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Effect of benzalkonium chloride stress on immune resistance and susceptibility to *Lactococcus garvieae* in the giant freshwater prawn *Macrobrachium rosenbergii*

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ABSTRACT: Addition of benzalkonium chloride (BKC) at 0, 0.3, 0.6 and 1.0 mg l^{-1} to tryptic soy broth (TSB) had no effect on growth of *Lactococcus garvieae*, a bacterial pathogen of the giant freshwater prawn *Macrobrachium rosenbergii*. However, injection of the cultured cells into prawns at a dose of 4×10^6 colony-forming units (cfu) $prawn^{-1}$ resulted in significantly higher mortality at 120 h ($p < 0.05$) in prawns injected with cells grown in the absence of BKC than in prawns injected with cells grown in the presence of BKC. In other experiments, prawns were injected with TSB-grown *L. garvieae* (4×10^6 and 3×10^5 cfu $prawn^{-1}$) and then held in water containing BKC at 0, 0.3, 0.6 and 1.0 mg l^{-1}. After 120 h, mortality was significantly higher in all the BKC treatments than in the control without BKC. Prawns showed no significant differences in total hemocyte count (THC) or differential hemocyte count (DHC) amongst treatment and control groups. However, 96 h exposure to 0.3 mg l^{-1} BKC or more resulted in a decrease in phenoloxidase activity and an increase in respiratory burst to levels considered to be cytoxic. In summary, exposure of *L. garvieae* to BKC at 0.3 mg l^{-1} or more decreased its virulence to *M. rosenbergii*, while exposure of *M. rosenbergii* to BKC at 0.3 mg l^{-1} or more increased its susceptibility to *L. garvieae* infection.

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

INTRODUCTION

The giant freshwater prawn *Macrobrachium rosenbergii* is commercially important worldwide, and in inland Taiwan is a primary cultured species (New 1995). Disease outbreaks caused by yeast infections in the cool season and by *Lactococcus garvieae* (Chen et al. 2001) in the hot season have resulted in declining production of farmed prawns in Taiwan (Cheng & Chen 1998a).

Benzalkonium chloride (BKC) is widely used in aquaculture as a disinfectant and as a very effective herbicide. It has been reported to inhibit the growth of several species of microalgae such as the genera *Chlorella*, *Tetraselmis*, *Chaetoceros* and *Isochrysis* without significantly changing the concentrations of orthophosphate, ammonia and nitrite in culture pond water (Lee et al. 1995). The concentration of BKC added varies from 0.5 to 2.0 mg l^{-1} (Lee et al. 1994). However, prawn farmers often apply excess amounts, and the potential effect of this on the disease resistance or immune functions of the prawns is of some concern. The 24 h LC_{50} (median lethal concentration) of BKC for *Macrobrachium rosenbergii* postlarvae has been reported to be 2.0 mg l^{-1} (Liao & Guo 1990). However, little is known about the effect of BKC on later growth stages and on the prawn's resistance to desease.

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 phagocytosis (Bayne 1990), coagulation and in the pro-

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duction of melanin by the prophenoloxidase system (Johansson & Söderhäll 1989, Söderhäll et al. 1996). Prophenoloxidase is located in semi-granular and granular cells and is activated by a prophenoloxidase-activating enzyme, a serine protease that is in turn activated by microbial cell components such as β-1,3-glucan or lipopolysaccharide from fungal cell walls (Söderhäll 1983, Smith et al. 1984).

Several reactive oxygen species (ROS) are produced during phagocytosis by hyaline cells. When pathogens or foreign particles enter the host, they activate host NADPH-oxidase which, in turn, produces several reactive oxygen intermediates such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·) (Holmblad & Söderhäll 1999). These compounds can be directly toxic to pathogens (Roch 1999). The generation of superoxide anion (known as a respiratory burst) plays an important role in microbicidal activity, and has been reported for hemocytes of the shore crab *Carcinus maenas* (Bell & Smith 1993), the tiger shrimp *Penaeus monodon* (Song & Hsieh 1994), the blue shrimp *Penaeus stylirostris* (also known as *Litopenaeus stylirostris*) (Le Moullac et al. 1998), and *Macrobrachium rosenbergii* (Cheng & Wang 2001).

Several physico-chemical parameters and environmental contaminants have been reported to affect the crustacean immune response (reviewed by Le Moullac & Haffner 2000). Environmental toxicants have been reported to cause a reduction in hemocyte counts 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). Environmental toxicants have been reported to cause a reduction in phenoloxidase activity for *C. crangon*, *C. maenas* and *Macrobrachium rosenbergii* (Truscott & White 1990, Smith & Johnston 1992, Smith et al. 1995, Cheng & Wang 2001, Cheng & Chen 2002).

