Vol. 47: 137–144, 2001 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published November 8

The susceptibility of the giant freshwater prawn *Macrobrachium rosenbergii* to *Lactococcus garvieae* and its resistance under copper sulfate stress

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ABSTRACT: Addition of copper sulfate (0.1 to 0.4 mg l^{-1}) to tryptic soy broth (TSB) had no effect on growth rate of the bacterial pathogen *Lactococcus garvieae*. Giant freshwater prawns *Macrobrachium rosenbergii* were injected with *L. garvieae* (4×10^6 colony-forming units [cfu] $prawn^{-1}$) grown in TSB or TSB containing copper sulfate at 0.1, 0.2, 0.3 or 0.4 mg l^{-1}. After 48 h, the cumulative mortality was significantly ($p < 0.05$) higher for prawns exposed to *L. garvieae* grown in 0.4 mg l^{-1} copper sulfate than at the lower concentrations examined. In other experiments, prawns were injected with TSB-grown *L. garvieae* (4×10^6 and 2×10^5 cfu $prawn^{-1}$), then held in water containing copper sulfate. After 8 h the mortality of *L. garvieae*-exposed prawns held in water containing 0.4 mg l^{-1} copper sulfate was significantly higher than prawns held in water containing 0.2 and 0.3 mg l^{-1} copper sulfate. At the lower *L. garvieae* density, cumulative mortality of prawns increased directly with ambient copper sulfate concentrations in the range of 0.2 to 0.4 mg l^{-1}. All prawns survived a 168 h exposure to 0.1 mg l^{-1} copper sulfate. Prawns exposed to different concentrations of copper sulfate were examined for hemocyte density, phenoloxidase activity and respiratory burst. No significant differences in hemocyte density were observed among treatments. In prawns following a 48 h exposure to 0.1 mg l^{-1} copper sulfate, phenoloxidase activity was decreased, but respiratory burst was increased. In conclusion, copper sulfate increased the virulence of *L. garvieae* to *M. rosenbergii* and modulated its immune system. Copper sulfate at 0.1 mg l^{-1} decreased susceptibility of *M. rosenbergii* to *L garvieae* infection, whereas at 0.2 mg l^{-1} the susceptibility was increased. The generation of superoxide anion by *M. rosenbergii* exposed to copper sulfate at a concentration higher than 0.2 mg l^{-1} was considered to be cytoxic.

KEY WORDS: *Macrobrachium rosenbergii* · *Lactococcus garvieae* · Copper sulfate · Hemocyte count · Challenge test · Phenoloxidase activity · Respiratory burst

INTRODUCTION

The giant freshwater prawn *Macrobrachium rosenbergii* is commercially important in Taiwan as well as the world 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 resulted in declined production of farmed prawns in Taiwan (Cheng & Chen 1998a,b). Recently, this causative bacterium has been identified as *Lactococcus garvieae* by polymerase chain reaction assay and 16s rDNA sequencing (Chen et al. 2001).

Hemocytes are involved in phagocytosis by eliminating microbes or foreign particles (Hose et al. 1990, Bachère et al. 1995). In decapoda crustaceans, 3 types of circulating hemocytes are commonly recognized: hyaline, semi-granular and granular cells (Tsing et al. 1989). Environmental contaminants have been reported to cause a reduction in hemocyte numbers in

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the common shrimp *Crangon crangon* (Smith & Johnston 1992) and shore crab *Carcinus maenas* (Truscott & White 1990, Victor et al. 1990, Le Moullac & Haffner 2000). Cheng & Chen (2000) reported from tests on exposure of *Macrobrachium rosenbergii* to different pH and temperatures that both total hemocyte count (THC) and penoloxidase activity were minimal at pH 9.0 to 9.5 and 33 to 34°C. This supports the observation of Cheng & Chen (1998b) that *M. rosenbergii* is vulnerable to *Lactococcus garvieae* under these environmental conditions.

Hemocytes are involved not only in coagulation but also in the production of melanin by the prophenoloxidase system (Johansson & Söderhäll 1989, Söderhäll et al. 1996). The prophenoloxidase system, which is contained in the granular cells, is activated by the prophenoloxidase activating enzyme, a serine protease that is in turn activated by microbial cell components such as β-1,3-glucan or lipopolysaccharides from fungal cell walls (Söderhäll 1983, Smith et al. 1984). The activity of the phenoloxidase system has been reported for brown shrimp *Penaeus californiensis* (Herãndez-López et al. 1996), and *Macrobrachium rosenbergii* (Cheng & Chen 2000). Environmental contaminants have been reported to cause a reduction of phenoloxidase activity of *Crangon crangon* and *Carcinus maenas* (Truscott & White 1990, Smith & Johnston 1992, Smith et al. 1995).

