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ORIGINAL ARTICLE

Aquaculture

Optimal dietary protein to energy ratio for juvenile peanut worm *Sipunculus nudus* Linnaeus

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Abstract The optimal dietary protein to energy (*P*/*E*) ratio for juvenile peanut worm *Sipunculus nudus* Linnaeus (initial average weight 46.16 ± 0.07 mg) was determined using practical diets in a 3 × 3 factorial experiment. Three dietary protein levels (38, 43, and 48 %) and three lipid levels (6, 9, and 12 %) were tested, yielding *P*/*E* ratios of 19.5–26.4 mg protein kJ^{-1}. Each diet was fed to juveniles in three plastic tanks (65 × 55 × 45 cm) for 56 days. *S. nudus* juveniles fed a diet containing 43 % protein and 9 % lipid, yielding a *P*/*E* ratio of 23.1 mg protein kJ^{-1}, presented the highest specific growth rate among the treatments ($P < 0.05$). The highest protease and lipase activities were observed in *S. nudus* juveniles fed a diet with a *P*/*E* ratio of 23.1 mg protein kJ^{-1}. Carcass moisture and ash contents were not significantly affected by the dietary *P*/*E* ratio ($P > 0.05$). Carcass protein content improved as dietary protein increased at each lipid level, and carcass lipid content improved as dietary lipid increased at each protein level. The results indicate that feeding *S. nudus* a diet containing 43 % protein and 9 % lipid, with a *P*/*E* ratio of 23.1 mg protein kJ^{-1}, leads to optimal growth performance.

Keywords *Sipunculus nudus* Linnaeus · Protein to energy ratio · Growth · Digestive enzyme activity · Body composition

Introduction

The peanut worm *Sipunculus nudus* Linnaeus, which belongs to the phylum Sipuncula, is one of the most economically valuable species in China. The body of the adult worm is around 15 cm in length but can reach up to 25 cm in some cases. In addition to its good flavor, *S. nudus* has long been used as a traditional Chinese medicine in folk remedies due to its capacity to alleviate the symptoms of various conditions, such as hypertension, neurosism, cough with dyspnea, and frequent urination. Because of its tender flesh, delicious taste, high nutrition, and medicinal value, *S. nudus* is also known as the marine *Ophiocordyceps sinensis*. A warm-water species, *S. nudus* is widely distributed in the Pacific, Indian, and Atlantic oceans, especially in the south-coastal areas of China such as Fujian, Guangdong, and Guangxi. Among these, Guangxi has abundant resources of *S. nudus* [1]. Following a breakthrough in artificial *S. nudus* breeding in 2004, aquaculture of this species has developed rapidly in China, especially in subtidal zones of the Beibu Gulf. This is an omnivorous species, but studies of its nutritional requirements and feed preparation for this species are urgently required. Research on *S. nudus* has thus far mainly focused on its reproductive biology, metabolism, physiology, and ecology [2]. However, limited research has been conducted on the nutritional

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requirements of *S. nudus*. Zhang et al. [3, 4] reported that the best growth performance of juvenile *S. nudus* was obtained when the dietary protein and lipid levels were 46.79 and 8.70 %, respectively.

Dietary protein and lipid are both important determinants of the growth rates of aquatic animals. Protein is a readily available source of dietary energy, and dietary protein is metabolized for energy instead of being used for tissue growth when non-protein energy sources are lacking in the diet. Lipid is a source of dietary energy and essential fatty acids, and can reduce the need for protein in the diet [5–7]. It is well known that protein utilization can be improved by partially replacing protein with lipid in some fish species [8, 9], and that the optimum dietary protein level for growth can sometimes be lowered if dietary energy is increased by boosting lipid intake. Therefore, the optimal dietary protein to energy (*P*/*E*) ratio should be taken into account when the diet of *S. nudus* is formulated. Bearing this in mind, the present study was conducted to evaluate the optimal *P*/*E* ratio for *S. nudus*.

Although the dietary protein and lipid requirements of *S. nudus* juveniles have already been studied, the interaction between dietary protein and lipid has not. Thus, the other objective of the present study was to determine whether there are any effects of the interaction between protein and lipid on growth performance, digestive enzyme activities, and body composition of *S. nudus* juveniles.

Materials and methods

Experimental diets

A 3 × 3 factorial design with three replicates was used in this study. Nine experimental diets were formulated to include three protein levels (38, 43, and 48 %) and three lipid levels (6, 9, and 12 %), producing a range of *P*/*E* ratios from 19.5 to 26.4 mg protein kJ^{-1}. The experimental diets were denoted diet 1 (38/6), diet 2 (38/9), diet 3 (38/12), diet 4 (43/6), diet 5 (43/9), diet 6 (43/12), diet 7 (48/6), diet 8 (48/9), and diet 9 (48/12), respectively. The formulation and proximate composition of each experimental diet are presented in Table 1.

