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Modulatory effects of ammonia-N on the immune system of *Penaeus japonicus* to virulence of white spot syndrome virus

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**Abstract**

To study response to white spot syndrome virus (WSSV) under ammonia stress, *Penaeus japonicus* were exposed to 5 mg l\(^{-1}\) ammonia-N and challenged orally with WSSV (NW). Controls consisted of an ammonia-N-exposed control group (N), a WSSV-challenged positive control group (W), and an untreated control group (control). Immune parameters measured were total haemocyte count (THC), haemocyte phagocytosis, plasma protein content and haemolymph enzymatic activities for prophenoloxidase (proPO), alkaline phosphatase (ALP), and nitric oxide synthase (NOS). THC and plasma protein had downward trends with time in all treatment groups (NW, N, and W) in contrast to the untreated control group (control). The percentage phagocytosis, NOS activity, and ALP and proPO activity of W and NW decreased initially then increased from 6 to 78 h (except for NOS and ALP, from 6 to 54 h) before declining thereafter until the end of the experiment. Compared with untreated controls (control), there was a downward trend for all measured parameters in the treatment groups (N, NW, and W), but the degree was W>NW>N. WSSV was detected at 78 h

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postchallenge in both W and NW. In conclusion, 5 mg l\(^{-1}\) ammonia-N reduced the immunocompetence of *P. japonicus* and may have decreased the virulence of WSSV.

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**Keywords:** *Penaeus japonicus*; White spot syndrome virus (WSSV); Ammonia-N; Immune parameter

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1. **Introduction**

Shrimp farming has had an impressive growth in many developing countries, where this activity has attained great economic and social importance during the 1980s (Bachère, 2000). However, throughout the last decade, we have become increasingly aware of the socio-economic and environmental unsustainability of shrimp aquaculture industry because shrimp farming has always been affected by infectious diseases, mainly of bacterial and viral epidemics, together with water quality (Kautsky et al., 2000), causing great loss to production. White spot syndrome virus (WSSV) is the causative agent of a new viral disease that has caused a high shrimp mortality and severe damage to shrimp cultures throughout the world (Peng et al., 1998). The disease spreads very rapidly and, by 1994, it had severely impacted most of the shrimp farming regions of Southeast Asia including Japan, China, India, and Korea (Chou et al., 1995; Wang et al., 1995; Cai et al., 1995). Subsequently, WSSV spread to many countries of the western hemisphere. It was detected in the United States, central America, and south America (Kautsky et al., 2000). For this reason, WSSV prevention and control are priorities and shrimp immunology has become a prime area of research. Furthermore, it is very important to notice that environmental stressors can affect shrimp immunity.

WSSV seems to be triggered or aggravated by changes in seawater quality including hardness, temperature, and dissolved oxygen (Kautsky et al., 2000). Ammonia, the end-product of protein catabolism, accounts for more than half of the nitrogenous wastes of decapod crustaceans (Regnault, 1987). The major part of ammonia is excreted mainly through the gill epithelium (Regnault, 1987; Kinne, 1976) through diffusion of NH\(_3\) and NH\(_4^+\) and Na/NH\(_4^+\) exchanges (Pequeux and Gills, 1981; Pressley et al., 1981; Cameron, 1986). Elevated environmental ammonia has been reported to reduce growth, enhance molting (Wickens, 1976; Chen and Kou, 1992), decrease the value of immune parameters such as total haemocyte count (THC), prophenoloxidase (prPPO), superoxide dismutase (SOD), and peroxidase (POD) (Sun and Ding, 1999), and even cause the death of penaeid shrimp (Kou and Chen, 1991).

Variations in immune reactivity within invertebrates have been shown in response to various external and artificial stimuli, including changes in temperature and salinity, pollutants, and natural or artificially induced infections (Vogan and Rowley, 2002; Le Moullac and Haffner, 2000). In this study, THC, phagocytosis of haemocytes, plasma proteins, and haemolymph enzymatic activities such as prophenoloxidase (proPPO), alkaline phosphatase (ALP), and nitric oxide synthase (NOS) have been employed to increase our knowledge of the roles that the above immune parameters play in the defence mechanism.
of *Penaeus japonicus* to WSSV and to evaluate the effects of ammonia-N on the immunocompetence of *P. japonicus* to WSSV.

