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Effect of nitrite on interaction between the giant freshwater prawn *Macrobrachium rosenbergii* and its pathogen *Lactococcus garvieae*

Winton Cheng[1], Chun-Hung Liu[2], Jiann-Chu Chen[3,*]

[1]Department of Aquaculture, and [2]Institute of Tropic Agriculture, National Pingtung University of Science and Technology, Pingtung, Taiwan 912, ROC

[3]Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan 202, ROC

ABSTRACT: Addition of nitrite-N at 1.5 mg l^{-1} in tryptic soy broth (TSB) significantly ($p < 0.05$) decreased the growth rate of the bacterial pathogen *Lactococcus garvieae* and significantly ($p < 0.05$) reduced mortality compared to zero nitrite controls when injected into giant freshwater prawns *Macrobrachium rosenbergii* at 5×10^5 colony-forming units (CFU) per prawn. In other experiments, whereby prawns were injected with TSB-grown *L. garvieae* (5×10^5 CFU $prawn^{-1}$) and then held in water containing nitrite-N, mortality at 72 h post-injection was significantly ($p < 0.05$) higher for prawns held in water containing 1.68 mg l^{-1} nitrite than at lower concentrations. Prawns exposed to different concentrations of nitrite-N were examined for THC (total hemocyte count), phenoloxidase activity, respiratory burst, phagocytic activity and bacterial clearance efficiency. No significant differences in THC and phenoloxidase activity were observed among treatments. With prawns exposed to nitrite-N for 168 h (7 d) at 1.59 mg l^{-1}, phagocytic activity and clearance efficiency decreased, while at 1.15 mg l^{-1} or more, respiratory burst increased, generating the superoxide anion at levels considered cytoxic to the host. We conclude that nitrite-N at 1.68 mg l^{-1} causes depression in the immune response and increased mortality in *M. rosenbergii* infected with *L. garvieae*.

KEY WORDS: *Macrobrachium rosenbergii* · *Lactococcus garvieae* · Nitrite · Challenge · Virulence · Hemocyte count · Phenoloxidase activity · Superoxide anion

INTRODUCTION

The giant freshwater prawn *Macrobrachium rosenbergii* is commercially important globally, and especially in Taiwan, as a primary inland-cultured species (New 1995). Disease outbreaks caused by yeast infections in the cool season and *Enterococcus*-like infections in the hot season have resulted in declining production of farmed prawns in Taiwan (Cheng & Chen 1998a). Recently, by polymerase chain reaction assay and 16S rDNA sequencing, the causative bacterium was identified as *Lactococcus garvieae* (Chen et al. 2001).

In decapod crustaceans, 3 types of circulating hemocytes are recognized: hyaline, semi-granular and large granular cells (Tsing et al. 1989). They are involved in cellular immune responses that include phagocytosis and constitute the primary method of eliminating microorganisms or foreign particles (Bayne 1990). Normal hemocyte counts have been established for the shore crab *Carcinus maenas* (Truscott & White 1990), common shrimp *Crangon crangon* (Smith & Johnston 1992), and *Macrobrachium rosenbergii* (Cheng & Chen 2000).

*Corresponding author. E-mail: jcchen@mail.ntou.edu.tw

In addition to phagocytosis, hemocytes are involved in coagulation and in the production of melanin via the prophenoloxidase system (Johansson & Söderhäll 1989, Söderhäll et al. 1996). Enzymes for the prophenoloxidase system are contained in the granular hemocytes, released as proenzymes upon stimulation by microbial cell components such as β-1,3-glucan or lipopolysaccharide from fungal cell walls, and activated by a serine protease (Söderhäll 1983, Smith et al. 1984, Söderhäll et al. 1996). The activity of phenoloxidase has been reported for many crustaceans (Söderhäll et al. 1996) including the brown shrimp *Penaeus californiensis* (Hernández-López et al. 1996), the tiger shrimp *P. monodon* (Sritunyalucksana et al. 1999) and *Macrobrachium rosenbergii* (Cheng & Chen 2002).

Several species of reactive oxygen intermediates (ROIs) are produced during phagocytosis. Once a pathogen or foreign particle enters a host, it activates the host's NADPH-oxidase which, in turn, produces several reactive oxygen intermediates such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$) (Holmblad & Söderhäll 1999). These compounds can be directly toxic to pathogens (Roch 1999). The generation of the superoxide anion is known as the respiratory burst, and it plays an important role in microbicidal activity. It has been reported in the hemocytes of the shore crab *Carcinus maenas* (Bell & Smith 1993), the tiger shrimp *Penaeus monodon* (Song & Hsieh 1994) and the blue shrimp *P. stylirostris* (Le Moullac et al. 1998).