Fish meal (crude protein: 70.7 % dry matter, crude lipid: 7.1 % dry matter), soybean meal (crude protein: 53.5 % dry matter, crude lipid: 1.9 % dry matter), shrimp shell meal (crude protein: 39.2 % dry matter, crude lipid: 8.2 % dry matter), and squid visceral meal (crude protein: 60.23 % dry matter, crude lipid: 4.82 % dry matter) were purchased from Guangdong Evergreen Industry Co. Ltd. (Zhanjiang, China), while other feed ingredients were purchased from Weifang Conqueren Bioscience & Technology Co. Ltd. (China). All solid ingredients were ground into fine powder and mixed thoroughly with soybean oil and cod oil in a feed mixer (SHJY0.02, Huida Tech., China). An appropriate amount of water was added to the homogeneous mixture until stiff dough was produced. The wet dough was then pelleted using a laboratory pellet machine (YK-90, Fenghao Tech., China) and the pellets were dried in a ventilated oven (DHG9000, Zhongkewantong Tech., China) at room temperature. When they were dry, the pellets were broken up and sieved to achieve the appropriate size. All diets were then stored at −20 °C in separate plastic-lined bags until they were used.

Animals and feeding trials

S. nudus juveniles were obtained from the New Species Hatchery of the Engineering Technology Research Center in Beihai (Guangxi, China). Prior to the experiment, the juveniles were reared in the Key Laboratory of Marine Biotechnology of Guangxi (Beihai, China) for 10 days to acclimate them to the experimental conditions. During this period, they were fed the same commercial feed purchased from Weifang Conqueren Bioscience & Technology Co. Ltd. as used previously at the hatchery. After the acclimation period, the juveniles (average body weight: 46.16 ± 0.07 mg) were randomly distributed into 27 plastic tanks (65 × 55 × 45 cm), and each tank was stocked initially with 500 juveniles. A thin layer of sand (about 3–4 cm thick) was placed at the bottom of the tank to act as a perch for the juveniles. *S. nudus* were group-weighed at the beginning and end of the experiment after digestion track evacuation. Mortality was recorded daily.

Prior to feeding, one-third of the plastic tank water was changed to ensure good water quality. *S. nudus* juveniles were hand-fed twice daily (0900 and 1700 hours) for 56 days. The plastic tank was cleaned thoroughly once a week. During the feeding trial, the water temperature ranged from 24 to 28 °C, the salinity from 18 to 22 ‰, and the dissolved oxygen level was maintained at ~5.0 mg l^{-1}.

Sample collection and chemical analysis

To facilitate digestion track evacuation, the juveniles were moved for 2 days to new plastic tanks with no sand at the bottom. The following calculations were performed:

$$\text{Survival rate } (\%) = N_t/N_0 \times 100$$

$$\text{Specific growth rate (SGR)} = (\ln W_t - \ln W_0) \times 100/t,$$

where N_t and N_0 are the final and initial numbers of *S. nudus*, respectively, W_t and W_0 are the final and initial weights of *S. nudus*, respectively, and t is the duration of the experiment in days.

Table 1 Formulation and proximate composition of each experimental diet (% dry matter)

Ingredient	Diet groups (protein/lipid)								
	38/6	38/9	38/12	43/6	43/9	43/12	48/6	48/9	48/12
Fish meal[a]	25.00	25.00	25.00	30.00	30.00	30.00	35.00	35.00	35.00
Shrimp shell meal[a]	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
Squid visceral meal[a]	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Macroalgae (*Sargassum thunbergii*) meal[a]	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Soybean meal[a]	19.05	19.95	20.75	19.25	20.25	21.25	18.25	19.05	19.85
Wheat meal	28.20	24.20	20.20	23.30	19.30	15.30	19.50	15.80	12.10
Soybean oil	0.35	1.90	3.50	0.20	1.70	3.20	0.10	1.55	3.00
Cod oil	0.35	1.90	3.50	0.20	1.70	3.20	0.10	1.55	3.00
Soybean lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin premix[b]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral premix[c]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Ethoxyquin	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Proximate composition									
Dry matter	93.3	92.5	92.8	91.9	91.2	92.3	92.7	94.1	93.8
Crude protein (CP)	37.6	37.8	38.2	43.2	43.4	43.3	48.1	48.5	48.6
Crude lipid	5.9	9.1	12.1	6.0	8.9	11.9	6.1	8.9	11.8
Ash	11.6	11.9	12.0	12.1	12.3	12.6	12.7	13.1	13.3
Gross energy (GE, MJ kg^{-1})	18.4	18.9	19.6	18.3	18.8	19.4	18.2	18.8	19.3
CP:GE (g MJ^{-1})	20.4	20.0	19.5	23.6	23.1	22.3	26.4	25.8	25.2

