Effects of Dissolved Oxygen Levels on Growth, Survival and Immune Response of Juvenile Pacific White Shrimp *Litopenaeus vannamei*

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ABSTRACT

A study on the effects of dissolved oxygen (DO) levels on growth, survival and immune response of Pacific white shrimp (*Litopenaeus vannamei*) was conducted under laboratory conditions. Two hundred seventy shrimp (6-8 g) were stocked into 9 (500-liter) tanks at a density of 30 shrimp/tank. Tests were carried out at three DO levels, namely > 4 ppm (group A), 2 - 4 ppm (group B), and < 2 ppm (group C) with three replicates/ treatment. Shrimp were fed with a commercial feed four times daily for 60 days. Water salinity was maintained at 25 ppt throughout the experimental period. After 60 days, group A shrimp had an average body weight of 28.16 ± 2.77 g, which is significantly higher ($P<0.05$) than group B shrimp (25.01 ± 1.81g) and group C shrimp (25.90 ± 2.51 g). Survival rates of shrimp reared in groups A and B ranged from 81.11 to 92.22%, which were significantly higher ($P<0.05$) than that in group C (56.67%). Immune parameters, including total hemocyte count, percentage phagocytosis, bactericidal activity, phenoloxidase activity and superoxide dismutase activity were significantly higher ($P<0.05$) in groups A and B than in group C. Shrimp in groups A and B had bactericidal activity at the serum dilution of 1:8 while those in group C had bactericidal activity at a dilution of 1:4. Shrimp in group A had the highest survival rate after an experimental injection of *Vibrio harveyi* but it was not significantly different ($p>0.05$) from shrimp in group B.

Keywords: dissolved oxygen, *Litopenaeus vannamei*, immune response

INTRODUCTION

Thailand has been the world's leading shrimp exporter for more than 20 years, using the intensive culture system with high stocking density. Due to high protein level feeding, Thai farmers have been faced with excessive organic matter accumulation on the pond bottom, resulting in an unsuitable environment during the culture period, particularly in closed-farming systems, with little or limited water exchange (Limsuwan and Chanratchakool, 2004). The key success factor in closed-farming systems is to culture shrimp with the optimum stocking density which suits the pond carrying capacity, and to achieve good water quality with sufficient plankton throughout the culture period.

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Particular attention must be paid to the feeding rate. It has to precisely match the stocking density. In addition, aerators should be installed and located where they can efficiently provide sufficient oxygen. Insufficient aeration in combination with high stocking densities cause slow growth problem, especially towards the end of the culture period. Dissolved oxygen (DO) is one of the most important factors in aquaculture. The bottom layer of pond water, where shrimp spend most of their time, may become hypoxic or even anoxic due to the organism's respiration and decomposition of accumulated organic matter, e.g., feed remains and feces, particularly at night (Zhang *et al*., 2006). Diaz and Rosenberg (1995) reported that hypoxia or low DO condition is characterized by DO concentrations of < 2.8 ppm. Over the past few years, much research has been done to evaluate the effect of hypoxia on growth, survival, feeding, molting, behavior, osmoregulatory capacity, and immune response of penaeid shrimp (Seidman and Lawrence 1985; Clark 1986; Madenjian *et al*., 1987; Aquacop *et al*., 1988; Allan and Maguire 1991; Charmantier *et al*., 1994; Paterson and Thorne 1995; Le Moullac *et al*., 1998; Harper and Reiber 1999; Rosas *et al*., 1999; Wu *et al*., 2002; Cheng *et al*., 2002; Cheng *et al*., 2003). It has been reported that lethal DO levels range from 0.2 to 1.27 ppm for a number of penaeid shrimp species.

Although the effects of dissolved oxygen in shrimp have been reported, most of them focus on the hypoxic condition. Little research about the effects of DO levels on the growth, survival and immune response in shrimp has been reported. The aim of this study was to investigate the effect of dissolved oxygen levels on growth, survival and immune parameters of *Litopenaeus vannamei*, and to gain more information on the mechanism of the shrimp immune system, thereby providing a scientific basis for water quality management in the shrimp culture industry.