Several physico-chemical parameters and environmental contaminants have been reported to affect the immune response in crustaceans and these have been reviewed by Le Moullac & Haffner (2000). Environmental toxicants have been reported to cause a reduction in hemocyte count in the common shrimp *Crangon crangon* (Smith & Johnston 1992) and the shore crab *Carcinus maenas* (Truscott & White 1990, Victor et al. 1990, Le Moullac & Haffner 2000). They have also been reported to cause reduction of phenoloxidase activity in *C. crangon*, *C. maenas* and *Macrobrachium rosenbergii* (Truscott & White 1990, Smith & Johnston 1992, Smith et al. 1995, Cheng & Chen 2002).

Ammonia, the end product of protein catabolism, accounts for more than half the nitrogenous waste released by decapod crustaceans in intensive culture ponds (Regnault 1987). Although ammonia is toxic to crustaceans, it can be removed by bacterial nitrification or bacterial denitrification via nitrite, an intermediate in the nitrogen cycle. However, elevated concentrations of nitrite have also been reported to have negative effects on growth and molting (Chen & Chen 1992), oxygen consumption and ammonia excretion (Cheng & Chen 1998a), hemolymph hemocyanin and protein levels (Cheng & Chen 1999), osmolality and ion concentration in penaeid shrimp and *Macrobrachium rosenbergii* (Chen & Lee 1997, Cheng & Chen 1998b). The 96 h median lethal concentration of nitrite-N for *M. rosenbergii* larvae and juveniles has been reported to be 8.6 and 8.5 to 12.9 mg l^{-1}, respectively (Armstrong et al. 1976, Chen & Lee 1997).

Ammonia has been reported to affect the immune response of *Penaeus stylirostris* (Le Moullac & Haffner 2000) and *Macrobrachium rosenbergii* (Cheng & Chen 2002). However, little is known regarding the effect of nitrite stress on the immune response and pathogen resistance of shrimp or prawns. Accordingly, this study examined the effects of nitrite on growth and virulence of *Lactococcus garvieae* and on the response of *M. rosenbergii* to *L. garvieae*.

MATERIALS AND METHODS

Lactococcus garvieae. *L. garvieae* was isolated from diseased *Macrobrachium rosenbergii* with opaque and whitish musculature (Cheng & Chen 1998a). It was cultured on tryptic soy agar (TSA, Difco) for 24 h at 30°C before being transferred to 10 ml tryptic soy broth (TSB, Difco) for 24 h at 30°C as a stock culture for tests. For challenge experiments, stock cultures were centrifuged at 7155 × *g* for 15 min at 4°C. The supernatant fluid was removed and the bacterial pellet was resuspended in saline solution (0.85% NaCl) at 2.5 × 10^7 CFU ml^{-1} or 10^9 CFU ml^{-1} as stock bacterial suspensions for injection challenges.

Macrobrachium rosenbergii. *M. rosenbergii* were obtained from a commercial farm in Pingtung, Taiwan, and acclimated in the laboratory for 2 wk before experimentation. For bacterial challenge experiments, test and control groups comprised 10 prawns each in triplicate and individual prawns were in the intermolt stage (Stage C) and weighed 8 to 12 g. After injection, each group of 10 prawns was kept in a separate 60 l glass aquarium containing 40 l aerated water. For immune parameter assays, prawns weighed 20 to 30 g and were in the intermolt stage. For immune activity assays, tests were carried out in triplicate or quadruplicate test groups consisting of 2 prawns each in separate 60 l glass aquaria containing 40 l aerated water. In all tests, prawns were fed twice daily with a formulated prawn diet (Shinta Feed Company) and observed for 168 h (7 d). During experiments, water temperature was maintained at 28 ± 1°C, pH 7.3 to 7.8, total hardness of 100 mg l^{-1} (as $CaCO_3$).

Effect of nitrite on growth of *Lactococcus garvieae.* Inoculum for the growth tests consisted of 0.5 ml of stock broth culture. The stock solution of nitrite was prepared by dissolving 4.93 g $NaNO_2$ (Merck reagent grade) in 1 l distilled water to achieve a concentration

of 1000 mg l^{-1} nitrite-N. Bacteria were incubated in 50 ml TSB with different concentrations of nitrite-N (zero control, 0.3, 0.6, 1.0 and 1.5 mg l^{-1}) in 250 ml flasks at 30°C. Each test was conducted in triplicate and bacterial growth was monitored at 12, 24, 48 and 120 h incubation by measuring the optical density (OD) at 601 nm using a Model U-2000 spectrophotometer (Hitachi).

