Effect of water temperature on the immune response of white shrimp *Litopenaeus vannamei* to *Vibrio alginolyticus*

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Received 28 November 2004; received in revised form 19 April 2005; accepted 24 April 2005

**Abstract**

White shrimp *Litopenaeus vannamei* held in 25\(^\circ\)C seawater at 27 \(^\circ\)C or 28 \(^\circ\)C were injected with TSB-grown *Vibrio alginolyticus* at 1 \(\times\) 10\(^5\) colony-forming units (cfu) shrimp\(^{-1}\) or 1 \(\times\) 10\(^5\) cfu shrimp\(^{-1}\), and then cultivated onward at water temperatures varying from 20 to 34 \(^\circ\)C. Over 24–144 h, mortality of *V. alginolyticus*-injected shrimp held at 34 \(^\circ\)C or 32 \(^\circ\)C was significantly higher than that of shrimp held at lower temperatures. In a separate experiment, shrimp held in 25\(^\circ\)C seawater at 28 \(^\circ\)C and then cultured onward at 20 to 32 \(^\circ\)C were examined for immune parameters at 24–96 h. THC, phenoloxidase activity, respiratory burst, and SOD activity decreased significantly at 24 h after transfer to 32 \(^\circ\)C. Shrimp held in 25\(^\circ\)C seawater at 27 \(^\circ\)C and then cultured onward at 20 to 34 \(^\circ\)C showed a significant reduction in phagocytic activity and clearance efficiency for *V. alginolyticus* at 24 h after transfer to 34 \(^\circ\)C. It was concluded that transfer of shrimp from 27 or 28 \(^\circ\)C to higher temperatures (32 and 34 \(^\circ\)C reduced their immune capability and decreased resistance to *V. alginolyticus* infection.

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**Keywords**: *Litopenaeus vannamei*; *Vibrio alginolyticus*; Temperature; Challenge; Phenoloxidase activity; Respiratory burst; Superoxide dismutase activity; Phagocytic activity; Clearance efficiency

1. Introduction

White shrimp *Litopenaeus vannamei* (also called *Penaeus vannamei*) is naturally distributed throughout the Pacific coast from Gulf of California to northern Peru. However, it has now become the primary penaeid shrimp currently being cultured in Pacific rim countries (Lin et al., 1990). Since 1998, shrimp farmers have experienced disease problems linked to production declines due to Taura Syndrome Virus (TSV) (Yu and Song, 2000) and vibriosis caused by *Vibrio alginolyticus* (Liu et al., 2004a). Disease outbreaks have been associated with increases in the proportion of potentially pathogenic species in the *Vibrio* population of culture pond waters (Lavilla-Pitogo et al., 1998; Sung et al., 2001).

In decapod crustaceans, three types of circulating hemocytes are recognized: hyaline, semi-granular and...
large granular cells (Tsing et al., 1989). They are involved not only in phagocytosis, an important process of eliminating microorganisms or foreign particles (Bayne, 1990), but also in the production of melanin via the prophenoloxidase (proPO) system, an important component of the cellular defense reaction (Söderhäll and Cerenius, 1998; Söderhäll et al., 1996).

During phagocytosis, contact with a pathogen activates the host’s NADPH oxidase which, in turn, increases oxygen consumption and produces several species of reactive oxygen intermediates (ROIs) such as the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), singlet oxygen (¹O$_2$) and hydroxyl radical (OH). Superoxide anion is the first product released by respiratory activity bursts, and plays an important role in microbicidal activity (Munoz et al., 2000). Superoxide dismutases (SODs), enzymes that scavenge superoxide anion are classified into three distinct groups depending on the metal content: iron SOD (Fe-SOD), manganese SOD (Mn-SOD) and copper/zinc SOD (Cu,Zn-SOD) (Brouwer et al., 2003). In decapod crustacean, the characterization of SOD and its role in immunolocalization has been reported in shore crab Carcinus maenas (Orbea et al., 2000), crayfish Pacifastacus leniusculus (Johansson et al., 1999), and blue crab Callinectes sapidus (Brouwer et al., 1997, 2003).