The mechanism involved in phagocytosis encompasses the generation of various reactive oxygen intermediates and has been observed in several species of decapod crustaceans (Bell & Smith 1993, Song & Hsieh 1994, Le Moullac et al. 1998). Once the pathogen infects the host, it activates the host's NADPH-oxidase, which in turn produces several reactive oxygen intermediates such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) (Homblad & Söderhäll 1999). These compounds can be directly toxic to pathogens (Roch 1999). This phenomenon, known as respiratory burst, plays an important role in microbicidal activity (Song & Hsieh 1994).

Copper sulfate is commonly applied in prawn ponds to eradicate filamentous algae. The application of copper sulfate is also very effective in reducing the abundance of phytoplankton including *Microcystis* and other blue-green algae. The application rate of copper sulfate varies from 0.025 to 2 mg l^{-1} (Boyd 1990). Since prawn farmers often apply excess amounts of copper sulfate in pond management, the concentrations of copper sulfate remaining in water and their effect on the resistance of cultured prawns are of primary concern.

The 24 and 96 h median lethal concentration of copper on *Macrobrachium rosenbergii* postlarvae has been reported to be 1.15 and 0.32 mg l^{-1}, respectively (Ismail et al. 1990). Copper has been reported to affect hemocyte number and induce immunomodulation in bivalves (Suresh & Mohandads 1990, Pipe et al. 1999). Little is known, however, of the effect of copper on the resistance of decapod crustaceans (Truscott & White 1990). This study is aimed at determining (1) the growth of *Lactococcus garvieae* in tryptic soy broth (TSB), (2) the virulence of *L. garvieae* to *M. rosenbergii*, (3) the resistance of *M. rosenbergii* to *L. garvieae* and (4) the immune parameters of *M. rosenbergii* under stress from copper sulfate. Hemocyte counts (THC and differential hemocyte counts [DHC]), phenoloxidase activity and respiratory burst (production of superoxide anion) were used as indicators.

MATERIALS AND METHODS

Effect of copper sulfate on the growth of *Lactococcus garvieae*. The bacterium *L. garvieae* isolated from diseased *Macrobrachium rosenbergii* was used in this study. Previously it had been shown to cause opaque and whitish musculature in experimental infections (Cheng & Chen 1998a). The bacterium was cultured on tryptic soy agar (Difco) for 24 h at 28°C before being transferred to 10 ml TSB (Difco) for 24 h at 30°C as a stock bacterial broth for growth tests. Inoculum for the growth tests consisted of 0.5 ml of this stock broth culture.

The concentration of copper sulfate was prepared by dissolving 1 g of anhydrous copper sulfate (Merck reagent grade) in 1 l of distilled water to make 1000 mg l^{-1} of stock solution. Bacteria were incubated in 50 ml TSB with different concentrations of copper sulfate (control, 0.1, 0.2, 0.3 and 0.4 mg l^{-1}) in 250 ml flasks at 30°C. Each test was conducted in triplicates 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, Tokyo, Japan).

Effect of copper sulfate on the virulence of *Lactococcus garvieae*. Bacteria from the TSB media containing different concentrations of copper sulfate (0, 0.1, 0.2, 0.3 and 0.4 mg l^{-1}) were tested for the effect of copper sulfate on the virulence of *L. garvieae*. After 24 h of cultivation, *L. garvieae* in each test medium were harvested by centrifugation at $7155 \times g$ for 15 min at 4°C. The pellet was re-suspended in saline solution (0.85% NaCl) at 2×10^8 colony-forming units (cfu) ml^{-1} as the stock bacterial suspension for injection.

Macrobrachium rosenbergii (9 to 13 g in the intermolt stage) were obtained from a commercial farm in Pingtung, Taiwan, and acclimated in the laboratory for 2 wk before experimentation. Twenty microliters of bacterial suspension was then injected into the ventral sinus of the cephalothorax of each prawn. Challenge

tests were conducted in triplicate using a dose of 4×10^6 cfu prawn^{-1} following the method of Cheng & Chen (1998b). The test and control groups comprised 10 prawns each. After injection, each group of 10 prawns was kept in a separate 60 l glass aquarium containing 40 l of aerated water. They were fed twice daily with a formulated prawn diet (Shinta Feed Company, Pingtung, Taiwan) and observed for 168 h. During the experiment, water temperature was maintained at 28 ± 1°C, pH 7.3 to 7.8, and total hardness 100 mg l^{-1}. Control prawns were injected with an equal volume of sterile saline solution (Table 1).