Effect of nitrite on virulence of *Lactococcus garvieae*. After 24 h of cultivation in TSB containing different concentrations of nitrite-N, *L. garvieae* was harvested by centrifugation at 7155 × *g* for 15 min at 4°C. The pellet was re-suspended in saline solution (0.85% NaCl) at 2.5×10^7 CFU ml^{-1} as the stock bacterial suspension for injection. The concentration of bacterial suspension was measured by its absorbance at 601 nm optical density, and was calculated as a standard curve based on a series of different concentrations of bacterial suspension.

Into the ventral sinus of the cephalothorax of each prawn, we injected 20 µl of bacterial suspension (5×10^5 CFU $prawn^{-1}$) following the method of Cheng & Chen (1998b). Control prawns were injected with an equal volume of sterile saline solution (see Table 1).

Effect of nitrite on resistance of *Macrobrachium rosenbergii* to *Lactococcus garvieae*. *M. rosenbergii* were injected with *L. garvieae* at 5×10^5 CFU $prawn^{-1}$ using 20 µl of bacterial stock solution (2.5×10^7 CFU ml^{-1}), and then kept in 60 l glass aquaria containing 40 l of water with different added concentrations of nitrite-N (zero control and 0.3, 0.6, 1.0 and 1.5 mg l^{-1}) that was renewed daily for 7 d (168 h). The actual mean measured concentrations of nitrite-N were 0.01 (control), 0.29, 0.66, 1.15 and 1.68 mg l^{-1}, respectively (Bendscheider & Robinson 1952). Prawns injected with an equal volume of sterile saline solution and kept in water containing 1.68 mg l^{-1} nitrite-N served as an unchallenged control (see Table 2).

Effect of nitrite on immune parameters of *Macrobrachium rosenbergii*. For hemocyte counts and enzyme activity assays, *M. rosenbergii* (20 to 30 g, intermolt stage) were placed in triplicate in aquaria (2 prawns per aquarium) with added nitrite-N (zero control and 0.3, 0.6, 1.0 and 1.5 mg l^{-1}) that was renewed daily for 7 d (168 h). The actual mean measured concentrations of nitrite-N in each test solution were the same as given in the preceding subsection for the studies on THC, phenoloxidase activity and respiratory burst (see Table 2); for the phagocytic activity and clearance efficiency studies, they were 0.01 (control), 0.31, 0.62, 1.08 and 1.59 mg l^{-1}.

Hemolymph was sampled individually at the beginning of the test and again at 168 h. Hemolymph (100 µl) was withdrawn from the ventral sinus of each prawn into a 1 ml sterile syringe (25 gage) containing 0.9 ml anticoagulant solution (0.114 M trisodium citrate, 0.1 M sodium chloride, pH 7.45, osmolality 490 mOsm kg^{-1}). A drop of hemolymph was placed on a hemocytometer, and THC was measured using an inverted phase-contrast microscope (Leica DMIL).

To measure phenoloxidase activity spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) (Hernãndez-López et al. 1996), diluted hemolymph was centrifuged at 300 × *g* at 4°C for 10 min; the supernatant fluid was then discarded and the pellet was rinsed, resuspended gently in 1 ml cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7.0) and centrifuged again. The pellet was then re-suspended in 200 µl cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 M magnesium chloride, pH 7.0), and a 100 µl aliquot was incubated with 50 µl trypsin (1 mg ml^{-1}), which served as an activator, for 10 min at 25 to 26°C; 50 µl L-DOPA was then added, followed by 800 µl cacodylate buffer 5 min later. The optical density at 490 nm was measured using a Hitachi U-2000 spectrophotometer. The control solution, which consisted of 100 µl cell suspension, 50 µl cacodylate buffer (to replace the trypsin) and 50 µl L-DOPA, was used to measure the background phenoloxidase activity in all test conditions. The optical density of the background phenoloxidase activity was in the range of 0.02 to 0.08. The optical density of the prawns, phenoloxidase activity for all test conditions 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 the superoxide anion, as described previously (Bell & Smith 1993, Song & Hsieh 1994). Briefly, 100 µl hemolymph in anticoagulant solution was deposited in triplicate on microplates previously coated with 100 µl poly-L-lysine solution (0.2%) to improve cell adhesion. Microplates were centrifuged at 300 × *g* for 15 min. Plasma was removed and 100 µl zymosan (0.1% in Hank's solution minus phenol red) was added and allowed to react for 30 min at room temperature. Zymosan was discarded and the hemocytes were washed 3 times with 100 µl Hank's solution and stained with 100 µl NBT solution (0.3%) for 30 min at room temperature. The NBT solution was removed and the hemocytes were fixed, washed 3 times with 100 µl 70% methanol and air-dried. Formazan was dissolved by the addition of 120 µl 2M KOH and 140 µl dimethyl sulfoxide. The optical density at 630 nm was measured using a microplate reader (Dynex Mrx II). Respiratory burst was expressed as NBT reduction 10 $µl^{-1}$ of hemolymph.

