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Original Article

Effect of dietary protein level on growth and immunity of *Litopenaeus vannamei*, Boone 1931.

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Abstract

This study was designed to evaluate the effect of feeding grade level of dietary protein (36, 32, 28 and 24%) on the growth and immune responses of pacific white shrimp for 51-100 days of culture period. In an outdoor pond trial, *L. vannamei* postlarvae-15 stage (0.0023 g) were stocked into a 50 m^2 polyethylene-lined pond at a density of 100 shrimp/m^2. This experiment was divided to four treatments with four replicates per treatment. In treatment-1, shrimp were fed 36%CP throughout the culture period. In treatment-2, -3 and -4, shrimps were fed 36%CP for the first 50 days, after that the shrimp were switched to 32, 28 and 24%CP feed, respectively, until day-100. The result showed the weight gain (WG), average daily gain (ADG), survival rate (SR) and specific growth rate (SGR) of shrimps were not affected by treatment ($P>0.05$). However, in terms of productivity, treatment-1 (74.94 kg.pond^{-1}) and treatment-4 (74.70 kg.pond^{-1}) were significantly lower as compared with treatment-2 (94.1 kg.pond^{-1}). Feed utilization, protein efficiency ratio (PER) and protein retention (PR) of treatment-1 was significantly lower than treatment-3 and -4. For the immune responses of shrimp, total hemocyte count (THC), phenoloxidase activity (PO) and the production of superoxide anion (SO), as happens during the respiratory burst in many immuno-competent cells, on day 60 and 100 of culture were not significant different among the treatments. From these results, it is concluded that the optimal dietary protein level for *L. vannamei*, after day 50 or body weight over 7.5 g can be lowered from 36% to 24-32%, because the growth performance and the immune responses of shrimp were not different from feeding only 36%CP diet throughout the culture period.

Keywords: *L. vannamei*, growth, low protein, immune response

1. Introduction

P. monodon culture used to be the most important aquaculture in Thailand. Nevertheless, *P. monodon* culture and export has reduced to 4% in recent years due to poor growth performance and disease problem. Currently, the shrimp farmer has converted from culturing *P. monodon* to *L. vannamei*, because this species is a fast growing species and gives higher production per rai (Noolit, 2006). The production cost of *L. vannamei* is cheaper than that of *P. monodon*, because *L. vannamei* can feed on low protein feed and use less chemical during culture period. The white leg shrimp can be culture in an intensive system, high stocking density (Limsuwan and Junratchakool, 2004). Typically, the shrimp diet is an important for growth and maintainance. It is composed with essential nutrient as protein, lipid, carbohydrate, minerals and vitamins. Each nutrient has its specific role for growth especially protein (Pandian, 1989). Protein is

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one of the major nutrients for shrimp growth and represents one of the primary costs in a compound feed formulation (Tacon and Akiyama, 1997). Protein requirement of shrimp change with respect to change in biotic factors (species, stage and size) and abiotic factors (salinity and temperature) (Guillaume, 1997). For juvenile white leg shrimp, Colvin and Brand (1977) reported that the protein requirement is less than 30% while Kureshy and Davis (2002) found that a maximum protein requirement at 32% and Wyban (1992) reported that at about 25-35% which were less than *P. chinensis* and *P. monodon*. In addition, protein content of the feed and its availability can effect to water quality via nitrogen excretion. Excess protein will be deaminated and used as an energy source and nitrogen metabolites will be release into the culture medium (Cho *et al*., 1994). The shrimp excretion and feed residue deposit in cultural pond can lead to the deterioration of pond bottom and the shrimp will be subject to stress condition thus weakening their immunity (Limsuwan, 2004). In order to reduce the excretion of nitrogenous compounds and their associated problems, a lower amount of protein should be input into the shrimp pond based on the protein requirements of the species, and adjusted for availability (Jesus *et al*., 2007). For these reasons there is an interest in optimizing feed input and feed management to improve the economic return in shrimp farms and to reduce the potential of environmental impact. Protein is the most expensive macro- nutrient in shrimp feed, thus to determine the optimal dietary protein level is important for formulate the cost effective feed (Wyban *et al*., 1988). On the other hand, reduction in hemocytes and immune function were registered when shrimp were fed sub-optimal dietary protein levels (Christina *et al*., 2004).

