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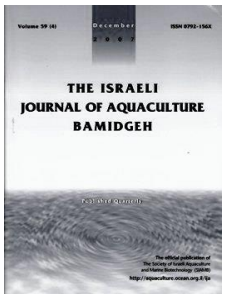

Optimal Dietary Protein Level for the White Shrimp (*Litopenaeus vannamei*) in Low Salinity Water

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Keywords: Protein; *Litopenaeus vannamei*; trypsin; growth performance; body composition

Abstract

In order to find the optimal dietary protein level for *Litopenaeus vannamei* in low salinity water, isolipid and isocaloric diets with different protein levels (25%, 30%, 35%, 40% and 45%) were tested to feed *L. vannamei* juveniles (mean weight 0.31 ± 0.02 g) for 56 days in salinity 2 g/L water. The results showed that: (1) as the dietary protein level increased, the final body weight, weight gain and specific growth rate increased at first and then decreased. In the 35% protein level group, significantly better results were obtained as compared to other groups ($P<0.05$). Through quadratic regression analysis of dietary protein level and weight gain and specific growth rate, we found that shrimps had the highest weight gain when dietary protein level was between 33.51%–34.35%; (2) as the dietary protein level increased, the shrimp moisture content decreased and the protein content increased while lipid and ash content did not significantly change; (3) as the dietary protein level increased, the activity of trypsin increased at first and then decreased, and the 35% protein level group had the highest trypsin activity, significantly higher than other groups ($P<0.05$). There were no significant differences in lipase or amylase activity.

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Introduction

White shrimp (*Litopenaeus vannamei*) are mainly distributed in the Pacific coastal waters from northern Peru to Mexico Sonora area. Due to their superior breeding characteristics, and tolerance to a wide range of salinity (0.5-40 g/L), *L. vannamei* together with *Penaeus monodon* and *Fenneropenaeus chinensis* are considered the world's three major shrimp candidates for aquaculture (Huai et al., 2009). Outbreaks of mariculture diseases, pollution of coastal waters, and increasing market demand have resulted in *L. vannamei* farming from coastal marine waters to inland low salinity waters, such as the Pearl river delta area, Yangtze river delta area, yellow sea region (Cheng et al., 2006) and southern Thailand (Saoud et al., 2003). Inland freshwater production of *L. vannamei*n reached 731,461 tons and accounted for 45.02% of the gross production of *L. vannamei* (China Fishery Statistical Yearbook 2016). Researchers have shown that salinity is an important factor affecting physiological responses of aquaculture animals, and nutrients including amino acids, fatty acids and minerals in feed play an important role in assisting shrimp to cope with unusual culture environments due to their effects on osmoregulation (Robertson et al., 1993; Davis et al., 2002; Huai et al., 2009). Growth and survival of shrimp reared at low salinity can be improved through manipulation of nutrients within formulated feeds (Gong et al., 2004).

Proteins which have numerous structural and metabolic functions play an important role in growth. Protein is considered the major dietary nutrient affecting growth performance of aquatic animals, however the cost of proteins in feed is high and their inclusion in aquaculture diets has had a significant impact on overall feed costs (NRC, 2011). For these reasons, attempts to optimize the amount of dietary protein in aquaculture feeds are necessary. Protein requirements for maximal growth of juvenile white shrimp have been reported to be between 30-36% in brackish or seawater (Kureshy & Davis, 2002). There are few reports on dietary protein requirements of *L. vannamei* juveniles at salinity <5 g/L. In this study, we investigated the effects of different dietary protein levels on growth and body composition of *L. vannamei* at salinity levels as low as 2 g/L. Our results will provide better understanding of the nutritional physiology of *L. vannamei* in low salinity water, and may lead to the development of a complete feed formula for freshwater farmed *L. vannamei*.

Materials and Methods

Experimental diets. Protein sources for the experimental diets were fish meal, soybean meal, casein, and gelatin. Five semi-refined isocaloric feeds with protein levels at 25%, 30%, 35%, 40%, 45%, were prepared. Actual protein levels of 25.55%, 30.07%, 35.38%, 40.87%, and 46.23% respectively (Table 1) were prepared in the laboratory. The ingredients were ground into a fine powder and sifted through 320 μm mesh, weighed and mixed together according to each recipe. Trace components were mixed, packed, numbered and stored at -20°C.