Effect of copper sulfate on the resistance of *Macrobrachium rosenbergii* to *Lactococcus garvieae*. The bacteria were cultured in TSB for 24 h at 30°C, then centrifuged at 7155 × *g* for 15 min at 4°C. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution (0.85% NaCl) at 2×10^8 cfu ml^{-1} as the stock bacterial suspension for injection challenges.

Macrobrachium rosenbergii were injected, held, fed and observed as described above. Challenge tests at 2 doses (4×10^6 and 2×10^5 cfu prawn^{-1}) were conducted in triplicates comprising 10 prawns for each replicate. After injection, prawns were kept in 60 l glass aquaria (10 prawns each) containing 40 l of water with different concentrations of copper sulfate (0, 0.1, 0.2, 0.3 and 0.4 mg l^{-1}). These test solutions were renewed daily. Prawns injected with an equal volume of sterile saline solution and kept in water containing 0.4 mg l^{-1} copper sulfate served as the unchallenged controls (Table 2).

Effect of copper sulfate on the immune parameters of *Macrobrachium rosenbergii*. *M. rosenbergii* (20 to 28 g in the intermolt stage) were obtained and acclimated as described above. Two prawns were kept in each 60 l glass aquarium containing 40 l of test solution at different concentrations of copper sulfate (0, 0.1, 0.2, 0.3 and 0.4 mg l^{-1}). The test solutions were renewed daily, and the experiment lasted for 96 h. Each test solution was conducted in triplicates. The prawns were fed as described above.

Hemolymph was sampled at the beginning of the test, at 48 h and at 96 h. Hemolymph (100 µl) was withdrawn from the ventral sinus of each prawn into a 1 ml syringe (25 gauge) containing 0.9 ml anticoagulant solution (trisodium citrate 0.114 M, sodium chloride 0.1 M, pH 7.45, osmolality 490 mOsm kg^{-1}). A drop of hemolymph was placed on a hemocytometer, and THC and DHC were measured using an inverted phase contrast microscope (Leica DMIL, Leica Microsystems Wetzlar GmbH, Germany).

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) (Hernãndez-López et al. 1996). The diluted hemolymph was centrifuged at 300 × *g* at 4°C for 10 min, the supernatant fluid was discarded and the pellet was rinsed, resuspended gently in 1 ml cacodylate-citrate buffer (sodium cacodylate 0.01 M, sodium chloride 0.45 M, trisodium citrate 0.10 M, pH 7.0) and then centrifuged again. The pellet was then resuspended with 200 µl cacodylate buffer (sodium cacodylate 0.01 M, sodium chloride 0.45 M, calcium chloride 0.01 M, magnesium chloride 0.26 M, pH 7.0). One hundred microliters of the cell suspension was incubated with 50 µl of trypsin (1 mg ml^{-1}), which served as an elicitor, for 10 min at 25 to 26°C. Fifty microliters of L-DOPA was added, followed by 800 µl of cacodylate buffer added 5 min later. The OD at 490 nm was measured using a Hitachi U-2000 spectrophotometer. The control solution, which consisted of 100 µl of cell suspension, 50 µl of cacodylate buffer (to replace the trypsin) and 50 µl of L-DOPA, was used to measure the background phenoloxidase activity in all test solutions. The background phenoloxidase activity OD values were subtracted from the phenoloxidase activity OD values of prawns for all test conditions and ranged from 0.02 to 0.08.