*L. vannamei* exhibits hyper-osmotic regulation in low salinity levels, and exhibits hypo-osmotic regulation at high salinity levels with an iso-osmotic point of 718 mosM kg$^{-1}$ (equivalent to 2500) (Castille and Lawrence, 1981). Best survival of juveniles is between temperatures of 20 and 30 °C and salinities above 20% (Ponce-Palafox et al., 1997). It has been reported that the growth of *L. vannamei* increases directly with temperature in the range of 23–30 °C at 33% (Wyban et al., 1995).

Seasonal range of water temperature in shrimp farms may vary from 15 to 32 °C (Chen, 1990). It is assumed that change in temperature may weaken the immune resistance of *L. vannamei*, and lead to its susceptibility to *Vibrio* infection. Accordingly, this study was aimed at examining (1) change in temperature on the susceptibility of *L. vannamei* to *V. alginolyticus*, and (2) change in temperature on the immune parameters of *L. vannamei*. For the latter purpose, we examined THC, phenoloxidase activity, respiratory burst (release of superoxide anion), SOD activity, phagocytic activity and clearance efficiency of shrimp infected with *V. alginolyticus*.

2. Materials and methods

2.1. *L. vannamei*

*L. vannamei* juveniles were obtained from a commercial farm in Ililan, Taiwan, and acclimated in the laboratory for 2 weeks before experimentation. Only shrimp in the intermolt stage were used for the study. The molt stage was determined by the examination of uropoda in which partial retraction of the epidermis could be distinguished (Robertson et al., 1987). For the susceptibility experiment, test and control groups composed of 10 shrimp each in triplicate. For the examinations of immune parameter assays, tests were carried out in eight replicate test groups consisting of one shrimp each in triplicate. For the examinations of immune parameter assays, tests were carried out in eight replicate test groups consisting of one shrimp each in triplicate. For the examinations of immune parameter assays, tests were carried out in eight replicate test groups consisting of one shrimp each in triplicate. 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2.3. Effect of temperature change on susceptibility of L. vannamei to V. alginolyticus

There were two tests. The first test was conducted when L. vannamei were transferred from 27 °C to 20, 27 (control) and 34 °C, and the second test was conducted when the shrimp were transferred from 28 °C to 20, 24, 28 (control) and 32 °C. Shrimp were injected with 20 μl of bacterial suspension of 0.5 × 10⁵ cfu ml⁻¹ or 0.5 × 10⁷ cfu ml⁻¹ resulting in 1 × 10⁴ cfu shrimp⁻¹ or 1 × 10⁵ cfu shrimp⁻¹ in the first or second test, respectively. Challenge tests were conducted in triplicate with 10 shrimp per replicate. After injection, each batch of 10 shrimp were kept in separate 60 l glass aquaria containing 40 l of aerated water (25%) at 20, 27 (control), 34 °C, and 20, 24, 28 (control) and 32 °C in the first and second tests, respectively. The experiment lasted 144 h. Shrimp injected with an equal volume of sterile saline solution and kept in 20, 27, 34 °C, and 20, 24, 28, 32 °C served as the unchallenged controls in the first and second tests, respectively (Table 1).

2.4. Effect of temperature change on immune response of L. vannamei

For hemocyte counts and enzyme activity assays, the test was conducted in eight replicates of 20 l PVC tanks (one shrimp per tank). Each tank contained 10 l of water at different temperatures. L. vannamei at 25% seawater and 28 °C were transferred to 20, 24, 28 (control) and 32 °C for 96 h. There were four treatments (20, 24, 28 and 32 °C) with four sampling times (24, 48, 72 and 96 h). Eight shrimp for each treatment and time were used for the study. In addition, eight shrimp were used as the initial group. Hemolymph was sampled individually at the beginning of the test, and after 24, 48, 72 and 96 h. Hemolymph (100 μl) was withdrawn from the ventral sinus of each shrimp into a 1 ml sterile syringe (25 gauge) containing 0.9 ml anticoagulant (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55, osmolality 780 mosM kg⁻¹). A drop of the anticoagulant–hemolymph mixture was placed on a hemocytometer, and THC was made under an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems, Wetzlar GmbH, Germany), while the remainder of the mixture was used for subsequent tests.