[a] Fish meal: crude protein 70.7 % dry matter, crude lipid 7.1 % dry matter; soybean meal: crude protein 53.5 % dry matter, crude lipid 1.9 % dry matter; shrimp shell meal: crude protein 39.2 % dry matter, crude lipid 8.2 % dry matter; squid visceral meal: crude protein 60.23 % dry matter, crude lipid 4.82 % dry matter; macroalgae (*Sargassum thunbergii*) meal: crude protein 12.8 % dry matter

[b] Composition (IU or g kg^{-1} vitamin premix): retinal palmitate, 1,500,000 IU; cholecalciferol, 300,000 IU; DL-α-tocopherol acetate, 20.0 g; menadione, 8.0 g; thiamin-HCl, 5.0 g; riboflavin, 5.0 g; D-calcium pantothenate, 16.0 g; pyridoxine–HCl, 4.0 g; meso-inositol, 200.0 g; D-biotin, 8.0 g; folic acid, 1.5 g; *para*-aminobenzoic acid, 5.0 g; niacin, 20.0 g; cyanocobalamin, 0.01 g

[c] Composition (g kg^{-1} mineral premix): $CoSO_4 \cdot 4H_2O$, 0.30; $CuSO_4 \cdot 5H_2O$, 10.0; $FeSO_4 \cdot 7H_2O$, 100.0; KCl, 100.0; KI, 0.2; $MgSO_4 \cdot 2H_2O$, 203.4; $MnSO_4 \cdot 4H_2O$, 36.0; NaCl, 160.0; $Na_2SeO_3 \cdot H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 40.0

For enzyme assays, a frozen sample of *S. nudus* was homogenized in ten volumes (W/V) of ice-cold double-distilled water by an electric homogenizer (T-25, IKA, Staufen, Germany). Homogenates were then centrifugated (KR25i, Thermo, Schwerte, Germany) at 15,000×*g* for 15 min at 4 °C to analyze digestive enzyme activity. After centrifugation, the supernatants were collected and then stored at 4 °C prior to analysis. All enzymatic assays were conducted within 24 h after extraction.

Protease activity was determined by the Folin-phenol reagent method [10]. Amylase activity was measured via the method of Worthington [11], using iodine solution to reveal nonhydrolyzed starch. Lipase activity was determined by measuring fatty acid release due to the enzymatic hydrolysis of triglycerides in a stabilized emulsion of olive oil [12]. Enzyme activities were expressed as specific activities (U mg^{-1} protein). One unit of protease activity was defined as 1 μg tyrosine liberated by hydrolyzing casein in 1 min at 37 °C. One unit of amylase activity was defined as 1 μg maltobiose liberated by starch in 1 min at 25 °C. One unit of lipase activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of fatty acids in 1 min at 37 °C. Specific activities were expressed as the enzyme activity per mg protein. Protein was determined via the method of Bradford [13], using bovine serum albumin as a standard. Glucose, salicylic acid, pyrogallic acid, tris(hydroxymethyl)aminomethane, and phenol were purchased from Sigma (St. Louis, MO, USA).

Proximate composition analysis of the feed ingredients, diets, and *S. nudus* samples was performed using the standard method of the AOAC [14]. Samples of diets and *S. nudus* were dried to a constant weight at 105 °C to determine moisture. Protein was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method (Kjeltec 8400, FOSS, Hillerød, Denmark); lipid by ether extraction using Soxhlet (Soxtec 2050, Gerber Instruments, Illnau-Effretikon, Switzerland); ash by combustion at 550 °C, and gross energy by an adiabatic bomb calorimeter (model 6400, Parr, Moline, IL, USA).

