99 ± 4.46	45.57 ± 5.47	35.12 ± 3.71	43.68 ± 4.51	0.783
TP	37.27 ± 0.30 ^{a}	37.68 ± 2.55 ^{a}	49.87 ± 1.55 ^{b}	38.48 ± 0.29 ^{a}	38.93 ± 1.39 ^{a}	42.77 ± 1.74 ^{a}	0.002
ALB	17.80 ± 1.33	16.65 ± 1.55	19.26 ± 0.83	17.93 ± 1.70	17.40 ± 1.17	18.78 ± 1.10	0.744
GLB	25.62 ± 1.95	23.88 ± 2.04	26.83 ± 0.61	26.03 ± 1.75	25.06 ± 1.24	27.78 ± 1.02	0.554
Urea	0.67 ± 0.05 ^{a}	0.82 ± 0.09 ^{ab}	0.85 ± 0.04 ^{ab}	0.90 ± 0.03 ^{ab}	1.13 ± 0.19 ^{b}	0.95 ± 0.07 ^{ab}	0.037
LDL	1.13 ± 0.18	1.55 ± 0.26	1.62 ± 0.16	1.91 ± 0.29	1.82 ± 0.31	2.01 ± 0.15	0.093
HDL	4.36 ± 0.16	4.20 ± 0.24	4.79 ± 0.20	4.77 ± 0.36	4.64 ± 0.32	5.00 ± 0.36	0.396
Crea	1008.54 ± 107.99	981.98 ± 45.56	1028.69 ± 50.62	964.60 ± 58.11	1066.96 ± 44.51	1116.52 ± 47.46	0.509
Trial 2 (initial body weight of 40.89 g)							
ALP	62.34 ± 8.98	50.23 ± 6.71	55.06 ± 5.97	61.54 ± 8.15	63.74 ± 7.36	47.81 ± 4.56	0.460
TP	26.44 ± 1.00 ^{a}	28.52 ± 1.20 ^{a}	33.83 ± 0.11 ^{b}	30.15 ± 1.16 ^{a}	28.33 ± 0.79 ^{a}	26.93 ± 1.75 ^{a}	0.009
ALB	4.17 ± 0.52	1.85 ± 0.10	2.96 ± 0.70	4.34 ± 0.64	3.28 ± 0.46	4.10 ± 0.36	0.087
GLB	27.59 ± 0.68	26.50 ± 1.51	26.50 ± 1.32	25.58 ± 1.39	25.33 ± 0.67	24.33 ± 2.73	0.611
Urea	0.44 ± 0.05	0.31 ± 0.01	0.45 ± 0.04	0.43 ± 0.05	0.44 ± 0.03	0.45 ± 0.03	0.123
LDL	1.21 ± 0.18	1.05 ± 0.07	1.04 ± 0.12	1.29 ± 0.14	1.02 ± 0.11	1.02 ± 0.15	0.611
HDL	3.01 ± 0.16	2.97 ± 0.26	2.79 ± 0.14	2.98 ± 0.10	2.77 ± 0.07	2.56 ± 0.18	0.356
Crea	38.79 ± 1.48	33.13 ± 0.90	38.47 ± 2.22	37.16 ± 1.37	34.87 ± 1.86	37.66 ± 1.15	0.113

Note: Values are presented as means ± SEM (n = 3). Values with different superscripts in the same row are significantly ($p < 0.05$) different.

3.3. Intestinal Digestive Enzyme Activity

As the dietary protein levels increased, the protease activity in the foregut of blunt snout bream at both growth stages exhibited an initial increase followed by a decrease (Table 4). Notably, the protease activity in the foregut at both fish growth stages peaked in the 32% protein group, which was significantly higher than that in the control group ($p < 0.05$). In the midgut of the larger fish, protease activity was highest in the control group, significantly higher than that of the 30% protein group ($p < 0.05$). Conversely, for the smaller fish, protease activity was highest in the 36% protein group, which was significantly higher than that in all other groups except the 32% protein group ($p < 0.05$). In the larger fish, hindgut protease activity was highest in the 34% protein group, significantly higher than that in the 30% protein group ($p < 0.05$), and not significantly different from other groups ($p > 0.05$). In smaller fish, no significant difference in hindgut protease activity was observed among all groups ($p > 0.05$).

Table 4. Effects of dietary protein levels on digestive enzyme activity in blunt snout bream at two growth stages.