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) following the procedures of Hernández-López et al. (1996). The details of the measurements were described previously (Liu and Chen, 2004). The optical density of the shrimp’s phenoloxidase activity

<table>
<thead>
<tr>
<th>Bacterial dose (cfu shrimp⁻¹)</th>
<th>Temperature (°C)</th>
<th>Cumulative mortality (%), time after challenge (h)</th>
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<tr>
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<td>24</td>
<td>48</td>
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<tr>
<td>Control</td>
<td>20–27</td>
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<td>Control</td>
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<td>3.3 ± 3.3</td>
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<td>1 × 10⁴</td>
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<td>1 × 10⁴</td>
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<td>Control</td>
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<td>Control</td>
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<td>1 × 10⁵</td>
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<tr>
<td>1 × 10⁵</td>
<td>32</td>
<td>40.0 ± 5.7a</td>
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Data in the challenge groups in the same column with different letters are significantly different (p < 0.05) among different temperatures. Values are mean ± S.E. (n = 30 shrimp in each case); cfu: colony-forming units.
was expressed as dopachrome formation in 50 μl of hemolymph.

Respiratory burst activity of hemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion, as described previously (Liu and Chen, 2004). The optical density at 630 nm was measured using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). Respiratory burst was expressed as NBT-reduction in 10 μl of hemolymph.

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical dependent reactions using the Ransod Kit (Randox, Crumlin, UK). The details of the measurements were described previously (Liu and Chen, 2004). The optical density was measured at 505 nm, 37 °C, and the rate of reaction was estimated from the absorbance readings 30 s and 3 min after adding xanthine oxidase. A reference standard SOD was supplied with the Ransod Kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units ml⁻¹.

For phagocytic activity and bacterial clearance tests, there were four treatments (20, 27 and 34 °C) with three sampling times (24, 48, and 72 h). Eight shrimp for each treatment and time were used for the study. In addition, eight shrimp were used as the initial group. Shrimp held in 25 °C and 27 °C were transferred individually to 20, 27 and 34 °C. After 0, 24, 48 and 72 h of transfer, we injected 20 μl bacterial suspension (2 × 10⁸ cfu ml⁻¹) into the ventral sinus resulting in 4 × 10⁶ cfu shrimp⁻¹. After injection, each shrimp was held in a separate tank containing one of the test solutions (20, 27, 34 °C) for 1.5 h. We then collected 100 μl of hemolymph from the ventral sinus, and mixed with 100 μl and 900 μl of sterile anticoagulant for the measurement of phagocytic activity and clearance efficiency, respectively.

Phagocytic activity was measured following the method described by Weeks-Perkins et al. (1995). Briefly, 200 μl of the diluted hemolymph sample was mixed with 0.2 ml 0.1% paraformaldehyde for 30 min at 4 °C to fix the hemocytes, and then centrifuged at 800×g (Model 5403, Eppendorf, Hamburg, Germany) at 4 °C. The details of the measurements were described previously (Liu and Chen, 2004). Two hundred hemocytes were counted. Phagocytic activity, defined as percentage phagocytosis was expressed as:

\[
\text{Percentage phagocytosis} = \frac{\text{(phagocytic hemocytes)}}{\text{(total hemocytes)}} \times 100.
\]

Clearance efficiency was measured following the method of Adams (1991). The 1 ml of volume of diluted hemolymph was further diluted to 100 ml with saline solution. Three 50 μl portions of each diluted hemolymph sample were spread on separate TSA plates and incubated at 27 °C for 24 h before colonies were counted using a colony counter. The number of colony of shrimp kept in 25% and 27 °C was expressed as the control group, and the number of colony of shrimp transferred to 20 °C and 34 °C after 24, 48 and 72 h was expressed as the test group. Clearance efficiency to V. alginolyticus, defined as percentage inhibition (PI), was calculated as:

\[
\text{PI} = 100 - \frac{\text{(cfu in test group)}}{\text{(cfu in control group)}} \times 100.
\]

2.5. Statistical analysis

Tukey’s multiple comparison test was used to compare the significant differences among treatments using the SAS computer software (SAS Institute Inc., Cary, NC, USA). The percentage data (susceptibility study) were normalized using an arcsin transformation before analysis. For statistically significant differences, it was required that \( p < 0.05 \).