Table 2 Growth responses of *Sipunculus nudus* juveniles that were fed diets with different *P*/*E* ratios

Diet no. (protein/lipid)	Initial body weight (mg)	Final body weight (mg)	Feed amount of each treatment (g)	Specific growth rate (% day^{-1})	Survival (%)
Diet 1 (38/6)	45.90 ± 0.16	263.01 ± 3.42^{a}	103.45	3.49 ± 0.03^{a}	88.92 ± 0.79
Diet 2 (38/9)	46.14 ± 0.10	278.68 ± 9.34^{ab}	106.23	3.59 ± 0.08^{ab}	90.58 ± 1.88
Diet 3 (38/12)	46.50 ± 0.34	271.84 ± 2.19^{ab}	102.94	3.53 ± 0.01^{ab}	87.33 ± 0.65
Diet 4 (43/6)	46.13 ± 0.19	298.10 ± 5.74^{bc}	107.92	3.73 ± 0.03^{bc}	91.00 ± 0.52
Diet 5 (43/9)	46.18 ± 0.32	315.04 ± 7.98^{c}	116.18	3.84 ± 0.04^{c}	89.67 ± 0.60
Diet 6 (43/12)	46.27 ± 0.29	298.13 ± 8.50^{bc}	112.64	3.72 ± 0.06^{bc}	90.42 ± 0.73
Diet 7 (48/6)	46.09 ± 0.02	262.40 ± 5.57^{a}	99.23	3.48 ± 0.05^{a}	87.75 ± 0.72
Diet 8 (48/9)	46.32 ± 0.22	264.55 ± 5.49^{a}	101.38	3.48 ± 0.03^{a}	88.17 ± 0.85
Diet 9 (48/12)	45.90 ± 0.16	257.71 ± 2.92^{a}	100.79	3.45 ± 0.02^{a}	88.92 ± 0.79
One-way ANOVA (*P* value)					
P/*E* effect	0.659	0.000		0.000	0.104
Two-way ANOVA (*P* value)					
Protein effect	0.864	0.000		0.000	0.036
Lipid effect	0.545	0.105		0.096	0.742
Protein × lipid effect	0.392	0.718		0.708	0.159

Means ± SEM ($n = 3$) that have the same superscripts and are in the same column are not significantly different ($P > 0.05$)

All the chemicals and reagents used in the enzyme assays and proximate composition analysis were of analytical grade and purchased from Qingdao Fulin Biochemical Co. Ltd. (China).

Statistical analysis

All data were analyzed by SPSS 15.0 for Windows. One-way analysis of variance (one-way ANOVA) was used to determine whether there were significant differences between the treatments. When overall differences were found, differences between means were determined and compared by Tukey's honest significant difference post hoc test. Two-way ANOVA was used to analyze the synergistic effects between protein and lipid on growth performance, digestive enzyme activities, and body composition of *S. nudus* juveniles. All differences were considered significant at $P < 0.05$. All data are presented as the mean ± standard error of the mean (SEM) of three replicates.

Results

Growth

Final body weights, feed amounts, SGRs, and survival rates of the juveniles fed graded ratios of protein to energy (*P*/*E*) are presented in Table 2. Two-way ANOVA testing showed that final body weight and SGR were significantly affected by the dietary protein level but not by the lipid level ($P > 0.05$). *S. nudus* juveniles that were fed diets with 43 % protein (*P*/*E* of about 23.0 mg kJ^{-1}) had a significantly higher final body weight and SGR than those fed diets with 38 % protein (*P*/*E* of about 20.0 mg kJ^{-1}) and 48 % protein (*P*/*E* of about 25.5 mg kJ^{-1}). There was no effect of the interaction between dietary protein and lipid levels on final body weight or SGR ($P > 0.05$). Among all the treatments, *S. nudus* juveniles fed 43 % protein and 9 % lipid (*P*/*E* of 23.1 mg kJ^{-1}) had the highest final body weight and SGR. Moreover, the survival rate of *S. nudus* ranged from 87.33 to 91.00 % and was not significantly affected by dietary protein level, lipid level, or *P*/*E* ratio during the whole experimental period.

Digestive enzyme activities

Two-way ANOVA testing revealed that protease activity was significantly affected by protein and lipid levels, and a significant interaction was found between the protein and lipid levels ($P < 0.05$). We first compared the protease activities that were observed when the protein level was fixed while the lipid level was varied. When the protein level was 38 %, the protease activities observed at three lipid levels were similar. When fed on 43 or 48 % protein, juveniles fed 9 % lipid showed greater protease activity than juveniles fed 6 and 12 % lipid (Table 3) ($P < 0.05$). Next we varied the protease activity while fixing the lipid level. When the lipid level was 6 %, the protease activity observed at a protein level of 48 % (*P*/*E* of 26.4 mg kJ^{-1}) was significantly higher than the protease activities observed at protein levels

Table 3 Digestive enzyme activities of *Sipunculus nudus* juveniles that were fed diets with different *P*/*E* ratios