Table 1 Dietary composition and nutrient levels

Ingredients	*content (%)*				
	25%	*30%*	*35%*	*40%*	*45%*
fish meal	20.0	20.0	20.0	20.0	20.0
casein	6.0	10.7	15.4	20.1	24.8
gelatin	1.5	2.7	3.8	5.0	6.2
soybean meal	7.0	7.0	7.0	7.0	7.0
wheat flour	20.0	20.0	20.0	20.0	20.0
fish oil	2.5	2.5	2.5	2.5	2.5
lecithin	2.5	2.5	2.5	2.5	2.5
stay-C	0.1	0.1	0.1	0.1	0.1
monocalcium phosphate	1.0	1.0	1.0	1.0	1.0
vitamin mix	1.0	1.0	1.0	1.0	1.0
mineral mix	1.0	1.0	1.0	1.0	1.0
corn starch	37.4	31.5	25.7	19.8	13.9
Proximate analysis (% dry matter basis)					
crude protein	25.55	30.07	35.38	40.87	46.23
gross energy（MJ/kg DM）	19.14	19.30	19.47	19.63	19.81
crude lipid	7.83	7.78	8.02	7.97	7.81
ash	8.66	8.37	8.44	8.57	8.63
moisture	8.31	8.12	8.29	8.33	8.18

L. vannamei and feeding management. L. vannamei were procured from Guangdong Haixing agriculture biotechnology Ltd., and cultured at the East Sea Island experiment base of Guangdong Ocean University. The water was natural seawater from the East Sea Island of Zhanjiang. Water temperature was 27.5 ± 1.5°C and initial salinity was 30 ± 0.5 g/L; during the nursing period (two weeks) lower salinity was adjusted by gradually adding tap water. The decrease in salinity was no more than 1–2 g/L per day until reduced from 30g/L to 2 g/L. Healthy shrimp with similar body length and weight were selected (average body weight 0.31 ± 0.02 g) and divided into five groups with dietary protein levels of 25%, 30%, 35%, 40% and 45%. Each treatment was replicated three times with 40 shrimps per replicate, reared in a 350 L polyethylene tank for eight weeks. The shrimps were fed 4 times per day, at 6:00, 11:00, 17:00, and 22:30 respectively. Rations were ad libitum. The remaining feed and waste were siphoned daily to keep water clean.

Sample collection and analysis. At the end of the experiment, shrimps were fasted for 24 h, and then weighed and the number of surviving individuals was counted. Body weight and other growth indicators were measured. For each replicate, five shrimps were randomly selected and dissected on ice. The liver and pancreas were collected, and excess fat was removed. The tissues were then washed with saline and dried with a paper towel. The samples were stored in a pre-chilled centrifuge tube (RNase free), and frozen at -80°C.

Whole shrimp composition was analyzed by standard methods (AOAC, 2003). Moisture content was measured after drying at 105°C; crude protein was measured by the micro Kjeldahl method; crude lipid was determined by Soxhlet extraction method. The organic extraction solvent was ether; ash was measured by heating at 550°C in a muffle furnace.

Frozen tissues were thawed at 4°C and 0.5g tissue sample was weighed. After adding 5 volumes of cold saline, tissue was homogenized in a glass homogenizer at 4°C. The homogenate was centrifuged at 9000 r/min for 30 min at 4°C, and the supernatant was saved and stored at 4°C. Trypsin and amylase activity assays were completed within 24 h. For preparation of the lipase enzyme solution, 3–4 volumes of pre-chilled 0.025 mol/L phosphate buffer was added to the tissue, then homogenized on ice. The homogenate was centrifuged at 3600 r/min for 20 min at 0–4°C and the supernatant was saved for lipase activity determination.

The enzyme concentration in the supernatant was analyzed by Uquant Microplate Reader and protein quantification kit (Coomassie brilliant blue method) from Nanjing Jiancheng Bioengineering Institute. Trypsin, lipase and amylase were measured with kits from Nanjing Jiancheng Bioengineering Institute. Bovine serum albumin was used as the standard, and units were in mg/ml. A unit of trypsin activity was defined as the amount of enzyme to hydrolyze 1μg casein into tyrosine in one minute at 37°C. A unit of amylase activity was defined as the amount of enzyme to hydrolyze 1 mg starch in 1 minute at 37°C. A unit of lipase activity was defined as the amount of enzyme to release 1 μmol fatty acids per minute under the experimental condition.