This study was aimed at determining the effect of BKC on (1) the growth of *Lactococcus garvieae* in tryptic soy broth (TSB), (2) the virulence of *L. garvieae* to *Macrobrachium rosenbergii*, (3) the resistance of *M. rosenbergii* to *L. garvieae*, and (4) the immune parameters of *M. rosenbergii*. For the latter purpose, hemocyte counts (total and differential), phenoloxidase activity and respiratory burst (production of superoxide anion) were used as indicators.

MATERIALS AND METHODS

Effect of BKC on growth of *Lactococcus garvieae*. *L. garvieae* was isolated from diseased *Macrobrachium rosenbergii* with opaque and whitish musculature (Cheng & Chen 1998a). The bacterium was cultured on tryptic soy agar (TSA, Difco) for 24 h at 30°C and transferred to 10 ml tryptic soy broth (TSB, Difco) for 24 h at 30°C for use as a stock bacterial broth. Inoculum for the growth tests consisted of 0.5 ml of this stock broth-culture.

A stock solution of BKC was prepared by dissolving 1.0 g of BKC (Sigma) in 1 l of distilled water (1000 mg l^{-1}). Bacteria were incubated at 30°C in 50 ml TSB with different concentrations of BKC (0 mg l^{-1} as control, and 0.3, 0.6 and 1.0 mg l^{-1} treatments) in 250 ml flasks. Each test was conducted in triplicate and bacterial growth was monitored after 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 BKC on virulence of *Lactococcus garvieae*. Bacteria from TSB media containing different concentrations of BKC were tested for virulence to *Macrobrachium rosenbergii*. After 24 h of cultivation, cells of *L. garvieae* were harvested by centrifugation at 7155 × *g* for 15 min at 4°C. The pellet was re-suspended in a saline solution (0.85% NaCl) at 2×10^8 colony-forming units (cfu) ml^{-1} as a stock bacterial suspension for injection.

Macrobrachium rosenbergii (8 to 12 g in the intermolt stage) were obtained from a commercial farm in Pingtung, Taiwan, and acclimated in the laboratory for 2 wk before experimentation, 20 µl of bacterial suspension was 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). 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, Pingtung, Taiwan) and observed for 16, 24, 48, 72, 96 and 120 h. During the experimental period, water temperature was maintained at 28 ± 1°C, pH 7.3 to 7.8, total hardness 100 mg l^{-1}, 10 prawns in each control group were injected with an equal volume of sterile saline solution (see Table 1).

Effect of BKC on susceptibility 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 1.5×10^7 and 2×10^8 cfu ml^{-1} as for the stock bacterial suspensions described above.

Macrobrachium rosenbergii were injected, held, fed and observed as described above. Challenge tests at 2 doses (3×10^5 and 4×10^6 cfu $prawn^{-1}$) were conducted in triplicate with 10 prawns per replicate. After injection, prawns were kept in 60 l glass aquaria (10 prawns each) containing 40 l of water with different concentrations of

BKC (0 mg l^{-1} as control, and 0.3, 0.6 and 1.0 mg l^{-1} treatments). These test solutions were renewed daily; the experiment lasted 8, 16, 24, 48, 72, 96 and 120 h. The LC_{50} of BKC for *M. rosenbergii* was determined using a computer program (Trevors & Lusty 1985). Prawns injected with an equal volume of sterile saline solution and kept in water with 1.0 mg l^{-1} BKC served as unchallenged controls (see Table 2).

Effect of BKC on immune parameters of *Macrobrachium rosenbergii*. In the intermolt stage, *M. rosenbergii* has the highest hemocyte count compared to other molt stages (Cheng & Chen 2001). The molt stage was determined based on the retraction of the epithelium within setae of the antennal scale (Peebles 1977). *M. rosenbergii* (20 to 30 g in the intermolt stage) were acclimated and fed as described above. Two prawns were kept in each 60 l glass aquarium containing 40 l of test solution at different concentrations of BKC (0 mg l^{-1} as control, and 0.3, 0.6 and 1.0 mg l^{-1} treatments). The test solutions were renewed daily; the experiment lasted for 96 h. Each test was conducted in triplicate.

Hemolymph (100 µl) was sampled individually at the beginning of each test and at 48 and 96 h. It was withdrawn from the ventral sinus of each prawn into a 1 ml sterile syringe (25 gage) 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 the anticoagulant-hemolymph mixture was placed on a hemocytometer to measure THC and DHC using an inverted-phase contrast microscope (Leica DMIL) while the remainder was used for subsequent tests.