2. Materials and methods

2.1. Animals

Healthy (WSSV-free) *P. japonicus* were obtained from Haiyang Yellow Sea Shrimp Farm in Yantai China with an average length of 14.43±0.86 cm (mean±S.D., *n*=100) and an average weight of 19.84±3.71 g (mean±S.D., *n*=100), respectively. They were fed with unaltered artificial diet twice a day and acclimated in four aerated flat-bed concrete tanks marked with control, N, W, and NW, each containing 2000 l of sand-filtered seawater (24-28 °C; 32 ppt) for 7 days prior to the experiment. Each tank held 50 *P. japonicus*. The unconsumed food and faeces were removed carefully with a siphon twice a day. All shrimps used in the experiment were intermoult males.

2.2. Chemicals and solutions

All chemicals were of analytical reagent grade. The glassware and solutions were pyrogen-free to avoid enzymatic interruption by endotoxin. The anticoagulant that avoids clotting after collection of haemolymph was prepared according to Vargas-Albores and Ochoa (1992): 450 mM NaCl, 10 mM KCl, 10 mM EDTA–Na2, 10 mM HEPES, pH 7.3, 850 mOsm kg⁻¹. A sodium cacodylate buffer (10 mM sodium cacodylate, 10 mM CaCl₂, pH 7.0) was used to determine haemocyte phenoloxidase activity. The commercial kits for ALP and NOS activity assay were purchased from NanJin JianCheng Bioengineering Institute.

2.3. Experimental treatments

After acclimation, all the shrimps in the four tanks were fasted for 24 h then assigned in four groups, as shown in Table 1. Shrimps in groups NW and W were fed with meat of WSSV-infected Chinese shrimp *Penaeus chinensis* with prominent white spot of

| Group design | Actual concentration of unionized ammonium-N [NH₃–N (mg l⁻¹)]a | Infection of WSSV |
|--------------|---------------------------------------------------------------|-------------------|
| NW           | 5.0±0.2                                                       | +                 |
| N            | 5.0±0.2                                                       | –                 |
| W            | <0.3                                                          | +                 |
| Untreated control | <0.3                                                      | –                 |

* The actual amount of total ammonia-N in seawater was measured with a spectrophotometer (UNICO-UV-2100) by the phenat method at 640 nm (Parsons et al., 1985), and actual concentrations of unionized ammonia-N (NH₃–N) were calculated according to the equation of Bower and Bidwell (1978) based on temperature, salinity, and pH of 24 °C, 32 ppt, and 7.85, respectively.
approximately 15% body weight, and shrimps in groups N and untreated control were fed with an equal amount of Chinese shrimp *P. chinensis* free of WSSV infection. The seawater used in groups N and NW was prepared with a stock solution of ammonium chloride of 10,000 mg l\(^{-1}\) ammonia-N. It was replaced once a day to make the concentration of ammonium relatively stable.

Shrimps were fed with artificial diet after infection. Food residue and faeces were removed carefully with a siphon after each feeding activity. The water temperature, salinity, pH, and ammonia-N were monitored everyday. No dramatic change of ammonia-N concentration occurred during the experiment.

The feeding behavior of shrimps in the four tanks was observed. The immune parameters of *P. japonicus* were assayed at 0, 6, 30, 54, 78, 102, 126, 150, 174, and 198 h postinfection. Three shrimps were sampled (n=3) then they were frozen for WSSV diagnosis at each sampling time.

2.4. Diagnosis of WSSV during the experiment

Diagnosis of WSSV was performed by Digoxigenin-Labeled DNA Probe DOT-BLOT Hybridization Kit purchased from Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. Following blood sampling, the gills of prawn were used in WSSV detection (Shi et al., 1999).