Respiratory burst activity of hemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production (Song & Hsieh 1994). One hundred microliters of hemolymph in anticoagulant solution was deposited in

Table 1. Susceptibility of *Macrobrachium rosenbergii* to *Lactococcus garvieae* incubated in tryptic soy broth with different $CuSO_4$ concentrations at 28 ± 1°C, pH 7.3 to 7.8 and total hardness 100 mg l^{-1}. Data in the same column with different letters are significantly different ($p < 0.05$) among the treatments. Values are means ± SE. cfu: colony-forming units

Bacteria (cfu prawn^{-1})	Ambient $CuSO_4$ (mg l^{-1})	No. prawns	Cumulative mortality (%) 16	24	48	72	96
Control	Saline	30	0	0	0	0	0
4×10^6	0	30	13.3 ± 3.3^a	26.7 ± 8.8^a	36.7 ± 3.3^b	36.7 ± 3.3^b	36.7 ± 3.3^b
4×10^6	0.1	30	6.7 ± 3.3^a	20.0 ± 10.0^a	30.0 ± 5.8^b	30.0 ± 5.8^b	30.0 ± 5.8^b
4×10^6	0.2	30	13.3 ± 6.7^a	26.7 ± 8.8^a	33.3 ± 6.7^b	36.7 ± 3.3^b	40.0 ± 5.8^b
4×10^6	0.3	30	6.7 ± 3.3^a	20.0 ± 0.0^a	33.3 ± 8.8^b	33.3 ± 8.8^b	33.3 ± 8.8^b
4×10^6	0.4	30	13.3 ± 6.7^a	40.0 ± 11.6^a	60.0 ± 6.7^a	63.3 ± 6.7^a	66.7 ± 3.3^a

Table 2. Susceptibility of *Macrobrachium rosenbergii* to *Lactococcus garvieae* at different $CuSO_4$ concentrations and at 28 ± 1°C, pH 7.3 to 7.8 and total hardness 100 mg l^{-1}. Data in the same column with different letters are significantly different ($p < 0.05$) among treatments. Values are means ± SE

Bacteria (cfu prawn^{-1})	Ambient $CuSO_4$(mg l^{-1})	No. prawns	Cumulative mortality (%) 8	16	24	48	72	96
Control	0.4	30	0	0	0	0	0	0
4×10^6	0	30	0^b	0^b	6.7 ± 3.3^b	20.3 ± 0.0^b	23.3 ± 3.3^b	26.7 ± 3.3^{bc}
4×10^6	0.1	30	0^b	0^b	3.3 ± 3.3^b	13.3 ± 6.7^b	16.7 ± 3.3^b	20.0 ± 0.0^c
4×10^6	0.2	30	3.3 ± 3.3^b	10.0 ± 0.0^b	20.0 ± 5.8^b	30.0 ± 5.8^{ab}	30.0 ± 5.8^{ab}	36.7 ± 3.3^b
4×10^6	0.3	30	3.3 ± 3.3^b	10.0 ± 5.8^b	16.7 ± 3.3^b	26.7 ± 3.3^b	26.7 ± 3.3^b	30.0 ± 0.0^{bc}
4×10^6	0.4	30	16.7 ± 6.7^a	33.3 ± 8.8^a	40.0 ± 10.0^a	46.7 ± 8.8^a	46.7 ± 8.8^a	50.0 ± 5.8^a
Control	0.4	30	0	0	0	0	0	0
2×10^5	0	30	0^a	0^a	0^b	0^c	3.3 ± 3.3^d	3.3 ± 3.3^d
2×10^5	0.1	30	0^a	0^a	0^b	0^c	0^d	0^d
2×10^5	0.2	30	0^a	0^a	0^b	0^c	10.0 ± 0.0^c	10.0 ± 0.0^c
2×10^5	0.3	30	0^a	3.3 ± 3.3^a	6.7 ± 3.3^b	16.7 ± 3.3^b	23.3 ± 3.3^b	23.3 ± 3.3^b
2×10^5	0.4	30	3.3 ± 3.3^a	10 ± 5.8^a	16.7 ± 3.3^a	36.7 ± 3.3^a	40.0 ± 0.0^a	40.0 ± 0.0^a

microplates previously coated with 100 µl of 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 of zymosan (0.1% in Hanks' 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 of Hanks' solution, then stained with 100 µl of NBT solution (0.3%) for 30 min at room temperature. The NBT solution was removed and the hemocytes were fixed and washed 3 times with 100 µl of 70% methanol and air-dried. Formazan was dissolved by the addition of 120 µl of KOH and 140 µl of dimethyl sulfoxide. The OD at 630 nm was measured in triplicate using an enzyme-linked immunosorbent assay reader (Dynex Mrx II, Chantilly, VA, USA). Respiratory burst was expressed as NBT reduction 100 µl^{-1} of hemolymph.

Statistical analysis. A multiple comparison (Tukey) test was conducted to compare the significant difference among treatments using the SAS computer software (SAS Institute Inc., Cary, NC, USA). For statistically significant differences, it was required that $p < 0.05$.