Item	Protein Level (% of Dry Diet)						
	26	28	30	32	34	36	*p* Value
Trial 1 (initial body weight of 153.69 g)							
Protease activity							
Foregut	1.13 ± 0.17 ^{a}	1.35 ± 0.11 ^{a}	2.28 ± 0.06 ^{bc}	2.84 ± 0.06 ^{c}	1.72 ± 0.14 ^{ab}	1.49 ± 0.14 ^{ab}	0.006
Midgut	2.22 ± 0.30 ^{b}	1.42 ± 0.21 ^{ab}	0.81 ± 0.20 ^{a}	1.29 ± 0.31 ^{ab}	1.66 ± 0.24 ^{ab}	1.75 ± 0.20 ^{ab}	0.022
Hindgut	1.66 ± 0.10 ^{ab}	1.63 ± 0.33 ^{ab}	0.71 ± 0.19 ^{a}	1.47 ± 0.20 ^{ab}	1.86 ± 0.16 ^{b}	1.56 ± 0.25 ^{ab}	0.033
Lipase activity							
Foregut	4.40 ± 0.52	4.00 ± 0.26	3.33 ± 0.32	4.12 ± 0.40	4.74 ± 0.44	4.98 ± 0.51	0.939
Midgut	5.44 ± 0.77	3.06 ± 0.24	4.87 ± 0.43	3.51 ± 0.48	3.20 ± 0.29	5.74 ± 0.52	0.109
Hindgut	4.90 ± 0.52	2.65 ± 0.37	3.00 ± 0.43	3.26 ± 0.36	4.41 ± 0.40	5.98 ± 0.63	0.251
Amylase activity							
Foregut	38.81 ± 3.85	37.41 ± 2.16	40.90 ± 4.38	52.46 ± 14.10	53.93 ± 14.20	47.84 ± 9.31	0.081
Midgut	47.24 ± 12.58	33.06 ± 5.76	74.50 ± 0.90	51.86 ± 9.56	37.72 ± 8.00	61.43 ± 11.33	0.067
Hindgut	45.41 ± 6.16	50.64 ± 6.66	42.41 ± 7.73	47.72 ± 10.67	56.75 ± 10.61	54.39 ± 9.19	0.842
Trial 1 (initial body weight of 40.89 g)							
Protease activity							
Foregut	1.27 ± 0.11 ^{a}	1.20 ± 0.19 ^{a}	1.57 ± 0.20 ^{ab}	1.91 ± 0.09 ^{b}	1.71 ± 0.28 ^{ab}	1.47 ± 0.10 ^{ab}	0.02
Midgut	1.29 ± 0.13 ^{a}	1.00 ± 0.23 ^{a}	1.36 ± 0.20 ^{a}	1.50 ± 0.23 ^{ab}	1.35 ± 0.29 ^{a}	2.74 ± 0.53 ^{b}	0.005
Hindgut	1.86 ± 0.27	1.55 ± 0.18	1.47 ± 0.08	1.55 ± 0.13	1.75 ± 0.10	1.78 ± 0.18	0.588
Lipase activity							
Foregut	9.19 ± 0.94	10.19 ± 1.12	12.26 ± 1.20	10.11 ± 1.12	9.46 ± 1.02	10.00 ± 1.09	0.727
Midgut	8.62 ± 1.01	7.70 ± 0.78	9.24 ± 1.04	8.73 ± 1.02	11.31 ± 1.25	11.26 ± 1.05	0.195
Hindgut	7.43 ± 0.77	5.17 ± 0.49	5.93 ± 0.68	5.09 ± 0.64	5.39 ± 0.71	6.48 ± 0.65	0.697
Amylase activity							
Foregut	48.35 ± 4.38	49.13 ± 4.23	61.18 ± 7.67	58.71 ± 4.33	54.10 ± 7.52	40.58 ± 2.57	0.137
Midgut	56.36 ± 6.43	47.07 ± 6.03	61.02 ± 3.62	47.37 ± 3.93	51.21 ± 6.54	67.19 ± 6.45	0.098
Hindgut	78.48 ± 6.56 ^{b}	72.40 ± 7.26 ^{ab}	38.97 ± 5.52 ^{a}	60.94 ± 12.44 ^{ab}	60.58 ± 9.72 ^{ab}	55.12 ± 5.53 ^{ab}	0.043

Note: Values are presented as means ± SEM (n = 3). Values with different superscripts in the same row are significantly ($p < 0.05$) different.

The dietary protein levels had no significant difference on the lipase activity in the foregut, midgut, or hindgut at either growth stage of fish ($p > 0.05$). Similarly, the dietary protein levels did not significantly affect the amylase activity in the foregut or midgut at either growth stage of fish ($p > 0.05$). However, in the hindgut of the smaller fish, the amylase activity was the highest in the control group, significantly higher than that in the 30% protein group ($p < 0.05$), with no significant difference compared to the other groups ($p > 0.05$). In contrast, no significant difference in the hindgut amylase activity was observed among the groups of larger fish ($p > 0.05$).