2.5. Specimen preparation

Haemolymph (0.4 ml) from each shrimp was collected from the ventral sinus using a 26-gauge needle and 1-ml syringe containing precooled (4 \(^{\circ}\)C) anticoagulant solution (0.4 ml) and injected into the Eppendorf microfuge containing 0.4 ml of anticoagulant solution; the solution was then mixed thoroughly. This mixture was named solution A for THC and phagocytosis assay. Solution A was then centrifuged at 600\(\times\)g for 10 min at 4 \(^{\circ}\)C. The supernatant was used as the cell-free haemolymph fraction sample named as solution B for the measurement of ALP, plasma protein content, and NOS. Haemocytes were washed and adjusted to a cell density of 4\(\times\)10\(^6\) cells ml\(^{-1}\) in ice-cold sodium cacodylate buffer. This suspension was homogenized with a sonicator equipped with a microtip at 50 W for 20 s, and centrifuged at 15,000\(\times\)g for 10 min at 4 \(^{\circ}\)C. The resultant haemocyte lysate supernatant named solution C was used for proPO activity determination.

2.6. THC

A drop of solution A was placed on a haemocytometer and the THC was measured using a microscope (Olympus BH-2, Japan) with magnification of \(\times\)400.

2.7. Phagocytic activity

Twenty-five microlitres of solution A was placed on a dichromate-cleaned glass slide and incubated for 30 min at room temperature. Subsequently, 25 \(\mu\)l of *Staphylococcus aureus* at a concentration of 1\(\times\)10\(^8\) cells ml\(^{-1}\) was added to each solution A sample and
the preparation was incubated for an additional 30 min. Then, each slide was washed with anticoagulant, fixed with 4% glutaraldehyde in the solution of anticoagulant for 1 min, rinsed in distilled water for 1 min, postfixed with 95% ethanol for 1 min, and air-dried. The slides were then stained with toluidine blue for 5 min and decolorized in running tap water. Numbers of ingested *S. aureus* and numbers of haemocytes that have ingested *S. aureus* were counted from any 200 haemocytes observed using a light microscope at a magnification of ×1000 (Olympus BH-2). Percentage phagocytosis was calculated as (Weeks-Perkins et al., 1995): Percentage phagocytosis = (number of cells ingesting bacteria/number of cells observed) × 100.

2.8. Plasma protein content

Plasma protein concentrations were determined by the method of Bradford (1976). All samples were measured in triplicate and calibrated against a bovine serum albumin (BSA) standard curve (0–200 mg ml⁻¹).

2.9. ALP activity

According to the instruction of the ALP kit, measurement of ALP is based on the mechanism that, under alkaline conditions, ALP reacts with phosphophenyl-2-sodium and releases phosphate and phenol that reacts with 4-aminoantipyrine (4-aminophenazone) to produce a compound, which is oxidized by potassium ferricyanide [K(Fe(CN)₄)] to produce a red derivative of quinone with an adsorption maximum at 520 nm. The detailed operation is described in the instruction of ALP kit. One unit of ALP activity is defined as: 100 ml of plasma produces 1 mg of phenol per 15 min.

2.10. NOS activity

The mechanism of the NOS kit is that the NOS oxidizes the guanidine group of L-arginine in a process that consumes five electrons and results in the stoichiometric formation of NO and L-citrulline within a certain time. The NO was then oxidized into nitrite and nitrate, which was determined by modified Griess method (Marzinzig et al., 1997). The detailed operation is described in the instruction of the NOS kit. One unit NOS activity is defined as: 1 ml of plasma produces 1 nmol of NO per minute.

2.11. Phenoloxidase activity

Phenoloxidase activity in solution C was evaluated spectrophotometrically using L-3,4-dihydroxyphenylalanine (L-DOPA; Sigma) as substrate (Söderhäll, 1981) and trypsin (Sigma) as elicitor, using a method adopted from Smith and Söderhäll (1991). A total of 100 µl of solution C was incubated with 100 µl of 0.4% trypsin in sodium cacodylate buffer at room temperature for 10 min. Subsequently, 100 µl of L-DOPA (0.3% in sodium cacodylate buffer) was added. Each reaction mixture was further diluted with 2.7 ml of sodium cacodylate buffer, then mixed, and the absorbance measured at 490 nm. Absorbance measurements were made against a blank consisting of sodium cacodylate
buffer at intervals of 2 min for 2 h, L-DOPA, and elicitor to control for spontaneous oxidation of the substrate alone. One unit of enzyme activity was defined as: an increase in absorbance of 0.001 min$^{-1}$.

2.12. Statistical analysis

All data are presented as mean±S.E. and subjected to ANOVA. Differences were regarded as statistically significant with a value of $P<0.05$.