To measure bacterial clearance and phagocytic activity, experimental parameters were as described above except that tests were carried out in quadruplicate. For bacterial clearance tests, prawns at 168 h exposure were injected in the muscle of the second abdominal segment with 20 µl bacterial suspension (10^9 CFU ml^{-1} in 0.85% NaCl), resulting in 10^7 CFU $prawn^{-1}$. After injection, the prawns were held in their respective test solutions for 3 h at 27 ± 1°C, before collecting 200 µl volume of hemolymph from the ventral sinus and mixing with 200 µl sterile anticoagulant (modified alsever solution [MAS] or MAS containing 0.8 g sodium citrate, 0.34 g EDTA, 80 10 µl Tween, 100 ml distilled water, pH 7.45, 490 mOsm kg^{-1}). This mixture was divided into 2 equal sub-samples, one to measure phagocytic activity and the other to measure clearance efficiency. With 2 prawns in each of 4 replicates, this gave 8 measurements per parameter for each treatment.

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. They were then centrifuged at 800 × *g* (Centrifuge Model Z323 K, Hermle at 4°C, washed and resuspended in 0.4 ml sterile phosphate buffer solution (PBS). Then, 50 µl of the suspension was spread on a glass slide. The slide was placed in a cytospin centrifuge (Model Cytospin 3, Shandon) and centrifuged at 113 × *g* for 3 min. The slide was then air-dried, stained with Diff-Quick stain, and observed using a light microscope; 200 hemocytes were counted and the phagocytic rate (PR) was expressed as:

PR = [(phagocytic hemocytes)/(total hemocytes)] × 100

Clearance efficiency was measured following the method of Adams et al. (1991). Three 50 µl portions of each diluted hemolymph sample were spread on separate TSA plates and incubated at 30°C for 24 h before colonies were counted using a colony counter. Clearance efficiency, defined as percentage inhibition (PI) of *Lactococcus garvieae* was calculated as:

PI = 100 – [(CFU in test group)/(CFU in control group)] × 100

Statistical analysis. A multiple-comparison Tukey test was used to examine for significant differences among treatments (SAS computer software). Differences were considered significant when $p < 0.05$.

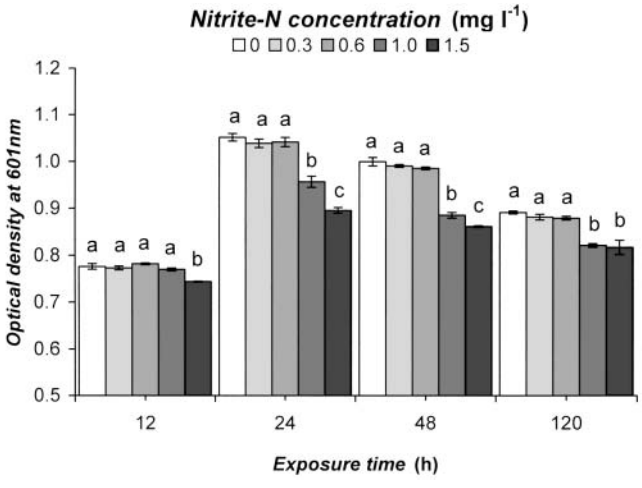

Fig. 1. *Lactococcus garvieae.* Effect of nitrite-N on growth at 30°C in TSB medium. Bacterial numbers were determined by absorbance of bacterial suspension at 601 nm OD at 12, 24, 48 and 120 h. Each bar represents the mean (±SE) of 3 determinations. Bars for same time with different letters are significantly different ($p < 0.05$)

RESULTS

Effect of nitrite on growth of *Lactococcus garvieae*

Lactococcus garvieae grew well in TSB medium containing nitrite-N concentrations of 0.3, and 0.6 mg l^{-1} but there was some retardation at 1.0 and 1.5 mg l^{-1}. Bacterial density reached a maximum at 24 h in all test media and declined thereafter (Fig. 1).