The optical density of phenoloxidase activity was measured by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) as described by Hernãndez-López et al. (1996). Briefly, 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). An aliquot of 100 µl of the cell suspension was incubated with 50 µl of a prophenoloxidase activator, trypsin (1 mg ml^{-1}) for 10 min at 25 to 26°C; 50 µl of L-DOPA was added, followed by 800 µl of cacodylate buffer added 5 min later. The optical density at 490 nm was measured using an Hitachi U-2000 spectrophotometer. The control solution consisting 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 conditions. The background phenoloxidase activity OD values were in the range of 0.02 to 0.08. The phenoloxidase activity optical density values of prawns for all test conditions were expressed as dopachrome formation in 50 µl of hemolymph.

The respiratory burst activity of hemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion, as described in other studies (Bell & Smith 1993, Song & Hsieh 1994). Briefly, an aliquot of 100 µl hemolymph in anticoagulant solution was deposited 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. The zymosan was discarded and the hemocytes were washed 3 times with 100 µl of Hank's solution, and 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 2 M 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). The respiratory burst was expressed as NBT reduction by 10 µl of hemolymph.

Statistical analysis. A multiple-comparison (Duncan) test was conducted to compare the differences among treatments using the SAS computer software (SAS Institute). Percent data (virulence study and susceptibility study) were normalized using arcsine transformation before analysis; $p < 0.05$ was regarded as indicating statistically significant differences.

RESULTS

Effect of BKC on growth of *Lactococcus garvieae*

Lactococcus garvieae grew well in TSB medium containing BKC at 0, 0.3, 0.6 and 1.0 mg l^{-1}. The optical density after 24 h of incubation was the same for all media (i.e. 1.45, 1.42, 1.45 and 1.52, respectively).

Effect of BKC on virulence of *Lactococcus garvieae*

All unchallenged control prawns survived. In contrast, mortality of the challenged prawns began at 16 h, and cumulative mortality after 120 h was significantly higher for prawns challenged with bacteria incubated in TSB medium without BKC (Table 1).

Effect of BKC on susceptibility of *Macrobrachium rosenbergii* to *Lactococcus garvieae*

All unchallenged control prawns survived. At challenge doses of 4×10^6 cfu prawn^{-1}, onset of mortality occurred earlier (8 h) in 0.3 and 0.6 mg l^{-1} BKC than in 0 and 1.0 mg l^{-1} BKC. After 120 h, the cumulative mortality of prawns was significantly higher in 0.3 (67 %), 0.6 (70 %) and 1.0 (77 %) mg l^{-1} BKC than in the control solution (40 %) (Table 2). The 24 h LC_{50} of BKC in *Macrobrachium rosenebergii* was calculated to be 0.44 mg l^{-1}. Reducing the challenge dose to 3×10^5 cfu prawn^{-1} delayed the onset of mortality (to 16 h for the 0.6 mg l^{-1} group, 24 h for the 0.3 mg l^{-1} group, and 48 h for the 1.0 mg l^{-1} group). After 120 h, cumulative mortality was significantly higher in 0.3, 0.6 and 1.0 mg l^{-1} BKC than in the control solution (Table 2).

Effect of BKC on immune parameters of *Macrobrachium rosenbergii*

No significant differences in THC, HC, SGC and GC were observed among the prawn treatment groups at the beginning of the tests and at 48 and 96 h. The mean (±SE) THC varied from $111 \pm 44 \times 10^5$ to $233 \pm 27 \times 10^5$ cells ml^{-1}. The hyaline cells varied from 103 ± 3 (mean ± SE) to 219 ± 72 $\times 10^5$ cell ml^{-1} (Table 3).

No significant differences in phenoloxidase activity were observed for prawns in the control solution at 0, 48 and 96 h. However, activity decreased with increasing exposure time in all BKC treatment groups. Phenoloxidase activity at 48 h ranged from 78 to 82 % of that at 0 h, while activity at 96 h ranged from 67 to 76 % (Fig. 1).

No significant differences in respiratory burst were observed for prawns in the control solution at 0, 48 and 96 h. However, it increased with increasing exposure time in all the BKC-treatment groups. Compared with the activity at 0 h, the relative respiratory burst in 0.3, 0.6 and 1.0 mg l^{-1} BKC at 48 h was 113, 122 and 113 %, respectively, while at 96 h it was 134, 149 and 135 %, respectively (Fig. 2).

DISCUSSION

As stated in the 'Introduction', the concentration of BKC added to prawn culture ponds varies from 0.5 to 2.0 mg l^{-1} (Lee et al. 1994). Our results suggest that concentrations of 1.0 mg l^{-1} would not affect the growth of *Lactococcus garvieae* in aquaculture ponds,

Table 1. Susceptibility (cumulative mortality, %) of *Macrobrachium rosenbergii* to *Lactococcus garvieae* grown in tryptic soy broth with different additions of benzalkonium chloride (BKC) at 28 ± 1°C and pH 7.3 to 7.8. Data in the same column with different letters are significantly different ($p < 0.05$) among treatments. Values are means ±SE (n = 30 prawns in each case). cfu = colony-forming units