Diet no. (protein/lipid)	Protease activity (U mg^{-1} protein)	Amylase activity (U mg^{-1} protein)	Lipase activity (U mg^{-1} protein)
Diet 1 (38/6)	0.77 ± 0.01^{a}	2.71 ± 0.03^{bc}	0.68 ± 0.02^{a}
Diet 2 (38/9)	0.78 ± 0.02^{a}	2.79 ± 0.04^{c}	0.75 ± 0.03^{a}
Diet 3 (38/12)	0.75 ± 0.01^{a}	2.77 ± 0.05^{c}	0.69 ± 0.01^{a}
Diet 4 (43/6)	0.87 ± 0.02^{a}	2.65 ± 0.04^{abc}	0.93 ± 0.04^{b}
Diet 5 (43/9)	1.20 ± 0.03^{d}	2.74 ± 0.03^{c}	0.97 ± 0.02^{b}
Diet 6 (43/12)	1.07 ± 0.05^{bc}	2.66 ± 0.01^{abc}	0.91 ± 0.02^{b}
Diet 7 (48/6)	1.08 ± 0.04^{bc}	2.64 ± 0.02^{abc}	0.76 ± 0.03^{a}
Diet 8 (48/9)	1.12 ± 0.02^{cd}	2.54 ± 0.05^{ab}	0.88 ± 0.01^{b}
Diet 9 (48/12)	0.99 ± 0.03^{b}	2.48 ± 0.03^{a}	0.74 ± 0.02^{a}
One-way ANOVA (*P* value)			
P/*E* effect	0.000	0.000	0.000
Two-way ANOVA (*P* value)			
Protein effect	0.000	0.000	0.000
Lipid effect	0.000	0.239	0.000
Protein × lipid effect	0.000	0.035	0.341

Means ± SEM ($n = 3$) that have the same superscripts and are in the same column are not significantly different ($P > 0.05$)

of 38 % (*P*/*E* of 20.4 mg kJ^{-1}) and 43 % (*P*/*E* of 23.6 mg kJ^{-1}) ($P < 0.05$). At a lipid level of 9 or 12 %, protein levels of 43 and 48 % yielded similar protease activities which were higher than that observed at 38 % protein ($P < 0.05$). Among all of the test diets, the *S. nudus* juveniles fed on 43 % protein and 9 % lipid (*P*/*E* of 23.1 mg kJ^{-1}) presented the highest protease activity ($P < 0.05$).

Amylase activity was significantly affected by dietary protein level ($P < 0.05$), but not by lipid level ($P > 0.05$). Moreover, there was a significant effect of the interaction between dietary protein and lipid levels on amylase activity ($P < 0.05$). Amylase activities of the juveniles fed diets with 38 % protein (*P*/*E* of about 20.0 mg kJ^{-1}) were higher than those of the juveniles fed diets with 43 % protein (*P*/*E* of about 23.0 mg kJ^{-1}) or 48 % protein (*P*/*E* of about 25.5 mg kJ^{-1}) at each lipid level (Table 3). At lipid levels of 9 and 12 %, the amylase activities of *S. nudus* juveniles fed diets with 48 % protein were significantly lower than those of the juveniles fed diets with 38 and 43 % protein. *S. nudus* juveniles that were fed 48 % protein and 12 % lipid (*P*/*E* of 25.2 mg kJ^{-1}) produced the lowest amylase activities, which were significantly different from those of the juveniles fed the diets with the lowest level of dietary protein (38 %) ($P < 0.05$).

Lipase activities of the juveniles were significantly affected by both protein and lipid levels ($P < 0.05$). Lipase activities of *S. nudus* juveniles that were fed diets with 43 % protein (*P*/*E* of about 23.0 mg kJ^{-1}) were significantly higher than those of juveniles fed 38 % (*P*/*E* of about 20.0 mg kJ^{-1}) or 48 % protein (*P*/*E* of 25.2 mg kJ^{-1}) at each lipid level ($P < 0.05$). Lipase activities of juveniles fed 9 % lipid were significantly higher than those fed 6 and 12 % lipid at a protein level of 48 %. However, there was no effect on the lipase activity of the interaction between protein level and lipid level ($P > 0.05$).

Body composition

Whole-body moisture and ash contents were not significantly affected by either dietary protein or lipid level ($P > 0.05$, Table 4), although ash content declined as the protein level increased.