The aim of this study was to determine the effect of dietary protein level on the growth performance, and immune response of *L. vannamei.*

2. Material and Methods

50 m^2 polyethylene ponds were randomly assigned to one of four treatments, with four replicates per treatment. Specific pathogen free *L. vannamei* postlarvae-15 (0.0023 g) were stocked into 16 outdoor ponds, at a density of 100 shrimps per square meter. Treatment-1: feed 36%CP diet throughout the 100 days culture period, and the others feed 36%CP only first 50 days, after that changed to feed 32, 28 and 24%CP diets for treatment-2, -3 and -4 respectively for another 50 days.

2.1 Feed and feeding

Four experimental diets were formulated with different dietary protein level at : 36, 32, 28 and 24%. The proximate analysis of the diet composition (Table 1) was performed by the standard method of Association of Official Analytical Chemists (AOAC, 1990)

Feed was administrated in the 1st week of culture at 1 kg/10^5 shrimps and increased 100 g every week thereafter, from the second to fourth week. After the first month, check trays were used to monitor the feed consumption of shrimp in each pond. Shrimp growth was monitored on a weekly basis by measuring the average body weight in a sample of 70-100 shrimps per pond. Shrimp samples were obtained using a cast net every week.

2.2 Pond management and water quality

The ponds were equipped with airlift from aerator. Two weeks before shrimp stocking, 27 ppt treated seawater were pumped into each pond. Natural food was prepared one week before stocking by applying fishmeal, molasses and rice bran into ponds. During the experiment, dissolved oxygen (DO), pH and temperature were measured twice a day at 6:00 and 14:00 and alkalinity, salinity, hardness, total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N) and nitrate (NO_3-N) were checked once a week. Pond water was exchanged according to the nitrogenous waste parameter.

2.3 Harvest

The study was terminated at 100 days, pond water was

Table 1. Ingredient composition (g/100g feed) and proximate analysis (%dry basis) of trial diets for *L.vannamei*

Ingredient	36%	32%	28%	24%
Fish meal	24.2	19	16	11.6
Shrimp head meal	10	10	10	10
Soybean meal	20	16	10	6.5
Wheat flour	38.5	37.5	42.5	53
Wheat bran		10	13	10
Mix oil *	4.1	4.3	4.3	4.7
Choline Choride	0.6	0.6	0.6	0.6
Vit. and Min. premix **	2	2	2	2
Mono NaP	0.6	0.6	0.6	0.6
Limestone			1	1
Total	100	100	100	100
Crude protein	36.00	32.90	28.20	25.40
Crude fat	5.43	6.35	6.28	6.30
Moisture	9.32	9.37	8.69	8.64
Fiber	2.11	3.50	3.63	3.40
Ash	10.33	10.40	9.66	8.44
Gross energy (kcal/100g)	5.02	5.12	5.12	4.98

* Mix oil = fish oil : lecithin (1 : 1)

** Vit. And Min. premix : Vit. A, 400 IU/g; B1, 24,000 mg/kg; B2, 16,000 mg/kg; DL Ca pantotenate, 30,000 mg/kg; B6, 30,000 mg/kg; B12, 80 mg/kg; C, 60,000 mg/kg; K3, 16,000 mg/kg; D3, 3200 IU/g; E, 60,000 mg/kg; niacin, 20,000 mg/kg; folic acid, 4000 mg/kg; Co, 2000 mg/kg; Mn, 16,000 mg/kg; Zn, 40,000 mg/kg; Cu, 20,000 mg/kg; Fe, 1 mg/kg; Se, 100 mg/kg; I, 2000 mg/kg

drained out for harvest. A random sample about 100 shrimp was collected from each pond for individual weight determination. These data were used to calculate the growth performance such as mean final body weight, survival rate and feed conversion ratio. Shrimp carcasses were analyzed for proximate composition to evaluate the feed utilization.

2.4 Immunity parameter

On day 60 and 100 of culture period, 5 surviving shrimps were collected from each pond and the same treatment shrimp was stocked in one bucket. Thus, there were 4 buckets for shrimp stocking of each treatment. Later on, 10 shrimps from each bucket were randomly chosen for checking immunity.