3. Results

The experiment lasted for 9 days. The shrimps in groups NW and W began to die 4 days after WSSV infection per os. No mortality of shrimp occurred in group N and untreated control. The shrimps in the control group and group N had similar feeding behavior. It seems that the ammonium-N concentration of 5 mg l$^{-1}$ had no dramatic impacts on the shrimps’ appetite.

3.1. THC

Fig. 1 shows that the THC of healthy $P. japonicus$ (control) in the experiment was in the range of $5.5±0.6×10^7$ and $4.9±0.4×10^7$ cell ml$^{-1}$ ($P>0.05$). From 0 to 6 h of experiment, the THC of $P. japonicus$ in N declined significantly to $4.5±0.2×10^7$ cells ml$^{-1}$ ($P<0.01$); afterward, it declined slowly in contrast to the controls. At 102 h, the haemocytes declined to the minimum ($2.3×10^7$ cell ml$^{-1}$) ($P<0.001$). At 6 h, the THC of $P. japonicus$ in W had decreased to $2.2±0.9×10^7$ cells ml$^{-1}$ ($P<0.01$) and that of $P. japonicus$ in NW to $2.9±0.2×10^7$ cells ml$^{-1}$ ($P<0.01$), then the THC of NW and W reduced further. At 102 h, the THC of W has decreased to $1.9±0.7×10^6$ cell ml$^{-1}$ and that of NW was $3.1±1.9×10^6$ cell ml$^{-1}$ ($P<0.001$). Afterwards, the THC of NW and W remained relatively stable but the THC of NW was higher than that of W ($P<0.05$).

![Fig. 1. THC in $P. japonicus$ from untreated control (control, —■—), ammonia-N stress (N, —□—), WSSV-infected+ammonia-N stress (NW, —□—), and WSSV-infected shrimp (W, —■—). Value±S.E.](image-url)
3.2. Phagocytic activity assay

The percentage phagocytosis of healthy *P. japonicus* (control) was in the range of 10.67 ± 3.21 to 16.33 ± 5.03% (*P* > 0.05). At 78 h, the percentage phagocytosis of *P. japonicus* in N reduced significantly from 14.33% to 4% (*P* < 0.01), but it increased significantly at 126 h (*P* < 0.01) then remained stable (*P* > 0.05). The percentage phagocytosis of *P. japonicus* in W and NW had similar trends; at 6 h, they declined sharply from 14.33 ± 1.15% to 2.33 ± 1.00% and from 14.33 ± 0.58% to 5.00 ± 0.58%, respectively (*P* < 0.01). From 6 to 78 h, both of them increased from 2.33 ± 1.00% (6 h) to 13.00 ± 4.00% (72 h) and from 5.00 ± 0.58% (6 h) to 18.67 ± 4.72% (72 h) (*P* < 0.01). Afterwards, they all declined until the end of experiment with values of 3.33 ± 1.53% and 6.00 ± 1.00%, respectively (*P* < 0.01) (Fig. 2). The percentage phagocytosis of W was lower than that of NW (*P* < 0.05).

3.3. Plasma protein content

The plasma protein concentrations of the control group was relatively stable from the beginning to the end of the experiment (79.54 ± 16.43 and 103.07 ± 8.37 mg ml⁻¹, *P* > 0.05). The plasma protein concentrations of *P. japonicus* in N remained relatively stable (*P* > 0.05) from the beginning of the experiment up to 54 h, then it decreased to 37.56 ± 11.46 mg ml⁻¹ at 102 h (*P* < 0.01). The plasma protein concentrations of *P. japonicus* in NW were generally downward from the beginning of the experiment, reaching a minimum of 34.26 ± 7.13 mg ml⁻¹ (*P* < 0.01) at 126 h, then it remained stable (*P* > 0.05). From 0 h of the experiment to 6 h, the plasma protein concentrations of *P. japonicus* in W increased, then declined toward the end of the experiment, reaching a minimum of 29.85 ± 7.61 at 174 h (*P* < 0.05). During the period of variations of plasma protein concentrations of *P. japonicus* with four treatments, the plasma protein concentrations of *P. japonicus* in W were higher than that of other groups from 6 to 78 h; that of the control group was higher than N and NW throughout the experiment from 102 h to the end of the experiment (*P* < 0.05) (Fig. 3).