Effect of nitrite on virulence of *Lactococcus garvieae*

All unchallenged control prawns survived. In contrast, deaths began to occur in the challenged prawns at 48 h. Cumulative mortality after 120 h was significantly lower with bacteria incubated in TSB containing nitrite-N at 1.0 to 1.5 mg l^{-1} than with bacteria incubated at lower concentrations (Table 1).

Effect of nitrite on resistance of *Macrobrachium rosenbergii* to *Lactococcus garvieae*

All unchallenged control prawns survived. In contrast, onset of mortality occurred at 12 to 24 h in the challenged prawns (Table 2). Onset of mortality of prawns in 1.68 mg l^{-1} nitrite-N was much earlier (12 h) than at lower concentrations, and mean cumulative mortality was always highest for this group, although the difference was not statistically significant until after 48 h. There were no significant differences in cumulative mortality between the challenged prawns at other concentrations of nitrite-N.

Effect of nitrite on immune parameters of *Macrobrachium rosenbergii*

There were no significant differences in THC for any of the prawns in the study as a function of nitrite-N concentration or time of exposure; the mean (±SE) THC varied from 82 ± 12 × 10^5 to 95 ± 5 × 10^5 cells ml^{-1} (Table 3). Neither were there any significant differences in phenoloxidase activity for any of the prawns in the study as a function of nitrite-N concentration or time. The mean (±SE) phenoloxidase activity varied from 0.39 ± 0.03 to 0.41 ± 0.04.

There were no significant differences in respiratory burst measured at 0 and 168 h for prawns placed in zero control water and 0.29 or 0.66 mg l^{-1} nitrite-N (Fig. 2). However, the respiratory burst at 168 h was significantly higher (41 to 46%) than that at 0 h for prawns exposed to nitrite-N at 1.15 and 1.68 mg l^{-1}.

With respect to phagocytic activity, no significant differences were observed for prawns placed in control water and water containing 0.31, 0.62, and 1.08 mg l^{-1} nitrite-N. However, there was significantly lower phagocytic activity (57%) for prawns at 1.59 mg l^{-1} nitrite-N than for prawns in the group with no added nitrite (Fig. 3A).

A similar trend was seen for clearance of *Lactococcus garvieae*, whereby the only significant difference was decreased clearance (155%) in the 1.59 mg l^{-1} nitrite-N group compared to the group with no added nitrite (Fig. 3B).

Table 1. *Macrobrachium rosenbergii* challenged with *Lactococcus garvieae*. Cumulative mortality (%) in prawns after injection of 5 × 10^5 CFU of the pathogen incubated in tryptic soy broth at different concentrations of nitrite-N. Controls were injected with equal volume of sterile saline solution. Data in same column with different letters are significantly different ($p < 0.05$) among the treatments. Values are means ± SE

Nitrite-N (mg l^{-1})	Time after challenge (h) 48	72	96	120	144	168
Unchallenged control	0	0	0	0	0	0
0	7 ± 7^a	13 ± 9^a	30 ± 6^a	30 ± 6^a	30 ± 6^a	30 ± 6^a
0.3	3 ± 3^a	17 ± 3^a	17 ± 3^{ab}	33 ± 3^a	33 ± 3^a	33 ± 3^a
0.6	10 ± 6^a	17 ± 9^a	20 ± 0^a	30 ± 6^a	30 ± 6^a	30 ± 6^a
1.0	7 ± 3^a	10 ± 6^a	13 ± 3^{ab}	17 ± 3^b	20 ± 0^{ab}	20 ± 0^{ab}
1.5	3 ± 3^a	7 ± 7^a	10 ± 6^b	17 ± 3^b	17 ± 3^b	17 ± 3^b

Table 2. *Macrobrachium rosenbergii*. Cumulative mortality (%) in prawns challenged with *Lactococcus garvieae* at 5 × 10^5 CFU $prawn^{-1}$, and then held at different concentrations of nitrite-N. Unchallenged controls were injected with equal volume of sterile saline solution and kept in water with 1.68 mg l^{-1} nitrite-N. Data in same column with different letters are significantly different ($p < 0.05$) among treatments. Values are means ± SE