Carcass protein content was significantly affected by both protein and lipid levels ($P < 0.05$). Carcass protein content improved with increasing protein level at each lipid level. *S. nudus* juveniles that were fed 48 % protein and 6 % lipid (*P*/*E* of 25.2 mg kJ^{-1}) presented the highest protein contents and were significantly different from groups fed 38 % protein at each lipid level and 43 % protein at the 6 or 9 % lipid level ($P < 0.05$). However, no interaction was found between dietary protein and lipid level ($P > 0.05$).

Carcass lipid content was significantly affected by both protein and lipid levels ($P < 0.05$). Carcass lipid content improved as dietary lipid level increased at each protein level, while carcass lipid content decreased with increasing dietary protein content at every lipid level. *S. nudus* juveniles that were fed 48 % protein and 6 % lipid (*P*/*E* of 26.4 mg kJ^{-1}) produced the lowest lipid contents and were significantly different from the groups that were fed the diets with the lowest level of dietary protein (38 %) and the diet with 43 % protein and 12 % lipid (*P*/*E* of 22.3 mg kJ^{-1}) ($P < 0.05$). However, there was no interaction between the dietary protein and lipid levels ($P > 0.05$).

Table 4 Whole-body compositions of *Sipunculus nudus* juveniles that were fed the seven diets with different *P*/*E* ratios (g kg^{-1} wet matter)

Diet no. (protein/lipid)	Moisture	Crude protein	Crude lipid	Ash
Diet 1 (38/6)	789.53 ± 2.77	146.48 ± 1.34^{a}	5.54 ± 0.10^{c}	36.38 ± 0.41
Diet 2 (38/9)	783.70 ± 2.93	143.57 ± 2.54^{a}	5.74 ± 0.04^{c}	36.19 ± 0.59
Diet 3 (38/12)	784.83 ± 4.36	142.25 ± 2.47^{a}	5.77 ± 0.09^{c}	36.35 ± 0.88
Diet 4 (43/6)	773.60 ± 6.99	149.36 ± 3.94^{ab}	4.58 ± 0.30^{ab}	36.35 ± 0.41
Diet 5 (43/9)	779.27 ± 3.45	146.07 ± 0.61^{a}	4.65 ± 0.15^{ab}	35.89 ± 0.56
Diet 6 (43/12)	781.90 ± 2.32	145.26 ± 4.05^{a}	5.18 ± 0.18^{bc}	34.19 ± 0.54
Diet 7 (48/6)	782.87 ± 7.10	160.75 ± 3.19^{b}	4.37 ± 0.12^{a}	35.27 ± 0.15
Diet 8 (48/9)	790.37 ± 1.02	152.88 ± 2.32^{ab}	4.51 ± 0.17^{ab}	34.46 ± 0.40
Diet 9 (48/12)	785.37 ± 6.01	148.74 ± 2.33^{ab}	5.12 ± 0.15^{abc}	34.08 ± 0.91
One-way ANOVA (*P* value)				
P/*E* effect	0.334	0.005	0.000	0.033
Two-way ANOVA (*P* value)				
Protein effect	0.079	0.001	0.000	0.008
Lipid effect	0.785	0.021	0.002	0.087
Protein × lipid effect	0.495	0.604	0.380	0.415

Means ± SEM ($n = 3$) that have the same superscripts and are in the same column are not significantly different ($P > 0.05$)

Discussion

Juvenile animals grow mainly by synthesizing proteins from dietary amino acids and dietary energy, so adequate protein and lipid supplies and a balanced *P*/*E* ratio are needed for marine fish [15] and other invertebrates, such as abalone [16], sea cucumber [17], and sea urchin [18]. An appropriate dietary *P*/*E* ratio is of great importance when preparing feed, as it exerts a strong influence on the feed conversion efficiency—especially the efficiency of protein and energy utilization [18, 19]—and it helps to lower the cost of farming animals and to reduce the deterioration in water quality caused by the presence of wasted feed [20]. In the present study, the growth performance of *S. nudus* juveniles was significantly affected by the dietary protein level and *P*/*E* ratio. *S. nudus* juveniles showed optimal growth when they were fed a diet containing 9 % lipid and 43 % protein (*P*/*E* ratio of 23.1 mg kJ^{-1}), which also led to a better survival rate. The worm we chose to study in the present work differs from fish in its lifestyle. Apparent satiation of the worms was measured in a similar way to a sea cucumber [21], as all groups were fed their respective diets at the same fixed rate (1 % of body weight per day) and less than 1 % of the feed was left in the sand by the worms. The amount of feed present at the beginning of the experiment and the amount at the end were weighed and there was no significant difference in those amounts for different treatments. This means that changes in growth rate can be largely attributed to dietary changes, i.e., variations in the protein/energy ratio. The optimum dietary *P*/*E* varies among aquatic species, particularly between cold-water and warm-water species. Cold-water species, which can utilize high levels of dietary lipid for energy, require lower dietary *P*/*E* ratios, e.g., 18 mg kJ^{-1} for *Salmo salar* [22]. In contrast, *P*/*E* ratios for warm-water species are relatively high, e.g., 31 mg kJ^{-1} for *Epinephelus malabaricus* [23], 28 mg kJ^{-1} for *Seriola dumerilii* [24], and 28 mg kJ^{-1} for *Sciaenops ocellatus* [25]. In marine abalone, different species showed different dietary lipid requirements but the same dietary protein level, i.e., various *P*/*E* ratios [26]. *S. nudus* is an evolutionarily primitive warm-water species with an optimum *P*/*E* ratio that is similar to the optimum ratios of 22.9 mg kJ^{-1} for *Labeo rohita* [27], 22.6 mg kJ^{-1} for *Pampus argenteus* [28], and 23.5 mg kJ^{-1} for *Heteropneustes fossilis* [29].