Calculation formulas and statistical analysis. Formulas for growth data are as follows:

WG: weight gain (%) = $100 \times (W_t - W_0) / W_0$

SGR: specific growth rate (%·d^{-1}) = $100 \times (\ln Wt - \ln W_0)/t$

SR: survival rate (%) = $100 \times (N_0 - N_t) / N_0$

PER: protein efficiency ratio (%) = $(Wt - W_0) / I \times$ Dietary protein level

FCR: feed conversion ratio (%) = $I / (W_t - W_0)$

W_t and W_0 denoted the final and initial wet body weight of the shrimp, respectively. N_0 and N_t denoted the number of shrimp at the start and end of the experiment, respectively. T was feeding time, and I was the total amount of feed.

The results were expressed as mean ± standard deviation. Using SPSS17.0 software, data were analyzed by univariate (one-way ANOVA) analysis. If significant differences were found, then the data were analyzed by Tukey's multiple comparisons. $P < 0.05$ indicated significant difference.

Results

Effects of dietary protein levels on L. vannamei growth performance and survival. Dietary protein level has a significant effect on the growth of *L. vannamei*, as shown in Table 2.

Table 2 Effects of dietary protein levels on L. vannamei growth performance and survival

Item	*25%*	*30%*	*35%*	*40%*	*45%*
Initial weight (g)	0.27±0.01	0.28±0.01	0.28±0.01	0.27±0.01	0.28±0.01
Final weight (g)	$5.57±0.32^{ab}$	$5.79±0.15^{bc}$	$6.06±0.06^{c}$	$5.51±0.27^{ab}$	$5.18±0.34^{a}$
WG(%)	$1937.91±128.37^{ab}$	$2005.98±34.90^{a}$	$2104.44±20.92^{a}$	$1924.69±101.59^{ab}$	$1783.49±112.39^{b}$
SGR(%/d)	$5.38±0.12^{ab}$	$5.44±0.03^{b}$	$5.52±0.02^{b}$	$5.37±0.09^{ab}$	$5.24±0.11^{a}$
SR(%)	$99.06±1.44^{a}$	$95.04±2.45^{b}$	$97.94±1.29^{ab}$	$99.22±1.59^{a}$	$83.11±3.00^{c}$
FCR	$1.42±0.05^{a}$	$1.29±0.05^{b}$	$1.07±0.02^{c}$	$1.12±0.03^{c}$	$1.11±0.02^{c}$
PER	$2.76±0.14^{a}$	$2.58±0.11^{a}$	$2.64±0.17^{a}$	$2.18±0.07^{b}$	$1.94±0.10^{b}$

Note: All values represent the mean ± S.D. (All treatments were conducted in triplicate of n=40 individuals). Values in the same column with different superscripts are significantly different. (P<0.05, Tukey's test).

When *L. vannamei* was cultured at salinity 2 g/L long-term, dietary protein levels strongly affected the shrimp final body weight, weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio and survival rate(p<0.05). With the increase of dietary protein levels, the shrimp final body weight, weight gain, and specific growth rate increased at first, and then decreased. The 35% group had the highest final body weight, weight gain and specific growth rate, significantly higher than 25%, 40% and 45% groups (p<0.05).Final body weight of the 45% group was significantly lower than the 30% group (p<0.05), and weight gain and specific growth rate of 45% group were significantly lower than 30% and 40% groups (p<0.05). There were no significant differences among the other groups. With the increase of dietary protein levels, feed conversion ratio and protein efficiency ratio mostly decreased. The 35% group had the lowest feed conversion ratio, significantly lower than 25% and 30% groups(p<0.05), but not significantly different from the 40% and 45% groups. For protein efficient ratio, the 45% group had the lowest ratio and the 40% and 45% groups were significantly lower than the 25%, 30% and 35% group. No significant difference (p<0.05) was found among the rest of the groups.