MATERIALS AND METHODS

Experimental shrimp

Pacific white shrimp weighing 6-8 grams were obtained from a farm in Chanthaburi province, Thailand, and acclimated at the Aquaculture Business Research Center Laboratory, Faculty of Fisheries, Kasetsart University for a week. During this period, shrimp were fed four times daily with commercial pellet feed. After that, shrimp were randomly stocked in 18 (500-liter) fiberglass tanks (9 fiberglass tanks for growth and survival study and another for immune study). Tests were carried out at three DO levels, namely above 4 ppm (A), 2-4 ppm (B), and less than 2 ppm (C), with three replicates per treatment. Each tank was stocked with 30 shrimp. Shrimp were fed four times daily to satiation, according to the standard feeding rate. The feeding rate was adjusted according to shrimp weight throughout the 60-day experimental period, following a published protocol (Limsuwan and Chanratchakool, 2004). Salinity, pH, and temperature during the acclimation and experimental periods were maintained at 25 ppt, 7.8–8.0, and 28°C, respectively. Water quality parameters such as dissolved oxygen, ammonia, and nitrite were measured weekly using a standard protocol (APHA *et al*., 1995). Leftover feed

and feces were siphoned daily, and 10% of the water was exchanged every 3 days. Every 10th day, shrimp from all treatment groups were counted and weighed. For immunology study, shrimp from each treatment were randomly sampled to evaluate immune parameters, including total hemocyte count (THC), phagocytic activity, phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, and bactericidal activity.

Immune parameters analysis

Preparation of hemolymph samples: A hemolymph sample of 0.5 ml was withdrawn from the base of the third walking leg of each shrimp by a syringe containing 1.5 ml anticoagulant (K-199 + 5% L-cysteine).

Total hemocytes: After collecting the hemolymph, hemocytes were counted using a hemocytometer and calculated as the number of blood cells (total hemocytes per cubic millimeter).

Phagocytotic activity: This was determined according to Itami *et al*. (1994). Two hundred microliters of hemolymph were collected from the base of the third walking leg of shrimp and mixed with 800 µl of sterile anticoagulant. The collected hemocytes were rinsed with shrimp saline and the viable cell number was adjusted to 1 x 10^6 cells/ml. The cell suspension (200 µl) was inoculated into a cover slip. After 20 minutes, the cell suspension was removed, then rinsed with shrimp saline three times. Heat-killed yeast (2 ml) was added and the suspension was incubated for 2 hours. After the incubation, heat-killed yeast was removed, and the suspension was rinsed five times with shrimp saline, fixed with 100% methanol and then the cover slip was stained with Giemsa stain and mounted with permount.

Two hundred hemocytes were counted. Phagocytic activity, defined as percentage phagocytosis was expressed as:

$$\text{percentage phagocytosis} = \frac{\text{phagocytic hemocytes}}{\text{total hemocytes}} \times 100$$

Phenoloxidase activity assay: This method was modified from Supamattaya *et al*. (2000). After the hemolymph was withdrawn, the hemocytes were washed three times with shrimp saline (1,000 rpm 4 ^{o}C 10 min). Hemocyte lysate (HLS) was prepared from hemocytes in a cacodylate buffer at pH 7.4 by using a sonicator at 30 amplitute for 5 seconds. The suspension was then centrifuged at 10,000 rpm, at 4 ^{o}C for 20 min. The supernatant was collected as HLS. Then 200 µl of 0.25% trypsin in cacodylate buffer was mixed to the 200 ml HLS, followed by 200 µl of L-dihydroxyphenylalanine (L-DOPA) at 4 mg/ml as the substrate. Enzyme activity was measured as the absorbance of dopachrome at a wavelength of 490 nm. The amount of protein in HLS was determined using the methode of Lowry *et al*. (1951). The phenoloxidase activity was calculated as the increasing of optimum density (OD) per minute per mg of protein as, expressed in this equation:

$$1 \text{ unit of phenoloxidase} = \triangle \text{OD490 /min/ mg protein}$$

Superoxide dismutase activity assay: This was carried out with the RANSOD kit (Randox, USA). This method is based on the formation of red formazan from the

reaction of 2-(4-iodophenyl)-3-(4-nitrophenol) -5-phenyltetrazolium chloride (INT) and superoxide radical, which was assayed in a spectrophotometer at 505 nm.