![Graph showing percentage phagocytosis over time](image-url)
3.4. ALP activity assay

The trends of ALP in W and in NW were similar with the time elapsed, namely it decreased sharply from 0 to 6 h \( (P<0.01) \) then increased from 6 to 54 h with a maximum of \( 8.00\pm0.82 \) (W) and \( 8.21\pm0.58 \) (NW), respectively. Afterwards, it decreased \( (P<0.01) \) until 174 h, reaching a minimum of \( 1.70\pm0.72 \) (W), and 150 h, with a minimum of 3.49\pm3.46 (NW). At the time of death of the shrimp (198 h), it increased significantly \( (P<0.05) \) again, reaching values of \( 7.37\pm1.93 \) (W) and \( 8.37\pm1.61 \) (NW). The value of ALP activity in the control group was in the range of \( 10.73\pm5.74 \) and \( 7.67\pm1.61 \) \( (P>0.05) \). The trend of ALP activity in N was downward from 0 to 198 h, reaching a minimum of \( 2.41\pm0.00 \) \( (P<0.01) \) (Fig. 4).
3.5. NOS activity determination

At 6 h, the NOS activities of \textit{P. japonicus} in W (19.86±0.41) and \textit{P. japonicus} in NW (20.10±3.18) were lower than those of the control (28.26±1.80) and \textit{P. japonicus} in N (21.17±6.13) \textit{(P}<0.05). Then NOS activity of W and NW increased with time \textit{(P}<0.01); at 54 h, it reached a maximum of 25.95±2.95 and 24.30±2.95, respectively. Afterwards, it decreased significantly from 54 to 150 h \textit{(P}<0.01), reaching its lowest value of 2.74±0.71 (W) and 13.49±7.66 (NW), respectively. Throughout the whole course of the experiment, the NOS activity of healthy prawns remained stable (25.00±2.09 to 31.37±3.19, \textit{P}=0.318>0.05), and from 6 to 126 h, the NOS activity of N (21.23±4.96 to 28.96±1.47, \textit{P}=0.06>0.05) remained stable; but at 150 h, it decreased significantly \textit{(P}<0.01) (Fig. 5).

3.6. Phenoloxidase activity assay

There was generally a reduction in proPO activity in the three treatment groups \textit{(P}<0.05). The proPO activity of W declined from 0 to 54 h and increased from 54 to 78 h significantly \textit{(P}<0.01), then it decreased significantly until 150 h \textit{(P}<0.01). Afterwards, it remained relatively stable \textit{(P}>0.05). During the period of the experiment, the trend of proPO activity in the control group remained stable from 20.17±2.96 to 18.92±3.33 \textit{(P}>0.05). ProPO activity of \textit{P. japonicus} in N declined from 0 to 30 h, then it increased until 78 h, reaching a maximum of 21.38±2.31; afterwards, it decreased until 150 h then increased again by 198 h \textit{(P}<0.01). proPO activity of \textit{P. japonicus} in NW is similar to that of N at 78 h with a maximum of 14.48±4.96. Afterwards, it declined until 150 h, reaching a minimum of 2.53±0.81 \textit{(P}<0.01), and increased at 174 h again, then remained stable up to 198 h (Fig. 6).

Fig. 5. NOS in \textit{P. japonicus} from untreated control (control, ———), ammonia-N stress (N, ——), WSSV-infected+ammonia-N stress (NW, ——), and WSSV-infected shrimp (W, —). Value±S.E.
3.7. Diagnosis of WSSV in P. japonicus during the experiment

*P. japonicus* used as controls and those used in the N experiment were diagnosed as WSSV-negative. *P. japonicus* in W and NW were diagnosed as WSSV-negative up to 78 h from the beginning of artificial infection; afterwards, both groups of shrimp were found to be positive for WSSV. The titres of W were higher than those of NW shrimp.

4. Discussion

Evaluation of cellular and humoral parameters of the immune response of penaeid shrimps has played a vital role for the development of immunoassays to explore the mechanisms of immunity. Ammonia originating from excretion by cultured animals and from ammonification of unconsumed food or organic detritus is one of the most common toxicants in aquaculture systems. Accumulation of ammonia may cause the deterioration of water quality and threaten the growth of cultured penaeids (Chen and Lin, 1992a,b,c). In the present study, six immune parameters (THC, percentage phagocytosis, plasma proteins, propenoloxidase, ALP, and NOS) were employed to assess the immunocompetence of WSSV-infected *P. japonicus* and WSSV-infected *P. japonicus* under 5 mg l\(^{-1}\) ammonia-N stress.