RESULTS

Effect of copper sulfate in the growth medium on the growth of *Lactococcus garvieae*

Lactococcus garvieae grew well in the TSB medium containing copper sulfate even at 0.4 mg l^{-1}. The log phase occurred at 12 to 24 h, and the bacterial density after 24 h of incubation was the highest in all test media.

Effect of copper sulfate on the virulence of *Lactococcus garvieae*

All the unchallenged control prawns survived. In contrast, in the challenged prawns deaths began to occur at 16 h. The cumulative mortality of prawns after 48 h was significantly higher for prawns challenged by the bacteria incubated in TSB medium containing 0.4 mg l^{-1} copper sulfate than for those challenged by bacteria at other concentrations (Table 1).

Effect of copper sulfate in the holding water on the resistance of *Macrobrachium rosenbergii* to *Lactococcus garvieae*

All the unchallenged control prawns survived. At a challenge dose of 4×10^6 cfu prawn^{-1}, onset of mortality occurred earlier at 0.2, 0.3 and 0.4 mg l^{-1} copper sulfate (8 h) than among prawns at 0.1 mg l^{-1} copper sulfate (24 h). After 24 h, cumulative mortality of prawns was significantly higher in 0.4 mg l^{-1} than at other concentrations. Cumulative mortality over 96 h was 26.7, 20.0, 36.7, 30.0 and 50.0% for the prawns placed in 0, 0.1, 0.2, 0.3 and 0.4 mg l^{-1} copper sulfate, respectively (Table 2). Reducing the challenge dose to 2×10^5 cfu prawn^{-1} delayed the onset of mortality (16 h for the 0.3 mg l^{-1} group and 72 h for the 0.2 mg l^{-1} group). After 72 h, cumulative mortality of prawns increased directly with ambient copper sulfate in the range from 0.2 to 0.4 mg l^{-1}. Cumulative mortality over the 96 h period was 10.0, 23.3 and 40.0% in 0.2, 0.3 and 0.4 mg l^{-1} copper sulfate, respectively. However, all prawns survived in 0.1 mg l^{-1} copper sulfate over 96 h (Table 2).

Table 3. Effect of different concentrations of copper sulfate on hyaline cells (HC), granular cells (GC), semi-granular cells (SGC) and total hemocyte count (THC). Data in the same category with the same letter are not significantly different ($p > 0.05$). Values are means ± SE

Hemocyte	Sampling time (h)	No. prawns	$CuSO_4$ (mg l^{-1}) Control	0.1	0.2	0.3	0.4
HC ($\times 10^5$ ml^{-1})	0	6	65.58 ± 9.17[a]	72.22 ± 11.80[a]	76.55 ± 27.16[a]	82.15 ± 16.70[a]	77.08 ± 15.44[a]
	96	6	107.28 ± 25.45[a]	75.77 ± 9.00[a]	85.72 ± 9.77[a]	78.58 ± 17.41[a]	76.95 ± 13.24a
GC ($\times 10^5$ ml^{-1})	0	6	3.75 ± 0.72[a]	3.95 ± 1.33[a]	4.59 ± 0.80[a]	4.03 ± 1.40[a]	3.88 ± 0.51[a]
	96	6	3.10 ± 0.63[a]	4.07 ± 1.28[a]	4.10 ± 1.23[a]	3.66 ± 1.31[a]	3.05 ± 1.08[a]
SGC ($\times 10^5$ ml^{-1})	0	6	4.31 ± 2.10[a]	3.54 ± 0.71[a]	4.18 ± 1.35[a]	3.70 ± 1.39[a]	4.02 ± 0.46[a]
	96	6	3.30 ± 0.60[a]	3.52 ± 1.33[a]	4.02 ± 1.28[a]	3.73 ± 0.42[a]	3.47 ± 1.12[a]
THC ($\times 10^5$ ml^{-1})	0	6	73.64 ± 6.81[a]	79.71 ± 12.68[a]	85.32 ± 29.39[a]	89.88 ± 17.78[a]	81.10 ± 15.47[a]
	96	6	113.68 ± 24.35[a]	83.35 ± 9.48[a]	93.83 ± 11.47[a]	89.33 ± 19.81[a]	83.47 ± 14.69[a]

Effect of copper sulfate on the immune parameters of *Macrobrachium rosenbergii*

No significant differences in THC and DHC were observed among the prawns at the beginning of the tests and at 48 and 96 h. The hyaline cells constituted 88 to 95% of the hemocytes, and varied from 65.6 ± 9.2 × 10^5 (mean ± SE) to 107.3 ± 25.5 × 10^5 cells ml^{-1}. The mean (± SE) of THC varied from 73.6 ± 6.8 × 10^5 to 113.7 ± 24.4 × 10^5 cell ml^{-1} for THC (Table 3).