3. Results

3.1. Effect of temperature change on susceptibility of L. vannamei to V. alginolyticus

In the first test, all the unchallenged control shrimp transferred from 27 °C to 20 and 27 °C (control) survived. Only 1 out of 30 shrimp died in the unchallenged control shrimp transferred to 34 °C. By contrast, the onset of mortality occurred at 24 h in the
challenged shrimp transferred to 34 °C. Over 24–144 h, cumulative mortality for the shrimp transferred to 34 °C was significantly higher than that of shrimp transferred to 20 °C and 27 °C (Table 1).

In the second test, all the unchallenged control shrimp transferred from 28 °C to 20, 24 and 28 °C (control) survived. Only 1 out of 30 shrimp died in the unchallenged control shrimp transferred to 32 °C. By contrast, the onset of mortality occurred at 24 h in the challenged shrimp transferred to 20, 24 and 32 °C. Over 24–144 h, cumulative mortality for the shrimp transferred to 32 °C was significantly higher than that of shrimp transferred to 20, 24 and 28 °C (Table 1).

3.2. Effect of temperature change on immune response of L. vannamei

There was no significant difference in THC for the shrimp held at 28 °C at different sampling times. The mean (± S.E.) THC varied from 107.1 ± 1.59 × 10^5 to 109.6 ± 2.1 × 10^5 cells ml⁻¹. For the shrimp transferred to 32 °C, the THC decreased significantly by 40% after 24 h. For the shrimp transferred to 20 °C, the THC decreased significantly by 12% and 16% after 24 and 48 h, respectively. However, no significant difference in THC was observed among the shrimp at 20, 24 and 28 °C after 72 and 96 h (Fig. 1A).

![Fig. 1. Mean (± S.E.) total hemocyte count (THC) (A) and phenoloxidase activity (B) Litopenaeus vannamei kept at a salinity of 25% and 28 °C at the beginning, and after 24, 48, 72 and 96 h transfer to 20, 24, 28 and 32 °C. Each bar represents the mean value from eight determinations with standard error. Data (mean ± S.E.) in the same exposure time with different letters are significantly different (p < 0.05) among different temperature levels.](image-url)
No significant difference in phenoloxidase activity was observed among the shrimp kept at 28 °C at different sampling times. For the shrimp transferred to 32 °C, phenoloxidase activity decreased significantly after 24–96 h. For the shrimp transferred to 20 °C, phenoloxidase activity decreased significantly by 12% and 14% after 24 and 48 h, respectively. For the shrimp transferred to 24 °C, phenoloxidase activity decreased significantly by 9% after 24 h. However, no significant difference in phenoloxidase activity was observed among the shrimp transferred to 20, 24 and 28 °C (control) after 72 and 96 h (Fig. 1B).

No significant difference in respiratory burst was observed among the shrimp kept at 28 °C at different sampling times. For the shrimp transferred to 32 °C, respiratory burst decreased significantly after 24, 48 and 72 h. For the shrimp transferred to 20 °C, respiratory burst decreased significantly by 15% and 13% after 24 h and 96 h, respectively. For the shrimp transferred to 24 °C, respiratory burst decreased significantly by 10% and 8% after 24 h and 72 h, respectively. However, no significant difference in respiratory burst was observed among the shrimp transferred to 24, 28 °C (control) and 32 °C after 96 h (Fig. 2A).

No significant difference in SOD activity was observed among the shrimp kept at 28 °C at different sampling times. For the shrimp transferred to 32 °C, the SOD activity decreased significantly after 24 h. For the shrimp transferred to 20 °C, the SOD activity decreased significantly by 20% and 20% after 24 and 48 h, respectively. For the shrimp transferred to 24 °C, the SOD activity decreased significantly by 15% after 24 h. However, no significant difference in SOD activity was observed among the shrimp transferred to 24, 28 °C (control) and 32 °C after 96 h (Fig. 2A).