Total Hemocyte Count (THC) : Hemolymph (100 µl) was withdrawn from the ventral sinus of each shrimp into a 1 ml syringe, containing 900 µl anticoagulant solution. A drop of the anticoagulant and hemolymph mixture was placed on a hemocytometer to count THC using an optical microscope without discrimination of the three types of hemocyte.

Prophenoloxidase activity (PO) : A sample of 50 µl of re-suspended sample containing degranulated hemocytes was incubated for 3 min at 25°C with 50 µl of 0.4% trypsin. Then, 50 µl 0.3% L-dihydroxyphenyalanine (L-DOPA) was added and incubated for 10 min. (Hernández-López *et al.*, 1996). Absorbance was measured at 490 nm in an ELISA reader.

Superoxide anion (SO) was quantified using the nitroblue tetrazolium (NBT) reduction to formazan (Song and Hsieh, 1994) 50 µl of hemolymph was placed in a well of microplate and centrifuged at 800xg for 10 min. Plasma was removed and hemocytes washed with 100 µl Hank's solution. Next, 100 µl zymosan (0.1% in Hank's solution) was added and incubated for 2 hrs. at room temperature. The zymosan was then removed and hemocytes washed three times with 100 µl Hank's solution and stained for 30 min with NBT solution (0.3%) at room temperature. After fixation hemocytes were washed three times with 100 µl methanol (70%) and dried for 5 min. Formazan was dissolved with 120 µl KOH and 140 µl dimethyl sulfoxide (DMSO), and absorbance was read at 630nm using a micro-plate reader.

2.5 Statistical analysis

Data were analyzed using one way analysis of variance (ANOVA) to determine of significant ($P<0.05$) difference existed among treatment means. Duncan's multiple range test was used to determine significant differences among treatment means.

3. Results and Discussion

3.1 Growth and feed utilization parameters

After 100 days of culture period, there were no significant differences ($P>0.05$) in growth performance among the treatments (Table 2). Final average body weight ranged

Table 2. Mean growth performance and feed utilization of shrimp fed different dietary protein levels during 51-100 days.

Parameter	Protein level in feed (%)				P-value	PSE
	36	32	28	24		
Weight gain (g)[1]	20.05[a]	21.01[a]	21.24[a]	19.21[a]	0.164	1.314
ADG ($g.d^{-1}$)[2]	0.20[a]	0.21[a]	0.21[a]	0.19[a]	0.255	0.013
SGR ($\% d^{-1}$)[3]	9.07[a]	9.12[a]	9.13[a]	9.03[a]	0.161	0.068
Survival rate (%)[4]	74.50[a]	89.59[a]	84.27[a]	77.76[a]	0.119	8.691
Productivity ($kg.pond^{-1}$)	74.94[b]	94.05[a]	89.51[ab]	74.70[b]	0.051	10.717
FCR[5]	1.50[a]	1.39[a]	1.44[a]	1.41[a]	0.11	0.064
FI ($g.shrimp100g^{-1}day^{-1}$)[6]	1.40[a]	1.33[a]	1.31[a]	1.47[a]	0.152	0.098
PER[7]	1.91[b]	2.13[ab]	2.29[a]	2.38[a]	0.013	0.138
PR (%)[8]	36.06[b]	40.11[ab]	45.30[a]	45.00[a]	0.0094	2.740

The different alphabets in the same row mean significant difference ($P<0.05$)

[1] Weight gain = final weight – initial weight

[2] Average daily growth (ADG) = (final weight – initial weight)/ time(days)

[3] Specific growth rate (SGR) = [ln(final weight)-ln(initial weight)] x 100/ time(days)

[4] Survival rate = final count) / initial count) x 100

[5] Feed conversion ratio = dry weight of feed offered / wet weight gained

[6] Feed intake = feed intake per day / average body weight x 100

[7] Protein efficiency ratio = body weight gain / protein intake

[8] Protein retention = [(mean final weight x protein in final shrimp) – (mean initial weight x protein in initial shrimp)] x 100 / protein intake

from 19.21-20.05 g and was numerically higher in treatment-3, whereas survival rate were between 74.5-89.6% and also numerically higher in treatment-2. However, there were significant differences ($P<0.05$) in productivity per pond, ranging from 74.70-94.05 kg.pond^{-1} or 2,390-3005 kg.rai^{-1}. Smith *et al*. (1985) studied the response of three sizes of *L. vannamei* (4.0, 9.8 and 20.8 g) fed diets containing 22, 29 and 36%CP for 30 days and found that dietary protein content only affected weight gain for 4-g shrimp, with a significant increase in weight gain corresponding to the increase in dietary protein content. Martin *et al*. (2007) reported a significantly lower survival of shrimp fed the higher protein diet (40%) compared to that of shrimp fed lower protein feeds (25-35%CP). This result is likely associated with higher concentration of total ammonia nitrogen in pond water when protein levels increased.