The SGR of *S. nudus* juveniles fed 43 % protein was significantly higher than those of *S. nudus* juveniles fed 38 and 48 % protein at all lipid levels, indicating that the optimum protein level is about 43 % for growth of *S. nudus* juveniles. This is slightly lower than the data reported by Zhang et al. [4], who suggested that the optimal dietary protein level for *S. nudus* juveniles is 46.79 %. This difference in optimal dietary protein level may have been caused by differences in the temperature of the water in which the worms were cultured, the type of protein fed to the worms, and the lipid and/or energy contents of the diets [30]. In the present research, the SGR of *S. nudus* juveniles fed 38 % protein (giving a *P*/*E* of about 20.0 mg kJ^{-1}) was significantly lower, mainly because insufficient dietary protein implies a lack of amino acids for the protein biosynthesis needed to maintain maximum animal growth. However, when 48 % protein was supplied, the SGR and final body weight were significantly lower than those seen when 43 % protein was supplied. This phenomenon has also been reported for fish

such as *Paralichthys olivaceus* [31], *Acipenser transmontanus* [32], *Oreochromis niloticus* [33], and yellow puffer [34], as well as other invertebrates such as sea cucumber [35]. Indeed, excess dietary protein results in extra feed costs and an increase in the nitrogen (N) load in the environment [36, 37]. In many aquatic studies, researchers have found that increasing protein levels beyond those required for growth frequently results in high levels of ammonia production [38]. So, it is likely that ammonia was excreted by *S. nudus* juveniles fed 48 % protein, leading to decreased growth performance and a reduced protein conversion rate [39]. Moreover, ammonia was excreted into the rearing system, negatively impacting the animals' immune systems and resulting in growth inhibition [40].

It is very important to feed *S. nudus* an appropriate level of dietary lipid due to its major role in fulfilling the energy needs of the worms and their essential fatty acid requirements. At all protein levels, the final body weight of *S. nudus* juveniles fed diets with 9 % lipid was higher than that of *S. nudus* juveniles fed 6 % lipid. This may be due to the lower dietary energy levels in the 38, 43, and 48/6 diets, as has also been noted for other marine invertebrates [16–18]. In addition, the SGR of *S. nudus* juveniles fed the 38/6 diet was significantly lower than that of the *S. nudus* juveniles fed the 43/6 diet, while the difference between the SGRs of the *S. nudus* juveniles fed the 38/9 and 43/6 diets was not significant, which may suggest a protein-sparing role of lipid in *S. nudus*. Increasing the dietary lipid level within a suitable range can increase the protein utilization rate, just as an inadequate dietary lipid supply will result in protein degradation, which natively impacts protein utilization and animal growth [41]. This protein-sparing effect of lipid has been found in many species, such as jundia fingerlings [41], Japanese seabass [42], and Nile tilapia [30]. To be precise, the protein-sparing effect observed in *S. nudus* juveniles was very limited and restricted to certain protein levels, as no protein-sparing effect of lipid was detected at higher protein levels. Moreover, excessive levels of lipid may be detrimental to the pelleting quality of feed, the growth of cultured animals, and the shelf-lives of animal products [43, 44], so the application of excess dietary lipid levels is not advised. In the present experiment, there was no significant difference between the *S. nudus* juveniles fed 12 % lipid and those fed 9 % lipid. *S. nudus* juveniles fed 43 % protein and 9 % lipid (*P*/*E* of 23.1 mg protein kJ^{-1}) showed the strongest growth. Thus, the optimum level of lipid for *S. nudus* juveniles appears to be about 9 %, in agreement with the data reported by Zhang et al. [3], who suggested that the optimum dietary lipid level for *S. nudus* juveniles is 8.70 %.