Fig. 2. Mean (± S.E.) respiratory burst (A) and superoxide dismutase (SOD) activity (B) in the hemocytes of *Litopenaeus vannamei* kept at a salinity of 25% and 28 °C at the beginning, and after 24, 48, 72 and 96 h transfer to 20, 24, 28 and 32 °C. See Fig. 1 for statistical information.
24 h. However, no significant difference in SOD activity was observed among the shrimp at 20, 24, 28 °C and 32 °C after 72 and 96 h (Fig. 2B).

At time 0 h, phagocytic activity was 51% in the control solution (27 °C). Phagocytic activity decreased significantly to 17%, 38% and 34% for the shrimp transferred to 34 °C after 24, 48 and 72 h, respectively, as compared to the shrimp at 27 °C (Fig. 3A). A similar trend was observed for clearance efficiency against *V. alginolyticus*. Clearance efficiency decreased by 42%, 39% and 33% for the shrimp transferred to 34 °C after 24, 48 and 72 h, respectively (Fig. 3B).

### 4. Discussion

Giant freshwater prawn *Macrobrachium rosenbergii* was more susceptible to *Lactococcus garvieae* when reared in 33 °C than when reared in 27 and 30 °C water (Cheng and Chen, 1998). Small abalone *Haliotis diversicolor supertexta* was more susceptible to *Vibrio parahaemolyticus* when the animals were transferred to 32 °C from 28 °C in 12 h (Cheng et al., 2004). Crayfish *P. leniusculus* were exposed to different temperatures after WSSV (white spot syndrome virus) injection, the pathogenicity of the WSSV was significantly higher at 22 °C as compared to temperatures at 4 and 12 °C (Jiravanichpaisal et al., 2004). In the present study, we found that *L. vannamei* was more susceptible to *V. alginolyticus* when the shrimp were transferred to 34 or 32 °C from 27 or 28 °C in 24 h. It can be concluded that increase in water temperature can trigger an outbreak of the disease by weakening the immune response of *L. vannamei*.

Circulating hemocytes are affected by extrinsic factors such as temperature, pH, salinity, dissolved oxygen and ammonia in several species of decapod crustaceans (Le Moullac and Haffner, 2000; Cheng and Chen, 2002). Lobster *Panulirus interruptus* reared in 4 °C had a significantly lower THC and phenoloxidase activity as compared to the lobster reared in 14 and 19 °C (Gomez-Jimenez et al., 2000). Giant freshwater prawn *M. rosenbergii* reared in 20 °C had significantly lower THC and phenoloxidase activity as compared to the prawn reared at 27 and 30 °C (Cheng and Chen, 2000). Blue shrimp *Litopenaeus stylirostris* reared in 18 °C had a significantly lower THC, as compared to the shrimp reared at 27 °C (Le Moullac and Haffner, 2000). A decrease in water temperature also caused a decrease in THC of shore crab *C. maenas* reared at 10 °C, as compared to the crab reared at 20 °C (Truscott and White, 1990), and caused a decrease in THC of crayfish *P. leniusculus* reared in 4 °C as compared to the crayfish reared at 18 °C (Jiravanichpaisal et al., 2004). A similar phenomenon was also observed in the present study: *L. vannamei* at 20 and 24 °C had a significantly lower THC and phenoloxidase activity after 24 h, as compared to the shrimp at 28 °C. However, *L. vannamei* transferred to 20 and 24 °C returned its normal THC and phenoloxidase activity after 72 h.
Several scientists reported decreases in both THC and phenoloxidase activity when animals reared in an optimal temperature condition were transferred to high temperature in a short time. For example, yellowleg shrimp *Farfantepenaeus californiensis* reared at 32 °C had a significantly lower phenoloxidase activity, as compared to the shrimp reared at 18 °C (Vargas-Albores et al., 1998). Shore crab *C. maenas* reared at 13 and 19 °C had significantly lower THC and phenoloxidase activity, as compared to the crab reared at 6 °C (Smith and Chisholm, 1992). Giant freshwater prawn *M. rosenbergii* reared at 33 °C had significantly lower THC and phenoloxidase activity, as compared to the prawn reared at 27 and 30 °C (Cheng and Chen, 2000). Crayfish *P. leniusculus* reared at 22 °C had lower THC, as compared to the crayfish reared at 18 °C (Jiravancihpaisal et al., 2004). A decrease in phenoloxidase activity was observed in small abalone *H. diversicolor supertexta* when transferred to 32 °C from 28 °C over 24 h (Cheng et al., 2004). In the present study, a decrease in THC, phenoloxidase activity and respiratory burst was also observed in *L. vannamei* when transferred to 32 °C from 28 °C over 24–76 h.