Feed conversion ratio (FCR) and feed intake (FI) were not significantly different among the treatments ($P>0.05$). The FCR ranged between 1.41-1.5; these values correspond to the FCR obtained by the majority farms in Southern of Thailand, which range from 1.4 to 2. According to the recent survey on the FCR achieved by a number of shrimp farmers in the South, it was found that a small number of farmers achieved an FCR as low as 1.2, but the majority of farmers still achieve on FCR higher than 2 (Lin, 1992). FI ranging from, 1.37-1.47 g.shrimp100g^{-1}day^{-1} indicating that feed consumption for all treatments were comparable. Results from this study demonstrate that feed inputs were adequately assimilated, allowing the shrimp to achieve consistent yields and low feed conversion ratio. Nevertheless, PER and PR were significantly different ($P<0.05$) and these values tended to be higher when shrimps were fed lower level protein feed. PER and PR ranged between 1.91-2.38 and 36.01-45.30%, respectively. PER of white shrimp fed commercial diet (30%CP) was 1.88±0.08 (Cruz-Suarez, 2007).

3.2 Immune Response

There were no significant differences among the treatments in THC, PO and SO (Figure 1). The mean value of THC was 2.5x10^{7} cell.ml^{-1} on day 60, and treatment 1 gave the highest THC and was slightly decreased corresponding to dietary protein level. Nevertheless, the mean value of THC on day 100 was 1.9x10^{7} cell.ml^{-1} and was not significantly different among treatments. Liu (2004) found that the THC was the highest at C stage and lowest at A and B stages, with mean values of 315, 226 and 250x10^{5} cell.ml^{-1}, respectively. Hemocytes play an important and central role in the cellular immune response, including clotting, non-self recognition, phagocytosis, melanization, encap-sulation and cytotoxicity, and each type of hemocyte in hemolymph has a different function into immune system (Söderhall, 1999). After detection of foreign material, hemocytes migrate to the site of invasion. The open circulatory system demands a rapid and efficient defense in which the proteolytic cascades play an important role (Sritunyalucksana and Söderhall, 2000). The proPO-activation system is an important immune defense system in crustaceans (Söderhall and Cerenius, 1998). Several components and associated with the proPO-activation system and induce to the reaction of melanisation. The mean values of PO at 490 nm on day 60 and day 100 were 0.053 and 0.047 respectively. The mean values of SO were 9.35 and 6.59 unit on day 60 and 100 respectively. SO of black tiger shrimp ranging from 6.5 to 12.925 units was reported by Areechon *et al*., 2004. They also found that the immunity of black tiger shrimp was stimulated by 3 ppm B-glucan after feeding 5 day per week for 4 weeks, and the SO was increased, ranged from 9.075-20.5 unit during 10 weeks. The lower immune responses obtained on day 60 compared to those values on day 100 may be due to the stress of the shrimp caused by the collection of hemolymph after harvest.