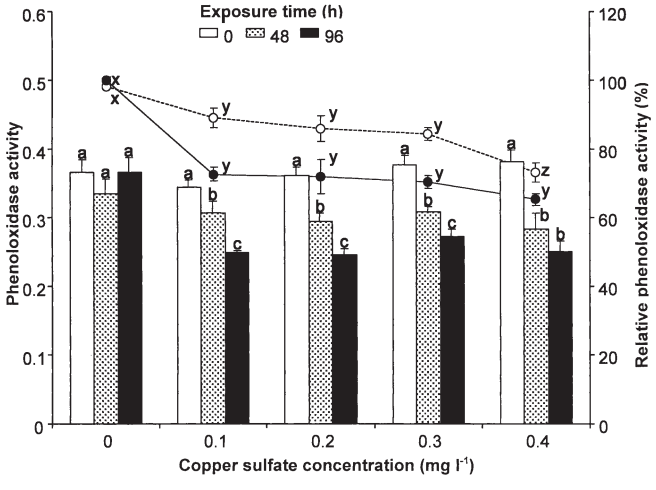

Fig. 1. Mean (±SE) phenoloxidase activity in the hemocytes of *Macrobrachium rosenbergii* at 0, 48 and 96 h exposure to copper sulfate, and relative phenoloxidase activity (compared with the activity at 0 h) of prawns exposed to different concentrations of copper sulfate. Each bar represents the mean value from 6 determinations with standard error. Data of phenoloxidase activity (in column) in the same copper sulfate concentration having different letters of a, b and c are significantly different ($p < 0.05$) among the prawns at 0, 48 and 96 h. Data of relative phenoloxidase activity (in dotted line and solid line) having different letters of x, y and z are significantly different ($p < 0.05$) among different copper sulfate concentrations after 48 and 96 h, respectively

No significant difference in phenoloxidase activity was observed among prawns placed in the control solution at 0, 48 and 96 h. Phenoloxidase activity decreased with exposure time at all copper concentrations. After 48 h, the relative phenoloxidase activity (compared with the activity at 0 h) at 0.1, 0.2, 0.3 and 0.4 mg l^{-1} copper sulfate was 89.1, 86.0, 84.4 and 73.2%, respectively. After 96 h, the relative phenoloxidase activity at 0.1, 0.2, 0.3 and 0.4 mg l^{-1} copper sulfate was 72.7, 71.8, 70.3 and 65.2%, respectively (Fig. 1).

No significant difference in respiratory burst was observed among the prawns placed in the control solution at 0, 48 and 96 h. Respiratory burst in prawns following 48 h exposure to copper sulfate was significantly higher than at 0 h. After 48 h, the relative respiratory burst (compared with the activity at 0 h) increased directly with copper sulfate in the range of 0.1 to 0.3 mg l^{-1}. The relative respiratory burst of prawns placed in 0.1, 0.2, 0.3 and 0.4 mg l^{-1} copper sulfate was 112.7, 156.1, 166.4 and 146.1%, respectively. After 96 h, the relative respiratory burst at 0.1, 0.2, 0.3 and 0.4 mg l^{-1} copper sulfate was 104.4, 112.6, 154.9 and 132.2%, respectively (Fig. 2).

DISCUSSION

Weinberg (1985) reported that media containing iron and manganese affected the growth of pathogen, and modulated the yields of bacterial enzymes and toxins. For example, in complex medium, the optimal iron concentration for maximal production of the neurotoxins of *Clostridium botulinum* was 5.4 µM (Siegel 1981). Cheng & Chen (1999) reported that *Lactococcus garvieae* incubated in brain heart infusion broths at pH 7 to 8 and temperature of 25 to 30°C resulted in significantly enhanced virulence to *Macrobrachium rosenbergii*. In our study, the fact that mortality was higher for *M. rosenbergii* challenged with *L. garvieae* previ-