Nitrite-N (mg l^{-1})	Time after challenge (h) 12	24	48	72	120	168
Unchallenged control (1.68)	0	0	0	0	0	0
0.01 (control)	0 ± 0^a	7 ± 3^a	7 ± 3^b	10 ± 0^b	27 ± 3^b	33 ± 3^b
0.29	0 ± 0^a	10 ± 6^a	10 ± 6^b	17 ± 3^b	30 ± 6^b	37 ± 3^b
0.66	0 ± 0^a	10 ± 6^a	20 ± 6^{ab}	20 ± 6^b	33 ± 7^b	40 ± 6^b
1.15	0 ± 0^a	7 ± 3^a	10 ± 6^b	13 ± 3^b	27 ± 3^b	40 ± 6^b
1.68	10 ± 6^a	20 ± 6^a	23 ± 3^a	40 ± 6^a	50 ± 6^a	57 ± 3^a

DISCUSSION

Culture media parameters can affect the growth of pathogens and their production of bacterial enzymes and toxins (Weinberg 1985, Arp 1988). Cheng & Chen (1999) reported that optimum conditions for *Lactococcus garvieae* growth in brain/heart infusion broth (BHIB) were pH 7 to 8 at 25 to 30°C. They also reported (Cheng & Chen 2002) that it grew well in tryptic soy broth (TSB) containing ammonia-N at concentrations as high as 5.14 mg l^{-1}. In the present study, we found that growth of *L. garvieae* was depressed in TSB containing nitrite-N at only ≥1.0 mg l^{-1} (about ≥ 0.07 mM). Doßmann et al. (1996) reported a minor effect of

Table 3. *Macrobrachium rosenbergii*. Effect of different concentrations of nitrite-N on THC and phenoloxidase activity. Differences in each category are not significant ($p > 0.05$). Values are means ± SE

Immune parameter	Exposure time (h)	Nitrite-N concentrations (mg l^{-1}) Control	0.29	0.66	1.15	1.68
THC (×10^5 ml^{-1})	0	86 ± 5	92 ± 12	91 ± 6	95 ± 5	93 ± 7
	168	94 ± 9	93 ± 9	93 ± 10	88 ± 10	82 ± 12
Phenoloxidase activity	0	0.39 ± 0.03	0.40 ± 0.04	0.40 ± 0.02	0.40 ± 0.04	0.41 ± 0.05
	168	0.41 ± 0.04	0.40 ± 0.05	0.41 ± 0.04	0.41 ± 0.04	0.40 ± 0.03

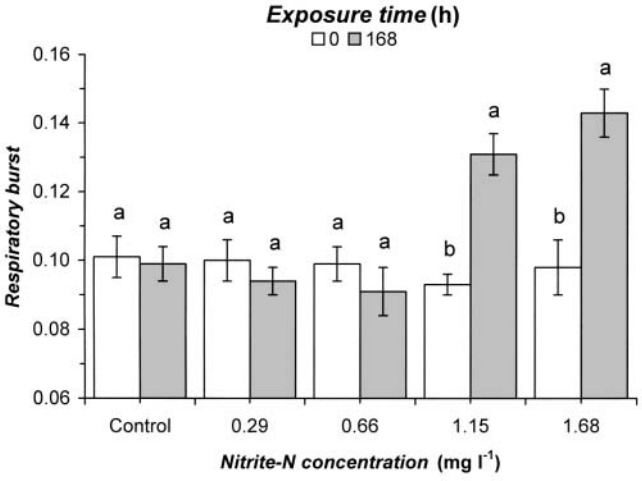

Fig. 2. *Macrobrachium rosenbergii*. Mean respiratory burst (as nitroblue tetrazolium reduction in 10 µl hemolymph) in hemocytes at 0 and 168 h exposure to different concentrations of nitrite-N. Each bar represents the mean (±SE) of 6 determinations. Bars for same nitrite-N concentration with different letters are significantly different ($p < 0.05$)

sodium nitrite on the growth of both *L. sake* and *L. pentosus*, although the concentration that affected growth was not given. Blasco et al. (2001) reported that *Rhodococcus* sp. was able to grow in media in the presence of high concentrations of nitrate (up to 0.9 M) and 60 mM nitrite (840 mg l^{-1}). This suggests that *L. garvieae* may be rather sensitive to the concentration of nitrite in growth media.

Cheng & Chen (1999) reported that incubation of *Lactococcus garvieae* under optimal conditions in BHIB containing 0.5 to 1.0 NaCl significantly enhanced its virulence for *Macrobrachium rosenbergii*. Cheng & Chen (2002) also reported that prior incubation of *L. garvieae* in TSB containing ammonia-N at 1.28 mg l^{-1} or more decreased its virulence when injected into *M. rosenbergii*. Herein we describe a similar effect upon prior incubation of *L. garvieae* in TSB containing 1.5 mg l^{-1} nitrite-N. However, the mechanism by which ammonia and nitrite may decrease the virulence of *L. garvieae* to *M. rosenbergii* is not known.