3.3 Pond management and water quality

Air stones were used to maintain the normal DO

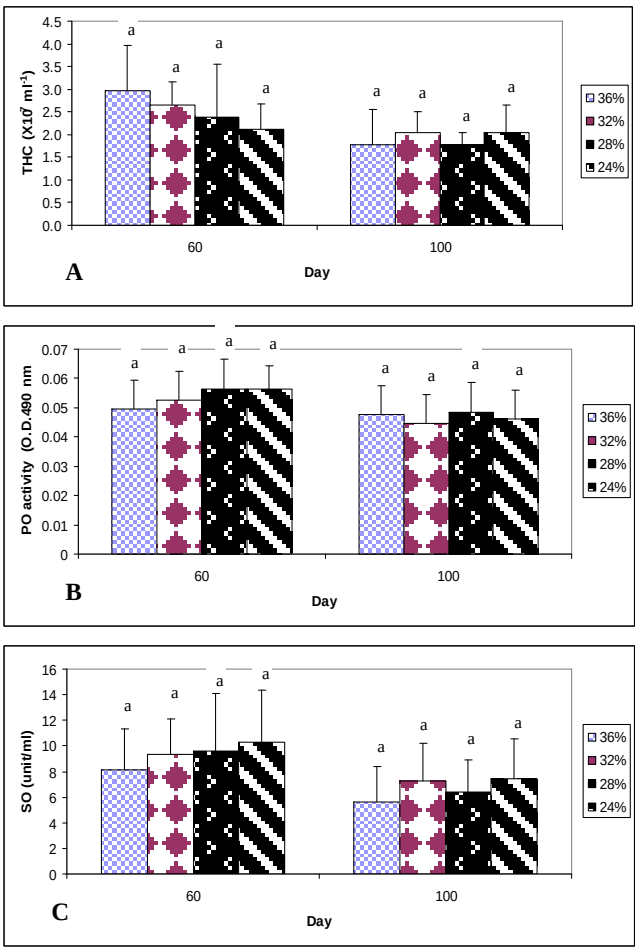

Figure 1. Total hemocyte count (A), phenoloxidase activity (B) and superoxide anion (C) of *L. vannamei* on day 60 and 100 of culture period. Each bar represents the mean value from 10 shrimp (2 replicates) with standard error. In the same exposure day with different letters are significantly different ($p<0.05$) among different dietary protein levels.

Table 3. Summary of water quality parameter observed over 100 days (Values are mean ± SD)

Parameter	Trt.1	Trt. 2	Trt. 3	Trt. 4
Temp (^oC)				
6:00	29.0±1.01	29.9±1.02	29.8±1.01	29.8±1.01
14:00	32.8±1.74	32.9±1.84	32.9±1.78	32.8±1.73
pH				
6:00	8.0±0.18	8.0±0.19	8.0±0.18	8.0±0.19
14:00	8.4±0.16	8.4±0.17	8.4±0.17	8.4±0.18
DO ($mg.L^{-1}$)				
6:00	4.9±0.61	4.8±0.63	4.8±0.61	4.9±0.58
14:00	8.4±1.13	8.4±1.05	8.6±1.23	8.6±1.09
Salinity ($g.L^{-1}$)	19±3.80	19±4.15	20±3.72	19±4.01
Alk. ($mg.L^{-1}$)	149±21.72	140±20.57	145±23.24	138±20.82
TAN ($mg.L^{-1}$)	0.7±0.55	0.76±0.80	0.69±1.03	0.37±0.51
NO_2-N ($mg.L^{-1}$)	0.31±0.44	0.4±0.51	0.18±0.33	0.10±0.54
NO_3-N ($mg.L^{-1}$)	0.22±0.55	0.27±0.54	0.12±0.54	0.12±0.54

values. Water quality parameters throughout the 100 days experimental period were maintained at suitable levels for adequate growth and survival of shrimp (Table 3). Analysis of mean values for water quality parameters showed no statistical difference among treatments. Nevertheless, nitrogen waste concentration of treatment-4 was numerically less than that of other treatments. The toxicity of TAN levels range from 2.6-3.95 $mg.l^{-1}$(Jiang *et al.*, 1999) and nitrite critical level reported for *L .vannamei* was 6.1 $mg.l^{-1}$ in 15 ppt (Lin and Chen, 2003). Therefore, the water quality in terms of the nitrogen excretion was in the range of suitable conditions.

4. Conclusion

From this trial, it was demonstrated that the dietary protein level can be lowered to 24-32% for *L. vannamei* after 50 days of culture or with body weight over 7.5 g although mean body weight of shrimp fed 24%CP was numerically lower than others. The production per pond was not significantly different among the treatments, suggesting that the dietary protein level can be reduce from 32% to 28% or even lowered to 24% according to this study. The nitrogen waste concentration of low protein feed tended to be less than that of high protein, suggesting a better assimilation of protein into shrimp biomass. Feed utilization was better in the group fed lower protein feed. Moreover, the immune responses of shrimp fed 36, 32, 28 and 24%CP after 50 cultured days were not markedly different.

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