Improving digestive enzyme activity can promote digestion and nutrient absorption, thus promoting growth [45]. Several studies of fish have suggested that the activities of the main digestive enzymes and their responses to different dietary compositions are likely to be the parameters that determine how effective a given diet is at optimizing growth and food utilization [46]. In this study, digestive enzyme activity was highest for *S. nudus* juveniles fed a diet containing 43 % protein and 9 % lipid, with a *P*/*E* ratio of 23.1 mg protein kJ^{-1}, which may be why those juveniles also presented the highest SGR. Protease activity improved significantly as the dietary protein level increased within a certain range, similar to results reported for *Epinephelus malabaricus* [23] and *Haliotis rufescens* [47]. *S. nudus* can adapt to elevated levels of feed protein and energy by increasing digestive enzyme activity. When the protein to energy ratio reached a certain level, protease activity decreased with further increases in the protein level, which is consistent with the findings of Lin et al. [48] for Chinese mitten crab, so feeding a suitable level of lipid can promote the excretion of aquatic animal protease.

The amylase activity of juveniles fed diets with 38 % protein was higher than those of juveniles fed 43 or 48 % protein at each lipid level, demonstrating that amylase activity decreased with increasing dietary protein, in agreement with findings for sea cucumber [49], *Channa punctatus* [50], and *Scortum barcoo* [51]. The group fed 48 % protein and 12 % lipid (*P*/*E* of 25.2 mg protein kJ^{-1}) exhibited the lowest amylase activity in the present study, and previous research on the effect of dietary lipid on juvenile *S. nudus* indicated that the level of dietary lipid did not have a significant effect on amylase activity [3]. The most likely explanation for this finding, when all factors are considered together, is the presence of superfluous protein in the diet.

Studies of *Morone chrysops* [52] and *Clupea harengus* [53] found that lipase activity was associated with the composition and quality of the diet, which was also confirmed by the present study. There have been many studies of the effects of dietary protein level on lipase activity, but the results obtained are inconsistent. In the present study, lipase activity significantly decreased when dietary protein was increased within a particular range. This result agrees with the results of a study on tilapia *Oreochromis niloticus* × *O. aureus* larvae [54], but not with results from studies of Indian carp *Catla catla* fry [55], white sea bream *Diplodus sargus* juveniles [56], and silver barb *Puntius gonionotus* fingerlings [57]. The inconsistent nature of these findings may be due to the different experimental species, protein sources, and culture conditions used in the studies. Lipase activity of *S. nudus* was significantly affected by the level of dietary lipid. When dietary lipid was increased, lipase activity first increased and then decreased. This is in good agreement with the results of Xiang et al. [58], who found that the lipase activity of *Erythroculter ilishaeformis*

increased as the lipid level was increased from 2.07 to 7.14 % and then decreased as the lipid level rose from 7.14 to 15.32 %.

Aside from their impact on growth and digestive enzyme activity, dietary protein and lipid (energy) levels were also observed to affect the body composition of *S. nudus.* In this study, the carcass protein content of *S. nudus* increased with increasing dietary protein. Similar results have been reported for other aquatic animals, such as abalone *Haliotis midae* [59], *Cyprinus carpio* [60], *Channa striata* [61], *Scortum barcoo* [51], and *Acipenser persicus* [62]. However, several researchers have found that carcass protein content is not affected by the dietary protein level [63–65]. Again, these inconsistent findings may be due to differences in the protein levels, protein sources, experimental species, and culture conditions used in the studies. Moreover, the lipid content of *S. nudus* juveniles increased with increasing dietary lipid level, indicating that *S. nudus* can deposit lipid in its body, which inevitably results in an increase in carcass lipid content. This result agrees with findings for blunt snout bream *Megalobrama amblycephala* fingerlings [66], but differs from findings reported for some fish fed high-lipid diets [27, 67]. Once again, the differences in the findings of these studies may be largely due to the different aquatic animal species investigated, as well as differences in the dietary energy and/or protein levels applied in the studies.

In conclusion, this study showed that a diet containing 43 % protein with 9 % lipid, with a *P*/*E* ratio of 23.1 mg protein kJ^{-1}, appears to lead to the highest SGR and digestive enzyme activities of *S. nudus* juveniles. The interaction between dietary protein and lipid level can significantly affect the protease and amylase activities in *S. nudus* juveniles. The present results will be of great interest to those involved in aquaculture research and the peanut worm farming industry.

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