4. Discussion

The results of this study indicate that the dietary protein levels had a significant impact on the growth performance of blunt snout bream, as evidenced by the changes in the final weight, WG, and SGR. The observed trend of an initial increase followed by a subsequent decrease in these growth parameters suggests an optimal dietary protein range for both larger and smaller fish. This pattern is consistent with previous research in aquaculture, which has identified protein requirement plateaus for various fish species [24]. For the larger fish, the peak values of WG and SGR were achieved with diets containing 30%, 32%, and 34% protein, with the highest values observed at 30%. This suggests that the protein requirement for optimal growth in larger fish is around 30%, and that increasing protein levels beyond this point does not provide additional benefits. This finding is similar to those of studies that have reported optimal protein levels for growth in other fish species, such as *Oreochromis niloticus* [25] and *Lepomis macrochirus* [24]. In the smaller fish, the highest WG and SGR were also observed with a 30% protein diet. This indicates that smaller fish have a similar optimal protein requirement, also around 30%. The similar response of the growth parameters to the dietary protein levels between the larger and smaller fish

is consistent with previous research that has reported similar protein requirements for different life stages of the same fish species. Specifically, no differences were found in the protein requirement for Dicentrarchus labrax with an initial body weight (IBW) of 99 or 160 g [26], humpback grouper *Cromileptes altivelis* (Valenciennes) with an IBW of 136, 175, or 225 g [27], and Scophthalmus maximus with an IBW of 4, 59, or 209 g [28]. In addition, according to broken-line regression analysis of the WG against dietary protein, the optimal dietary protein contents for the larger fish and smaller fish were determined to be 30.45% and 29.95%, respectively. These results further support the notion that the optimal protein requirement for growth is relatively consistent across different life stages and sizes within the same fish species. The broken-line regression analysis provided a more precise estimation of the optimal protein level, and the slight difference between the larger and smaller fish may be due to variations in their metabolic rate, digestive efficiency, or other physiological factors. The overall trend is clear: an intermediate level of dietary protein is beneficial for optimal growth in both larger and smaller fish, while excessive protein does not confer additional advantages. This has important implications for aquaculture practices, as it suggests that feed formulations can be optimized to meet the protein requirements of fish without unnecessary over-supplementation. Such optimizations can lead to more cost-effective production, reduced environmental impact, and improved animal welfare.

Interestingly, the dietary protein levels did not affect the feed conversion ratio (FCR), viscerosomatic ratio (VR), hepatosomatic index (HSI), or condition factor (CF) of the two sizes of fish. Our results suggest that while protein levels affected WG, they did not significantly alter the efficiency of feed utilization or the overall health status of the fish, as indicated by the VR, HSI, and CF. The absence of significant differences in these parameters across treatments imply that the fish were able to efficiently utilize the protein provided in the diets, and that the protein levels tested were not so high as to cause adverse effects on the fish's health or metabolism. This finding is supported by previous research that has reported similar results in terms of feed utilization and health status in fish fed diets with different protein levels [27,29,30].

The consistent plasma levels of alkaline phosphatase (ALP), albumin (ALB), globulin (GLB), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) across all the various treatments administered indicate that the protein levels tested did not have any adverse effects on the metabolic processes associated with these biochemical parameters. This suggests that the tested protein levels maintained normal metabolic functions without causing any significant disruptions [31]. However, the total protein (TP) content was highest in both sizes of fish when fed a diet containing 30% protein. This suggests that a 30% protein diet may be optimal for promoting protein synthesis and maintaining plasma TP levels in blunt snout bream, irrespective of fish size. TP in the blood is a critical indicator of overall health and nutritional status in fish, as it reflects the balance between protein synthesis and degradation [32]. The observed increase in TP levels with a 30% protein diet aligns with findings in other species, where dietary protein levels have been shown to directly influence plasma TP concentrations [33,34]. Moreover, the relationship between dietary protein intake and TP levels is crucial for understanding the metabolic demands of fish during growth and development [15,35]. In juvenile fish, higher protein diets have been associated with enhanced growth rates and improved feed conversion efficiency, which are correlated with increased TP levels [36]. The maintenance of normal TP levels is also indicative of a well-balanced diet that supports the immune system and the overall health of the fish. Therefore, the observed increase in TP levels with a 30% protein diet not only reflects enhanced protein synthesis but also suggests improved immune function and health status.

In the present study, the observation of elevated plasma urea content in the larger fish that were fed diets with higher protein levels indicates that protein metabolism might be more efficient and perhaps more optimal at lower protein levels for this particular size group of fish. This is evidenced by the fact that the urea levels did not escalate further in the fish fed diets containing up to 34% protein, suggesting a plateau or an upper limit to the efficiency of protein metabolism at these higher levels [31]. Additionally, the higher plasma creatinine levels observed in the larger fish could be attributed to their greater muscle mass and overall metabolic activity when compared to their smaller counterparts. The increased muscle mass in larger fish likely results in higher metabolic rates and, consequently, higher creatinine production [37].