Kautsky et al. (2000) reported that fluctuations in normal environmental conditions such as oxygen, temperature and salinity had a significant effect on the virulence of the luminous bacterium *Vibrio harveyi*, with higher salinity increasing virulence to shrimp more than higher temperatures. On the other hand, Prayitno & Latchford (1995) demonstrated that exposure of *V. harveyi* to low salinity (10 and 15 ppt) for 12 h prior to immersion challenge tests with larvae of the tiger shrimp *Penaeus monodon* resulted in higher mortalities, while exposure to pH 5.5 reduced pathogenicity.

With respect to the prawns themselves, Cheng & Chen (1998b) reported that *Macrobrachium rosenbergii* was most susceptible to *Lactococcus garvieae* when reared at pH 8.8 to 9.5 and 33 to 34°C. They also showed (Cheng & Chen 2002) that *M. rosenbergii* was most susceptible to *L. garvieae* when the rearing water contained ammonia-N at 1.68 mg l^{-1} or more. Herein, we found a similar phenomenon, with *M. rosenbergii* being more susceptible to *L. garvieae* when reared in water containing nitrite-N at 1.68 mg l^{-1}. We may conclude that the susceptibility of *M. rosenbergii* to *L. garvieae* is increased by high pH, high temperature and the presence of relatively low levels of ammonia-N and nitrite-N in water.

The abundance of circulating hemocytes of *Macrobrachium rosenbergii* at different body sizes, seasons, body weights and molting cycle stages have been reported by Cheng & Chen (2001). *M. rosenbergii* displayed the highest and lowest THC in autumn and winter, respectively. There were no significant differences between male and female prawns or among individuals with body weights ranging from 7 to 115 g. *M. rosenbergii* displayed the lowest THC at the D_3 molt stage and the highest at Stage C (Cheng & Chen 2001). In the present study the *M. rosenbergii* used

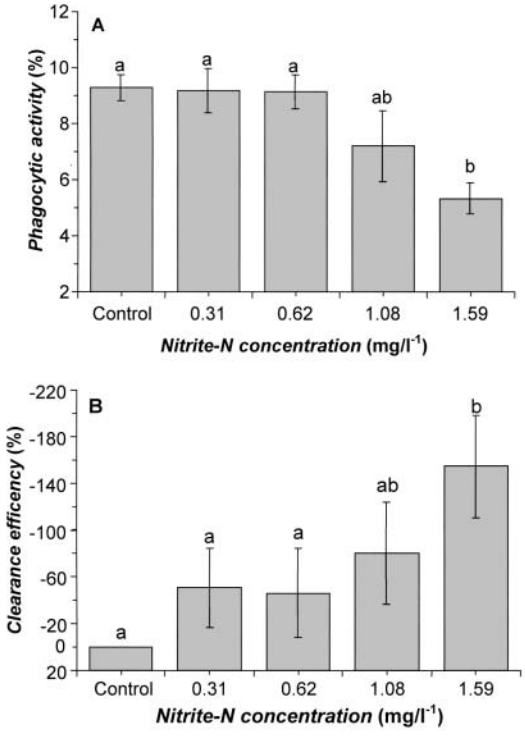

Fig. 3. *Macrobrachium rosenbergii*. Mean phagocytic activity (A) and clearance efficiency (B) of prawns infected with *Lactococcus garvieae* after the prawns had been placed in different concentrations of nitrite-N for 168 h. Each bar represents the mean (±SE) of 8 determinations. Bars with different letters are significantly different ($p < 0.05$)

were at Stage C, and therefore were considered to have a similar host defense capability.

Circulating hemocytes can be affected by extrinsic factors such as temperature, pH, salinity, dissolved oxygen and ammonia in several species of decapod crustaceans (Truscott & White 1990, Le Moullac et al. 1998, Le Moullac & Haffner 2000, Cheng & Chen 2001). *Macrobrachium rosenbergii* reared at temperatures of 27 to 28°C and 30 to 31°C had significantly higher THC than those reared at 20 to 21°C or 33 to 34°C. Prawns reared at pH 7.5 to 7.7 had significantly higher THC than those reared at pH 4.6 to 5.0 and 9.0 to 9.5 (Cheng & Chen 2000). In contrast, there were no significant differences in THC among prawn groups exposed to ammonia-N concentrations in the range of 0.07 to 3.18 mg l^{-1} for 168 h (Cheng & Chen 2002), and similar results were obtained herein for nitrite-N concentration in the range of 0.01 to 1.68 mg l^{-1}.