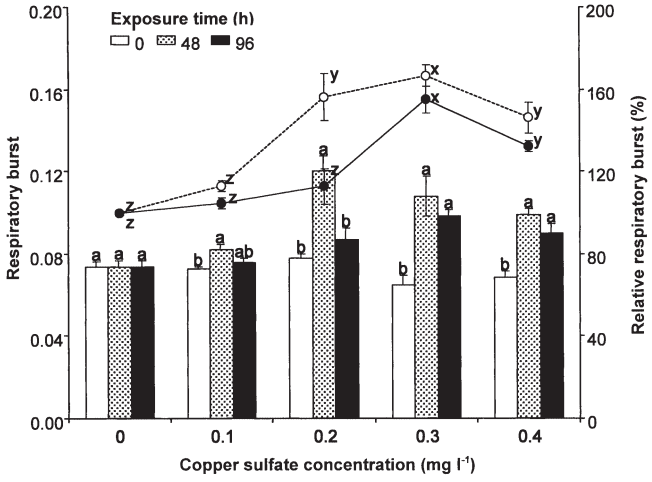

Fig. 2. Mean (±SE) respiratory burst in the hemocytes of *Macrobrachium rosenbergii* at 0, 48 and 96 h exposure to copper sulfate, and relative respiratory burst (compared with the activity at 0 h) of prawns exposed to different concentrations of copper sulfate. See Fig. 1 for statistical information

ously incubated in TSB medium with 0.4 mg l^{-1} copper sulfate indicates that copper sulfate enhanced the virulence of *L. garvieae* to *M. rosenbergii*.

Our previous research indicated that *Macrobrachium rosenbergii* were most susceptible to *Lactococcus garvieae* when they were reared at pH 8.8 to 9.5 and temperatures of 33 to 34°C (Cheng & Chen 1998b). In the present study, *M. rosenbergii* were most susceptible to *L. garvieae* when reared in water containing copper sulfate at 0.4 mg l^{-1}, as compared to those exposed to 0.2 and 0.3 mg l^{-1} copper sulfate. However, the fact that *M. rosenbergii* challenged with 2×10^5 cfu $prawn^{-1}$ and placed in 0.1 mg l^{-1} copper sulfate survived 96 h suggests an increase in resistance. In conclusion, the susceptibility of *M. rosenbergii* to *L. garvieae* is greatly affected by high pH level, high temperature and the presence of copper sulfate in water.

In decapod crustaceans, the number of hemocytes that are associated with cellular defense varies with species (Hose et al. 1990). Effects of intrinsic factors such as season, sex, size and body weight on the THC of *Macrobrachium rosenbergii* have been studied (Cheng & Chen 2001). *M. rosenbergii* displayed the highest and lowest THC in autumn and winter, respectively. There was no significant difference between male and female prawns or among animals with body weights ranging from 7 to 115 g. *M. rosenbergii* displayed the lowest THC at the D_3 stage and the highest at C stage (Cheng & Chen 2001). In the present study, the *M. rosenbergii* used were at the C stage and were therefore considered to be similar with regard to defense.

Extrinsic factors such as temperature, salinity and dissolved oxygen have been reported to affect THC in several species of decapod crustaceans (Truscott & White 1990, Le Moullac et al. 1998, Le Moullac & Haffner 2000, Cheng & Chen 2001). In the blue shrimp *Penaeus stylirostris* exposed to low temperature (18°C), a significant drop in THC (40%) was observed compared to that for prawns at 27°C (Le Moullac & Haffner 2000). An increase of temperature from 10 to 20°C and from 18 to 32°C has been reported to increase THC of *Carcinus maenas* and *Penaeus californensis*, respectively (Truscott & White 1990, Vargas-Albores et al. 1998). Brazilian shrimp *Penaeus paulensis*, reared at 34‰ had a significantly higher THC (20% more) than shrimp reared at 22 and 13‰ (Le Moullac & Haffner 2000). Decreased THC in *P. stylirostris* was observed following 24 h exposure to dissolved oxygen as low as 1 mg l^{-1} (Le Moullac et al. 1998). These studies indicated that the decrease of THC was due mainly to the decrease of hyaline cells.