The decreases of both phenoloxidase activity and respiratory burst are also well correlated with THC for *L. vannamei* when transferred to 32 °C over 24–76 h. This fact indicated that decreases of phenoloxidase activity and respiratory burst were a consequence of decreases in THC, hyaline cells and granular cells. The expression of peroxinectin cDNA obtained form the hemocytes of *L. vannamei* was significantly reduced when the shrimp were transferred to high temperature (34 °C) (Liu et al., 2004b). In the present study, phenoloxidase activity decreased when the shrimp were transferred to 32 °C from 28 °C after 24 h. It is expected that the transcript encoding pro-phenoloxidase may also reduce for the *L. vannamei* under high temperature stress.

In the present study, the THC decreased together with decreases in both phenoloxidase activity and respiratory burst after 24, 48 and 72 h transfer to 32 °C. Phenoloxidase is stored in the secretory granules of the semi-granular and granular hemocytes, whereas agranular hemocytes are involved in phagocytosis and the release of superoxide anion and other ROIs (Bachère et al., 1995). It is expected that differential hemocyte count may differ for the shrimp under different temperature stresses.

We found that *L. vannamei* when transferred to 20, 24 and 32 °C decreased the respiratory burst and SOD activity in 24 h, as compared to the shrimp at 28 °C. This fact indicated that the activity of NADPH oxidase, responsible for the release of superoxide anion decreased together with a decrease in the activity of superoxide dismutase (SOD) responsible for scavenging superoxide anion. Further research is needed to examine the activities of catalase and peroxidase (Holmblad and Söderhäll, 1999) for *L. vannamei* at different temperature levels.

Phagocytosis can be affected by environmental parameters in invertebrates (Bayne, 1990). For example, elevated temperature has been reported to decrease phagocytic activity of freshwater prawn *M. rosenbergii* against *L. garvieae* (Cheng et al., 2003). The antibacterial activity of shore crab *C. maenas* against *Planococcus citreus* was significantly lower at 13 °C and 19 °C as compared to the crab at 6 °C, which correlated well with lower THC and phenoloxidase activity (Smith and Chisholm, 1992). The phagocytic activity of *V. parahaemolyticus* decreased for small abalone *H. diversicolor supertexta* when transferred to 32 °C from 28 °C in 24 h, which correlated well with increase in susceptibility to *V. parahaemolyticus* (Cheng et al., 2004). In the present study, we found that phagocytic activity and clearance efficiency of *V. alginolyticus* decreased for *L. vannamei* when transferred to 34 °C from 27 °C in 24 h. This correlated well with increase in susceptibility of *L. vannamei* to *V. alginolyticus*.

In conclusion, the present study documented that *L. vannamei* transferred from 27 °C to 34 °C (or from 28 °C to 32 °C) showed a higher susceptibility to *V. alginolyticus*, together with lower THC, phenoloxidase activity, respiratory burst, SOD activity, and decreases in phagocytic activity and clearance efficiency against *V. alginolyticus*, indicating a reduction in immunity ability.

**Acknowledgements**

The study was supported by a grant from the National Science Council (NSC 92-2313-B-019-
042), Republic of China. We appreciate Mr. S.T. Yeh for his assistance in the experiment.

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