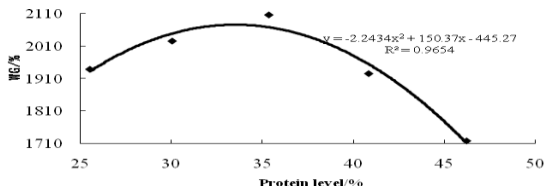

Fig 1. Relationship between the weight gain of *L. vannamei* and the protein level in feed

Quadratic regression analysis between the dietary protein levels and the shrimp final body weight, weight gain, and specific growth rate was performed. With the final body weight, weight gain, specific growth rate set as the dependent variable Y, and dietary protein level set as the independent variable X, quadratic regression equations were derived: weight gain $y = -2.2434x^2 + 150.37x - 445.27$ ($R^2 = 0.9654$) (Figure 1), growth rate $y = -0.0016x^2 + 0.1096x + 3.6342$ ($R^2 = 0.9237$) (Figure 2). When dietary protein levels were 33.51%, 34.35%, the shrimp final body weight, weight gain, and specific growth rate respectively, were the highest.

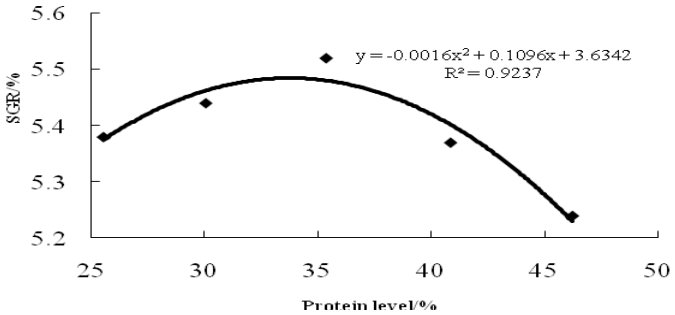

Fig 2 Relationship between the SGR of *L. vannamei* and the protein level in feed

Effects of dietary protein levels on the body composition of L. vannamei. As shown in Table 3, with the increase of dietary protein level, the moisture content decreased. Lowest moisture content was found in the 40% group; both 40% and 45% groups were significantly lower than the 25%, 30%, 35% groups(p<0.05); there was no significant difference among the rest of the groups (p>0.05). Shrimp protein content increased with increasing dietary protein levels. Protein content of the 45% group was the highest at 72.99% (of dry weight). Protein content of the 40% and 45% groups were significantly higher than the 30% and 25% groups (p<0.05). No significant differences were found among the other groups. Shrimp body fat content and ash generally decreased with increasing dietary protein levels, but there were no significant differences among the groups.

Table 3 *Effects of dietary protein levels on the body composition of L. vannamei*

Item	*Protein levels (%)*				
	25%	*30%*	*35%*	*40%*	*45%*
Body moisture (%)	77.82±0.06^{a}	76.93±0.90^{a}	77.25±0.37^{a}	75.52±0.62^{b}	75.74±0.68^{b}
Body protein (%, DM)	70.16±0.82^{a}	70.37±0.44^{a}	71.49±1.46^{ab}	72.76±1.36^{b}	72.99±1.63^{b}
Body lipid (%, DM)	4.62±0.25^{a}	4.47±0.35^{a}	4.67±0.64^{a}	4.58±0.48^{a}	4.68±0.71^{a}
Body ash (%, DM)	12.54±0.33^{a}	11.70±0.77^{a}	11.69±0.90^{a}	11.40±0.79^{a}	12.16±0.32^{a}

Note: All values represent the mean ± S.D. (All treatments were conducted in triplicate of 40 individuals). Values in the same column with different superscripts are significantly different. (P<0.05, Tukey's test).

Effects of dietary protein levels on digestive enzymes of L. vannamei. The effects of dietary protein levels on the trypsin, lipase, and amylase activities of *L. vannamei* are shown in Table 4. Dietary protein levels had a significant effect on *L. vannamei* trypsin activity (p<0.05). With the increase of the dietary protein level, trypsin activity first increased and then decreased. Maximum activity was observed in the 35% group and it was significantly higher than the 25%, 30% and 45% group (p<0.05). In the 25% group, activity was significantly lower than 35%, 40% and 45% groups (p<0.05). No significant differences were found among the other groups. Dietary protein levels had no significant effect on lipase and amylase activities.