The observed trends in protease activity within the gastrointestinal tract of blunt snout bream in response to varying dietary protein levels provide intriguing insights into the digestive physiology of this species. The initial increase, followed by a subsequent decrease in protease activity in the foregut of both the small and large fish, suggests an adaptive response to the protein content in the diet [38]. The peak protease activity at the 32% protein level in the foregut indicates an optimal dietary protein concentration for protease induction in blunt snout bream, which aligns with the notion that protein intake is a critical regulator of proteolytic enzyme production [38]. Interestingly, the midgut protease activity in the larger fish peaked in the control group, which was significantly higher than that of the 30% protein group. This finding implies that lower protein levels could potentially stimulate higher protease activity in the midgut of larger fish, possibly as a compensatory mechanism to enhance protein digestion [39]. Conversely, in the smaller fish, the 36% protein level resulted in the highest protease activity, suggesting that higher protein diets could also stimulate protease production, albeit with a different optimal concentration than observed in the foregut.

In the present study, we observed that the hindgut protease activity was highest in the larger fish fed a diet containing 34% protein, which was significantly higher than that in the group receiving 30% protein. This finding suggests that the dietary protein level had a significant impact on the protease activity in the hindgut of the larger fish. The increased protease activity in the higher protein group could be attributed to the greater demand for protein digestion, as higher dietary protein levels require more efficient enzymatic breakdown to meet the nutritional requirements of the fish [40]. However, no significant differences in the hindgut protease activity were observed among the groups of smaller fish. Our result suggests that the protein level may have a less pronounced effect on protease activity in the hindgut of smaller individuals. Similar results were also found in *Haliotis laevigata* [41]. Moreover, the optimal protein level for protease induction in the hindgut may differ from that in the foregut and midgut. This difference could be due to the distinct physiological roles and digestive capacities of these gut segments. It is plausible that the hindgut may require a higher protein level to stimulate protease production, particularly in larger fish, to ensure the efficient digestion and absorption of dietary proteins. The differences in protease activity responses between small and large fish further highlight the complexity of dietary protein regulation in fish digestion. The adaptive mechanisms observed in the foregut, midgut, and hindgut suggest that the digestive system of blunt snout bream is capable of fine-tuning protease production in response to dietary protein levels. This suggests that the digestive system of blunt snout bream is not only responsive to dietary protein levels but also exhibits spatial regulation of protease activity within different gastrointestinal regions. In a study of yellowtail kingfish (*Seriola lalandi*), it was also found that under the action of nutrients, the protease activity of different intestinal segments also showed different adaptive changes [23].

The absence of significant differences in the lipase activity across different dietary protein levels in both sizes of fish suggests that the dietary protein concentration does not significantly influence lipase production or activity in blunt snout bream. Similar results were also found in studies of *Apostichopus japonicus* [42], *Rhamdia quelen* [43], *Eriocheir sinensis* [44], and *Macrobrachium nipponense* [45]. Similarly, the lack of significant effects on the amylase activity in the foregut and midgut of both sizes of fish indicates that dietary protein levels do not substantially impact amylase production or activity in these gut regions. However, in the hindgut of the smaller fish, the control group exhibited the highest amylase activity, which was significantly higher than that in the 30% protein group. Our results suggest that lower protein diets may support amylase activity in the hindgut of smaller fish, potentially due to a compensatory mechanism to utilize carbohydrates more efficiently when protein intake is limited [36].

Therefore, the differential responses observed among the foregut, midgut, and hindgut, as well as between the different sizes of fish, underscore the importance of considering the digestive physiology of specific gut regions and life stages when formulating diets for optimal nutrient utilization.

5. Conclusions

In summary, the results show that dietary protein levels affect the growth performance of blunt snout bream at two growth stages. Based on broken-line regression analysis, the optimal dietary protein levels for larger and smaller fish are recommended to be 30.45% and 29.95% of the diet, respectively. The dietary protein levels maintained normal metabolic functions, and a 30% protein diet might be optimal for maintaining plasma TP levels. The digestive system had adaptive responses to the protein levels. They impacted the protease activity in the gastrointestinal tract, with the optimal protein concentrations for protease induction differing among the gut regions. The larger fish had significant differences in the protease activity with higher-protein diets, especially in the foregut and hindgut, while the smaller fish showed the same in the foregut but less pronounced effects in the hindgut. The dietary protein levels had no significant impact on the lipase activity and minimally influenced the amylase activity in the foregut and midgut, but lower-protein diets might support amylase activity in the hindgut of smaller fish.

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