Bactericidal activity: Serum was separated from each hemolymph sample and diluted with 2.6% NaCl at 1:2, 1:4, 1:8, 1:16 and 1:32. Then 0.5 ml of each serum dilution and 0.5 ml of NaCl as the control were used in the study. The *V. harveyi* suspension of 0.1 ml (prepared according to the method in 3) was added into each serum dilution and the control. The treatments were incubated at room temperature for 3 h before enumerating the number of bacteria by a spread plate technique. This dilution could decrease *V. harveyi* by 50% compared to the control.

The effect on survival of L. vannamei shrimp experimentally infected by V. harveyi

At day 60 of the growth trial, 30 shrimp were randomly sampled from each treatment and challenged with a virulent strain of *V. harveyi* isolated from a diseased *L. vannamei*. *V. harveyi* was cultured in tryptic soy agar, supplemented with 1.5% NaCl (w/v) for 24 h at 35 °C. After 24 h of growth, bacterial colonies were transferred to 10 ml tryptic soy broth supplemented with 1.5% NaCl and incubated for 24 h at 35 °C. Next, the bacterial culture was centrifuged at 1000 rpm for 15 min at room temperature. The supernatant was removed, and the bacterial pellet was suspended again in saline solution at a concentration of 4.0×10^6 CFU/ml. All shrimp were injected with *V. harveyi* suspension at 9.6×10^6 CFU/ml for two consecutive days. Animals injected with 2% saline served as control. Mortalities were recorded up to 96 h post-injection.

Statistical analysis

The data were subjected to a one-way analysis of variance followed by Duncan's multiple range test. Differences were considered significant at $P < 0.05$.

RESULTS

The effect of dissolved oxygen levels on growth and survival of Pacific white shrimp under laboratory conditions

After 60 days of experimentation, shrimp reared at DO level above 4 ppm (group A) had an average body weight of 28.16 ± 2.77 g which was significantly higher ($P<0.05$) than shrimp reared at DO levels of 2-4 ppm (group B) (25.01 ± 1.81 g) and DO of less than 2 ppm (group C) (25.90 ± 2.51g) (Table 1) respectively. The survival rates of shrimp in groups A and B ranged from 81.11 - 92.22%, which was significantly higher ($P<0.05$) than that in group C (56.67%), (Table 2).

Table 1. Average body weight of *L. vannamei* after 60 days of experiment

Treatment	Average body weight (g)
A	28.16 ± 2.77^{a}
B	25.01 ± 1.81^{b}
C	25.90 ± 2.51^{b}

Average values with different superscripts in the same column are statistically significantly different (P<0.05)

Table 2. Percentage survival of *L. vannamei* after 60 days of experiment

Treatment	Percentage survival
A	92.22 ± 3.85^{a}
B	81.11 ± 13.47^{a}
C	56.67 ± 8.82^{b}

Average values with different superscripts in the same column are statistically significantly different (P<0.05)

Effects of DO on immune characteristics of Pacific white shrimp under laboratory conditions

Immune parameters such as total hemocyte count, percentage phagocytosis, bactericidal activity, phenoloxidase activity, and superoxide dismutase activity were significantly higher (P<0.05) in the shrimp from groups A and B than those in group C.

The THC and phagocytic activity of shrimp are shown in Tables 3 and 4. Shrimp in group A had the highest THC and percent phagocytosis. No difference was found between groups A and B, but THC and the phagocytosis rate of these groups were statistically significantly higher than that of group C.

Table 3. Total hemocyte count (THC) of *L. vannamei* after 60 days of experiment

Treatment	THC (x 105 cells/ml)
A	200.63 ± 5.85^{a}
B	198.67 ± 5.97^{a}
C	161.17 ± 5.97^{b}

Average values with different superscripts in the same column are statistically significantly different (P<0.05)

Table 4. Percentage phagocytosis of *L. vannamei* after 60 days of experiment

Treatment	Percentage phagocytosis
A	37.25 ± 8.94^{a}
B	37.00 ± 6.07^{a}
C	26.33 ± 3.10^{b}

Average values with different superscripts in the same column are statistically significantly different (P<0.05)

After 60 days of experimentation, shrimp in group A showed the highest PO activity. Although no difference was found between groups A and B, PO activity in these groups was significantly higher than that in group C (Table 5). Shrimp raised in group A showed significantly higher SOD activity than group C. However, no significant difference in SOD activity was observed among shrimp in groups A and B (Table 6).