Table 4 Effects of dietary protein levels on digestive enzymes of *L. vannamei*

Item	*Trypsin U/mg pro*	*Lipase U/mg pro*	*Amylase U/mg pro*
25%	125.02±4.58^{a}	9.63±0.32^{a}	1.60±0.53^{a}
30%	129.63±1.72^{acd}	9.47±0.14^{a}	1.45±0.28^{a}
35%	143.30±2.48^{b}	9.53±0.22^{a}	1.57±0.47^{a}
40%	136.70±4.90^{bcd}	9.54±0.41^{a}	1.50±0.41^{a}
45%	132.26±4.74^{d}	9.58±0.57^{a}	1.53±0.36^{a}

Note: All values represent the mean ± S.D. (All treatments were conducted in triplicate of 40 individuals. Values in the same column with different superscripts are significantly different. (P<0.05, Tukey's test).

Discussion

Previous studies have investigated growth and survival of *L. vannamei* at low salinity levels similar to those of inland low-salinity water; <5 g/L (Li et al., 2015). When *L. vannamei* were cultured at salinities of 2, 4 or 8 g/L for 10 weeks, no differences were found in final weight and biomass load between all treatments (Samocha et al. 1998). In the present study, shrimp were cultured at salinity level of 2 g/L; survival rate was over 90% and there was no significant difference when dietary protein level was 25%-40%, but significantly decreased in the 45% protein group. This may be due to high protein levels leading to high ammonia levels in hemolymph metabolites (Rosas et al., 1995), and increased nitrogen catabolism (Vergara et al., 1996).

Results of this study also showed that dietary protein levels significantly affected growth performance; the optimum protein requirement for *L. vannamei* in low salinity was 33.51-34.35%. In another report, the optimum level of dietary protein for *L. vannamei* at 2 g/L was 26.7%, which is lower than that in water of salinity of 28 g/L (33%) (Huang et al., 2003). A recommendation was made of 40% dietary protein to be used in shrimp culture at low salinity to improve growth and health (Liu et al. 2005). The growth and condition factor was found to increase by increasing the level of dietary protein, but low survival at low salinity was not improved (Li et al., 2008). Contradictory results on the impact of dietary protein requirement on *L. vannamei* from these studies may be partially due to different protein sources used in these experiments. These results suggest that the optimum protein requirement for *L. vannamei* is affected by salinity. Therefore formulators need to adjust feed protein content according the farming water salinity, feed protein source, and a balanced mixture of essential and nonessential amino acids.

Body composition of aquaculture animals has been found to correlate with dietary nutrients. Some studies showed the protein level of aquaculture animals initially increased then decreased with increasing dietary protein levels (Mohanta et al., 2007). The results of this study showed that dietary protein levels significantly affected some nutrient compositions of whole shrimp. Crude protein content generally increased with increasing dietary protein levels. This is consistent with previous studies on the *Pampus argenteus* (Hossai et al., 2010), *Scylla serrata* (Unnikrishnan and Paulraj, 2010) and *L. vannamei* (Hu et al., 2008). However, some studies showed that crude protein content was not affected by dietary protein levels in *Scylla serrata* (Catacutan, 2002) and *Eriocheir sinensis* (Mu et al., 1998).Changes in moisture content of shrimp was opposite to protein content; moisture content decreased with increasing dietary protein levels. This is consistent with a previous study of *Scylla serrata* (Sheen and Wu 1999). Some reports indicate that moisture content of the *Scylla serrata* was not affected by dietary protein levels (Catacutan, 2002; Hu et al., 2008; Unnikrishnan and Paulraj, 2010). Dietary protein level did not affect whole body lipid and ash content of *L. vannamei* with increasing dietary protein levels, which is similar to *Barbodes altus* (Adrian, 1997).

In this study, trypsin activity in liver and pancreas first increased then decreased with increasing dietary protein levels. These results differ from a study on *Penaeus japonicus*, probably due to the different size of shrimps and different protein diets (Rodriguez 1994). A study on the relationship between the amount of the trypsin mRNA in *Dicentrarchus labrax* and the ingredients in the diet, showed that at the molecular level the responses of trypsin and amylase to the diet were regulated independently (Peres et al 1998). Future studies are needed on the expression of the genes encoding digestive enzymes and endocrine regulation pathways to further explore the mechanisms for the regulation of *L. vannamei* digestive enzymes.

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