For crustaceans, some information exists on the importance of THC in pathogen and environmental stress resistance. For instance, Persson et al. (1987) reported in *Pacifastacus leniusculus* a relationship between haemocytes and its resistance to the parasitic fungus, *Aphanomyces astaci*. They demonstrated that a decrease in the haemocyte number of crayfish harboring *A. astaci* as a latent infection resulted in an acute infection with incomplete melanization of fungus hyphae, leading to the death of the crayfish. Le Moullac et al. (1998) observed that *Penaeus stylirostris* with low THC resulting from exposure to hypoxia became more sensitive to infections with highly virulent *Vibrio alginolyticus*. In respect of environmental stress, Smith et al. (1995) reported that common shrimp *Crangon crangon* exposed to the dredge spoils were found
to display an elevation in recoverable haemolymph volume and a reduction in THC. *P. stylorosiris*, following exposure to ammonia at 3 mg l$^{-1}$, decreased its THC by 30% (Le Moullac and Haffner, 2000). Plasma protein plays a vital role in the immunity of crustaceans; it not only correlates with the infection of pathogen (Vogan and Rowley, 2002; Song et al., 2003) but also with environmental stress (Chen et al., 1994a,b); thus, it is a very important immunological tool of penaeid shrimp because several immune molecules have been identified and purified in crustaceans such as LPS-binding protein, β-glucan-binding protein, and clotting protein (Sritunyalucksana and Söderhäll, 2000). Zhang et al. (2004) isolated two peptides with molecular masses of 73 and 75 kDa that had nonspecific antiviral properties and no cytotoxicity against host cells from *Penaeus monodon* hemocyanin. The data from the present study show that both ammonia-N and WSSV caused the plasma protein and THC of *P. japonicus* to decrease significantly. The reasons for the phenomena may be as follows: there may be two pathways that cause THC to decline: (1) WSSV invades and destroys the haematopoietic tissue to cause the obstruction of haematopoiesis, and (2) WSSV invades and degrades haemocytes directly. Here, pathway (1) above may cause the general decline of plasma protein concentrations, but at an early stage of WSSV infection, the plasma protein concentrations of W are higher than other treatments, maybe due to WSSV causing haemocyte lysing and releasing protein into the plasma. In addition, exposure of shrimp to ammonia-N caused accumulation of haemolymph ammonia and urea, and caused the catabolism of haemocyanin and protein to free amino acids. This is the main reason for the decrease in plasma protein concentrations caused by high ammonia-N of water (Chen et al., 1993, 1994a,b).