Table 5. Phenoloxidase activity of *L. vannamei* after 60 days of experiment

Treatment	Phenoloxidase activity (unit/min/mg protein)
A	298.83 ± 5.56^{a}
B	289.22 ± 9.87^{a}
C	268.22 ± 6.01^{b}

Average values with different superscript in the same column are significantly different ($P<0.05$)

Table 6. Superoxide dismutase activity of *L. vannamei* after 60 days of experiment

Treatment	Superoxide dismutase (SOD units/ml)
A	47.87 ± 7.62^{a}
B	45.07 ± 10.23^{a}
C	35.97 ± 6.01^{b}

Average values with different superscript in the same column are significantly different ($P<0.05$)

Shrimp in DO above 4 ppm (group A) and DO 2-4 ppm(group B) had bactericidal activity at the serum dilution of 1:8 while shrimp at DO of less than 2 ppm (group C) had bactericidal activity at the dilution of 1:4 (Figure 1).

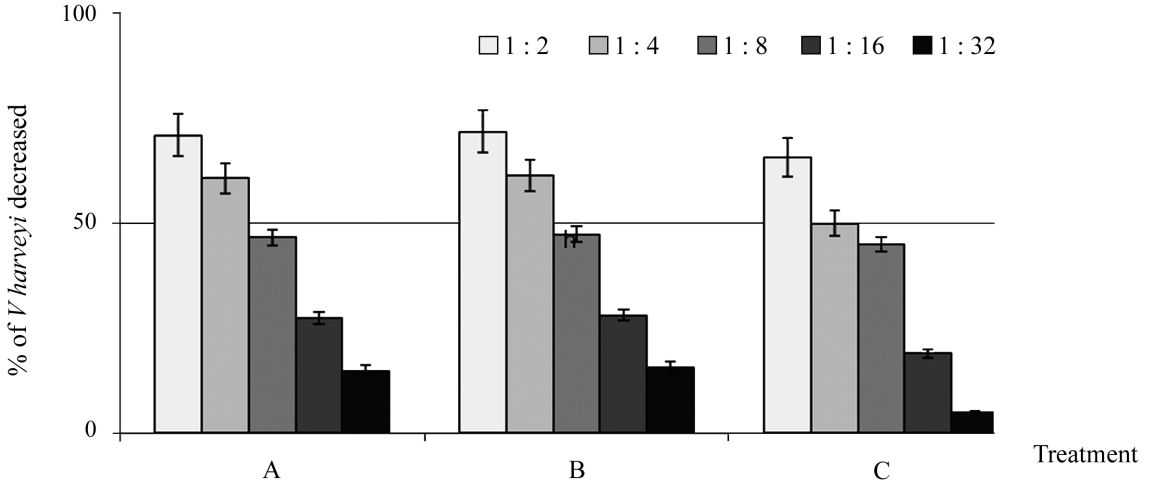

Figure 1. Bactericidal activity of *L. vannamei* serum after 60 days of experiment

The effect of DO level on the survival of L. vannamei after challenged with V. harveyi

The effect of dissolved oxygen (DO) levels after challenge with *V. harveyi* at a concentration of 9.6×10^6 CFU/ml for two consecutive days was evaluated. Shrimp in group A showed a significantly higher survival rate compared to shrimp in group C, but not significantly different ($p>0.05$) from the shrimp in group B (Figure 2).