Smith & Johnston (1992) reported that exposure of the common shrimp *Crangon crangon* to PCB-15 (polychlorinated biphenyl-15) resulted in significantly decreased THC and phenoloxidase activity. Cheng & Chen (2000) reported that both the phenoloxidase activity and THC of *Macrobrachium rosenbergii* were significantly higher at pH 7.5 to 7.7 and 30 to 31°C than at pH 4.6 to 5.0 or pH 9.0 to 9.5 and 33 to 34°C. Cheng & Chen (2002) indicated that phenoloxidase activity of *M. rosenbergii* was significantly decreased by exposure to ammonia-N at 0.55 mg l^{-1} or more, in spite of the fact that ammonia-N did not affect THC. Le Moullac & Haffner (2000) suggested that phenoloxidase and peroxinectin activity were reduced by ammonia at the level of gene expression in *Penaeus stylirostris*. Although we expected that nitrite-N exposure might reduce phenoloxidase activity in *M. rosenbergii*, this was not found to be the case.

Song & Hsieh (1994) reported that β-glucan had the strongest stimulative effect on hemocytes in *Penaeus monodon* for generating O_2^- (superoxide anion) and H_2O_2 (hydrogen peroxide) that were considered to play a more important role in shrimp microbicidal activity than OCl^- (hypochlorites) and MPO (myeloperoxidase). Le Moullac et al. (1998) proposed that the decreased production of the superoxide anion in hypoxic *P. stylirostris* was due to the decrease in THC, and that the NADPH oxidase responsible for production of the superoxide anion was not affected by hypoxia. Cheng & Chen (2002) reported that exposure of *Macrobrachium rosenbergii* to 0.55 mg l^{-1} ammonia-N for 168 h stimulated the production of the superoxide anion. Herein, we found that exposure of *M. rosenbergii* to 1.15 and 1.68 mg l^{-1} nitrite-N for 168 h also increased the superoxide anion. Thus, exposure of *M. rosenbergii* to appropriate concentrations of ammonia-N or nitrite-N may increase cytotoxic immunity (Muñoz et al. 2000). A small increase in the superoxide anion is considered to be beneficial with respect to increased immunity. However, too great an increase may be cytoxic to the host (Cheng & Wang 2001). From our experiments, it is not possible to distinguish whether the increase in the superoxide anion resulted from increased NADPH oxidase activity or from decreased activity of superoxide dismutase (SOD) responsible for scavenging the superoxide anion. Further research is needed to examine the activities of superoxide dimutase, catalase and peroxidase (Holmblad & Söderhäll 1999) in response to ammonia and nitrite stress.

Phagocytosis is an important cellular defense mechanism in crustaceans (Ratcliffe et al. 1985) that can be affected by environmental parameters. A significant reduction in phagocytosis of *Bacillus cereus* was observed in the shore crab *Carcinus maenas* following 14 d exposure to 500 µg l^{-1} Cd^{2+} and 10 d exposure to 100 µg l^{-1} Cu^{2+} (Truscott & White 1990). Direkbusarakom & Danayadol (1998) reported that phagocytosis of *Saccharomyces cerevisiae*, and clearance efficiency of *Vibrio harveyi* decreased in *Penaeus monodon* following exposure to 1.8 to 2.0 mg l^{-1} O_2 for 6 h, compared to control shrimp. Similarly, we found that phagocytic activity and clearance efficiency of *Lactococcus garvieae* decreased in *Macrobrachium rosenbergii* following exposure to 1.59 mg l^{-1} nitrite-N. This correlated with increased susceptibility of *M. rosenbergii* to *L. garvieae* when the prawns were under nitrite stress. In addition to these effects, it would be interesting to determine whether ammonia and/or nitrite stress affect the production of antimicrobial peptides such as penaeidins, which have been observed in the white shrimp *Penaeus vannamei* (Bachère et al. 2000).

In conclusion, the present study documented that nitrite retarded growth of *Lactococcus garvieae* and decreased its virulence to *Macrobrachium rosenbergii*. Nitrite exposure also increased the susceptibility of *M. rosenbergii* to *L. garvieae*, at least partially, by a reduction in immune responses such as decreased phagocytic activity and decreased bacterial clearance efficiency together with increased superoxide anions, possibly to cytotoxic levels for the host.

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