The proPO activity, NOS activity, ALP activity, and percentage phagocytosis of W and NW have similar trends with the time elapsed, namely, the percentage phagocytosis, ALP activity, NOS activity, and proPO activity of W and NW decreased when they were infected with WSSV initially, but from 6 to 78 h (with the exception of NOS and ALP at 54 h), they had an upward trend then decreased until the end of the experiment (Figs. 2, 4–6). The trends of proPO activity, NOS activity, and percentage phagocytosis of N are similar (Figs. 2, 5, and 6)—all had an upward trend then declined. The variations of immune parameters of *P. japonicus* under the stimulation of WSSV and stress of 5 mg l$^{-1}$ ammonia-N stress imply that 5 mg l$^{-1}$ ammonia-N stress affects the immune system of *P. japonicus* and the components of the immune system produce resistant response to the stress, in respect of WSSV, at the early stage of infection, WSSV stimulates the immune system of *P. japonicus*, upregulating then downregulating them. During the period of these immune parameter upregulation, *P. japonicus* was diagnosed as WSSV-negative by DNA dot blot hybridization; therefore, the implication is that *P. japonicus* has immune responses to WSSV and can resist it; but with the time of infection, WSSV destroys it. The immune parameters we employed in the present study are very important components of the immune system of invertebrates. The proPO cascade system can cause melanin formation, stimulation of phagocytosis, nodule formation, encapsulation, and clotting (Söderhäll and Cerenius, 1998; Sritunyalucksana and Söderhäll, 2000; Gillespie et al., 1997), and mediate to generate proteins like antibacterial peptides (Söderhäll and Smith, 1986; Lee and Soderhall, 2002). Rojtinmakorn et al. (2002) reported some proteins, including proPO, that are involved in the defence mechanism of *P. japonicus* to WSSV invasion.
Nitric oxide produced by NOS is associated with diverse actions in neurotransmission, vascular systems, and immunity, including antimicrobial and antiviral activities by way of inhibiting DNA as well as protein and lipid synthesis (Bredt and Snyder, 1994; Karupiah et al., 1993; Howe et al., 2002; Lepoivre et al., 1990). Thus, the NOS system is a very important component in innate immune system that is related directly to virus killing, such as poliovirus (López-Guerrero and Carrasco, 1998), picornavirus (Sanders et al., 1998), flavivirus (Kreil and Eibl, 1996), coronavirus (Lane et al., 1997), arenavirus (Campbell et al., 1994), rhabdovirus (Bi and Reiss, 1995), and ACID virus (Raber et al., 1996). As an innate immune effector, NOS can be induced to produce upregulation of NO in many invertebrates by the invasion of bacteria and parasites (Luckhart and Li, 2001; Dimopoulos et al., 2001; Nappi et al., 2000). As a response of the host to the virus, viral haemorrhagic septicemia virus induced turbot (Scophthalmus maximus) kidney macrophages to produce an upregulation of NO (Tafalla et al., 1999). Hepatitis virus and poliovirus infection induced human cell lines to produce an upregulation of NO (López-Guerrero and Carrasco, 1998; Majano et al., 1998). Although the role of NO in the pathogenesis of viral infection has been demonstrated on many occasions (Kreil and Eibl, 1996; Adler et al., 1997), invertebrate organisms also contain nitric oxide and appear to use this multidimensional molecules in a similar manner as noted for mammals (Stefano and Ottaviani, 2000). As to the NOS activity of penaeid shrimps in response to viral infections has not been reported, our data show that the NOS of P. japonicus seems to defend against WSSV at the early stage of infection, but with the time of infection, this type of action has been decreasing. As to the role of NOS in the immune system of penaeids, this need further evidence to verify it.

The ALP is an important component of lysosomal enzymes that originate from haemocytes to destroy extracellular “invaders” (Cheng and Rodirick, 1975), so phagocytic competence and ALP activity are related to the quantity and quality of haemocytes.

Le Moullac and Haffner (2000) indicated that the defence functions belong to phenoloxidase activity and the peroxinectin decreased at the level of gene expression in P. stylirostris when exposed to ammonia. Our data also indicated that 5 mg l⁻¹ ammonia-N affects the immune parameters, but less seriously than WSSV. As we observed, the ammonium-N concentration of 5 mg l⁻¹ had no dramatic impacts on the shrimp’s appetite, so to our surprise, 5 mg l⁻¹ ammonia-N as a toxicant concentration did not strengthen but rather reduced the susceptibility of P. japonicus to WSSV. Environmental parameters are considered to relate to disease outbreaks by affecting not only the health and defence mechanisms of the host, but also the virulence and density of pathogens. WSSV became completely noninfectious after 60 min of UV irradiation [9×10 (5) μW s cm⁻¹ (2)], by 55 and 70 °C heat treatment within 90 and 5 min, by high acidity (pH 1 for 10 min and pH 3 for 1 h) and high alkalinity (pH 12 for 10 min at 25 °C), by ozone (0.5 μg ml⁻¹ as a total residual oxidant for 10 min at 25 °C), and by 100 ppm of sodium hypochlorite and povidone iodine and 75 ppm of benzalkonium chloride for 10 min at 25 °C (Chang et al., 1998). In our present studies, we adopted oral infection; thus, ammonia-N reduced the immunocompetence of P. japonicus and may decrease the virulence of WSSV, too.

The data from our studies presenting information on penaeid immune parameters in response to virus invasion and environmental stress are helpful in clarifying the
mechanism of the penaeid immune system and the interaction among the host, pathogen, and environmental factors. This is very useful in making a prophylactic strategy of controlling epidemics of penaeid diseases. But this needs further research to optimize it.

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