In addition to physico-chemical parameters, environmental pollutants such as heavy metals have been reported to affect THC in several species of invertebrates. Cheng (1988) reported that Eastern oyster *Crassostrea virginica*, after 3 to 14 d of exposure to 1 mg l^{-1} Cd^{2+}, showed increased THC, and indicated that the increase was due to an increase in number of hyaline cells. The freshwater prawn *Macrobrachium idea* following 30 d exposure to 1 µg l^{-1} mercuric chloride exhibited hyperplastic gill lamella engorged with hemocytes (Victor et al. 1990). Truscott & White (1990) reported that no significant change in hemocyte count was observed in *Carcinus maenas* following 30 d exposure to 50 µg l^{-1} Hg^{2+}. *Penaeus stylirostris* following exposure to ammonia at 1.5 and 3.0 mg l^{-1} showed decreased THC by 15 and 50%, and decreased phenoloxidase activity by 60 and 50%, respectively (Le Moullac & Haffner 2000). In the present study, no significant difference in THC and DHC was observed among the prawns exposed to copper sulfate in the range of 0 to 0.4 mg l^{-1}.

Generation of various reactive oxygen intermediates from hemocytes responding to invasive pathogens or foreign particles has been observed in several species of mollusks (Adema et al. 1991, Pipe 1992, Anderson 1994). The hemocytes from *Crassostrea virginica* heavily infected with protozoan parasites produced significantly higher levels of reactive oxygen intermediates than hemocyte withdrawn from lightly infected oysters (Anderson 1994). NBT staining in *Penaeus stylirostris* following exposure to low concentrations of dissolved oxygen (1 mg l^{-1}) was decreased (Le Moullac et al. 1998) but the activity of NADPH oxidase, which produces superoxide anion from molecular oxygen, was not affected under hypoxia. In white shrimp *Penaeus*

vannamei, following injection of the fungicide propiconazol, the respiratory burst increased at Day 6 but decreased at Day 13 (Le Moullac & Haffner 2000). Elevated superoxide concentrations in *Drosophila* during melanotic encapsulation of parasites has been observed (Nappi et al. 1995). In an *in vitro* experiment using *Carcinus maenas*, superoxide anion production has been reported in hyaline cells (Bell & Smith 1993).

Pipe et al. (1999) indicated that the release of reactive oxygen intermediates from hemocytes increased directly with ambient copper but decreased significantly when the concentration was 0.5 mg l^{-1}. They suggested that a threshold of copper between 0.2 and 0.5 mg l^{-1} inhibited the production of reactive oxygen intermediates. In this study, the phenoloxidase activity of *Macrobrachium rosenbergii* decreased but the respiratory burst increased at 0.1 mg l^{-1} copper sulfate. A challenge test at a low dose also indicated that *M. rosenbergii* survived for 96 h at 0.1 mg l^{-1} copper sulfate. These findings may suggest that the NADPH oxidase is activated to produce superoxide anion under the stress of copper sulfate. The observation that in *M. rosenbergii* following 48 h exposure to 0.2 mg l^{-1} or more copper sulfate superoxide anion increased significantly but also phenoloxidase activity decreased significantly suggests that copper sulfate promoted cytoxicity and acted as an immunodepressor in *M. rosenbergii*.

It is known that copper induces immunomodulation in the marine mussel *Mytilus edulis* (Pipe & Coles 1995, Pipe et al. 1999) and increases the susceptibility to *Vibrio tubiashi* infection (Pipe & Coles 1995). Cheng & Chen (1998b) reported that the impact of *Lactococcus garvieae* on *Macrobrachium rosenbergii* was exacerbated by high pH (8.8 to 9.5) and high temperature (33 to 34°C) but was reduced by low salinity (5 to 10‰) indicating enhanced susceptibility of *M. rosenbergii* to *L. garvieae*. Copper sulfate increased respiratory burst but decreased phenoloxidase activity in *M. rosenbergii*, suggesting a possible reason for increased susceptibility to *L. garvieae* and indicating that copper sulfate might work as an immunomodulator in *M. rosenbergii*.

In conclusion, the immune system of *Macrobrachium rosenbergii* is modulated by copper sulfate. There is an increased virulence of *Lactococcus garvieae* to *M. rosenbergii* when it is first exposed to copper sulfate, and the susceptibility of *M. rosenbergii* to *L. garvieae* is enhanced by the stress of copper sulfate.

Acknowledgements. This research was supported by the Council of Agriculture (89-Ke-Chi-1.2-Yu-04-29) and National Science Council (NSC 90-2313-B-020-019) of the Republic of China. We thank Dr S. C. Sheu, Associate Professor of the Department of Food Science in our university, for the use of the ELISA reader, and Messrs C.-H. Liu and C.-H. Chang for assistance in the experiment.

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Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: August 16, 2000; Accepted: March 13, 2001
Proofs received from author(s): October 24, 2001