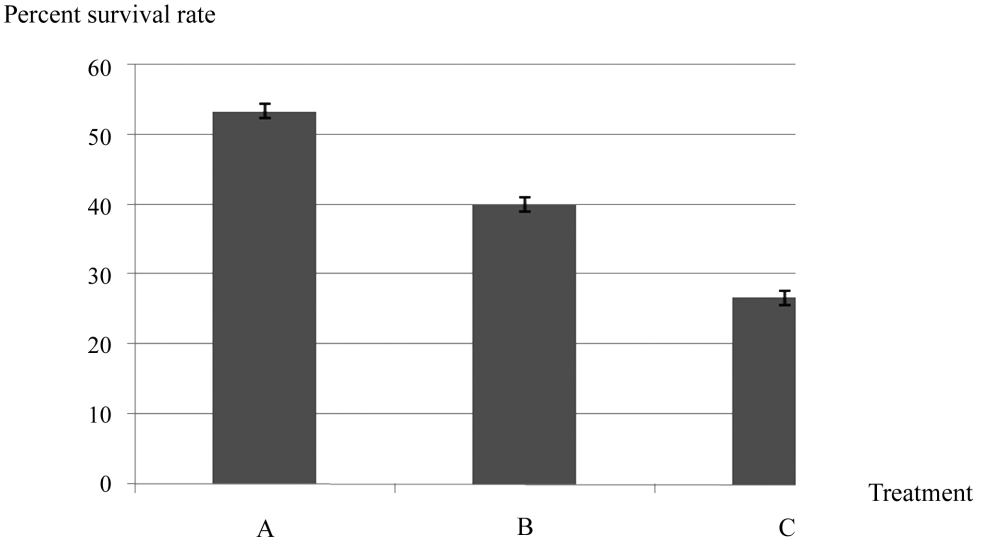

Figure 2. Percent survival of *L. vannamei* upon challenge with *Vibrio harveyi*

DISCUSSION

In the present study, shrimp reared in DO above 4 ppm showed the highest growth rate, and decreased significantly in the group reared in DO levels of 2-4 ppm and less than 2 ppm. This result is similar to the previous reports by Aquacop *et al*. (1988) and Ross and Lawrence (1985). Shrimp survival rate observed in this study suggests that DO level of less than 2 ppm had severely affected shrimp survival. Perez-Rostro *et al*. (2004) reported that a DO level of 0.2 ppm was lethal for the shrimp *L. vannamei* after 1 h of exposure, with the lethal DO level at about 1 ppm (Hopkins *et al*. 1991). Zhang *et al*. (2006) reported that *L. vannamei* subjected to a gradual reduction in DO showed the following locomotory responses: initially, an increased activity and frequent random vertical or horizontal swimming movements; then, lower activity and slower swimming speeds with obvious attempts to seek the surface; and lastly, keeping still. Moreover, large shrimp exhibited higher locomotory activity compared with smaller shrimps. Similar responses were observed in this study.

Immune parameter levels measured in this study such as total hemocyte count, percentage phagocytosis, bactericidal activity,

phenoloxidase activity and superoxide dismutase activity of *L. vannamei* reared in low DO conditions (less than 2 ppm) decreased significantly. This is different from a previous report by Jiang *et al.* (2005) and Zhang *et al.* (2006) which demonstrated that when *L. vannamei* was exposed to hypoxia conditions, their phenoloxidase activity increased significantly. This is because the present study was designed to keep shrimp under a low DO condition for a longer period, i.e. up to 60 days. Therefore, shrimp were able to adapt their physiological responses when exposed to constant DO levels. DO concentration is one of the most important environmental stress factors in aquaculture. The effect of low DO has been reported to reduce the resistance of *Penaeus monodon* to *V. harveyi* and *P. stylirostris* to *V. alginolyticus* (Direkbunsarakom and Danayadol, 1998; Le Moullac *et al.* 1998). This was observed similarly in this study.

The present study aimed to determine the effects of dissolved oxygen (DO) levels on growth, survival and immune responses in Pacific white shrimp (*Litopenaeus vannamei*), and survival against the challenge with *V. harveyi.* Our data showed that DO above 4 ppm is able to support good growth and survivability.

CONCLUSION

In conclusion, dissolved oxygen (DO) levels have an affect on the growth, survival and immunity of shrimp. Shrimp raised in DO above 4 ppm could achieve good growth and survivability.

ACKNOWLEDGMENTS

This research was supported by funding from The National Research Council of Thailand.

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