Dose (cfu prawn^{-1})	BKC (mg l^{-1})	Time after challenge (h) 16	24	48	72	96	120
Control	Saline	0	0	0	0	0	0
4×10^6	0	20 ± 6^a	33 ± 3^a	33 ± 0^a	37 ± 3^a	43 ± 3^a	47 ± 3^a
4×10^6	0.3	17 ± 3^{ab}	30 ± 0^a	33 ± 3^a	33 ± 3^a	37 ± 3^{ab}	37 ± 3^{ab}
4×10^6	0.6	17 ± 9^{ab}	27 ± 7^{ab}	27 ± 7^{ab}	27 ± 7^{ab}	30 ± 6^b	30 ± 6^b
4×10^6	1.0	10 ± 6^b	20 ± 0^b	23 ± 3^b	27 ± 3^b	30 ± 6^b	30 ± 6^b

Table 2. Susceptibility (cumulative mortality, %) of *Macrobrachium rosenbergii* to *Lactococcus garvieae* for prawns held in different concentrations of BKC at 28 ± 1ºC and pH 7.3 to 7.8. Data in the same column with different letters are significantly different ($p < 0.05$) among treatments. Values are means ± SE (n = 30 prawns in each case)

Dose (cfu prawn^{-1})	BKC (mg l^{-1})	Time after challenge (h) 8	16	24	48	72	96	120
Control	1.0	0	0	0	0	0	0	0
4×10^6	0	0 ± 0^b	13 ± 7^b	27 ± 3^b	33 ± 7^b	40 ± 6^b	40 ± 6^b	40 ± 6^b
4×10^6	0.3	27 ± 9^a	40 ± 6^a	47 ± 9^a	67 ± 3^a	67 ± 3^a	67 ± 3^a	67 ± 3^a
4×10^6	0.6	20 ± 0^a	37 ± 3^a	53 ± 3^a	70 ± 6^a	70 ± 6^a	70 ± 6^a	70 ± 6^a
4×10^6	1.0	0 ± 0^b	23 ± 3^a	57 ± 7^a	77 ± 7^a	77 ± 7^a	77 ± 7^a	77 ± 7^a
Control	1.0	0	0	0	0	0	0	0
3×10^5	0	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^b	0 ± 0^b	0 ± 0^b	0 ± 0^b
3×10^5	0.3	0 ± 0^a	0 ± 0^a	3 ± 3^a	3 ± 3^{ab}	3 ± 3^{ab}	7 ± 3^a	10 ± 0^a
3×10^5	0.6	0 ± 0^a	3 ± 3^a	3 ± 3^a	7 ± 3^a	7 ± 3^a	10 ± 0^a	10 ± 0^a
3×10^5	1.0	0 ± 0^a	0 ± 0^a	0 ± 0^a	3 ± 3^{ab}	3 ± 3^{ab}	10 ± 6^a	10 ± 6^a

Table 3. *Macrobrachium rosenbergii.* Effects of different concentrations of BKC on THC (total hemocyte count), HC (hyaline cell count), SGC (semi-granular cell count) and GC (granular cell count). Data in the same category with different letters are significantly different ($p < 0.05$). Values are means ± SE (n = 6 prawns in each case)

Sampling time (h)	Control	BKC (mg l^{-1}) 0.3	0.6	1.0
THC ($\times 10^5$ ml^{-1})				
0	233 ± 27[a]	194 ± 36[a]	111 ± 44[a]	190 ± 47[a]
48	210 ± 07[a]	174 ± 29[a]	112 ± 07[a]	173 ± 24[a]
96	227 ± 16[a]	165 ± 26[a]	121 ± 40[a]	163 ± 14[a]
HC ($\times 10^5$ ml^{-1})				
0	219 ± 72[a]	167 ± 35[a]	100 ± 19[a]	177 ± 20[a]
48	197 ± 08[a]	165 ± 29[a]	103 ± 03[a]	162 ± 10[a]
96	214 ± 48[a]	155 ± 26[a]	111 ± 40[a]	152 ± 12[a]
GC ($\times 10^5$ ml^{-1})				
0	8 ± 04[a]	7 ± 03[a]	6 ± 00[a]	7 ± 05[a]
48	8 ± 02[a]	5 ± 03[a]	4 ± 01[a]	6 ± 01[a]
96	7 ± 02[a]	5 ± 01[a]	5 ± 01[a]	5 ± 05[a]
SGC ($\times 10^5$ ml^{-1})				
0	7 ± 03[a]	6 ± 02[a]	6 ± 02[a]	6 ± 04[a]
48	6 ± 03[a]	4 ± 03[a]	4 ± 01[a]	5 ± 01[a]
96	6 ± 05[a]	5 ± 01[a]	5 ± 02[a]	6 ± 05[a]

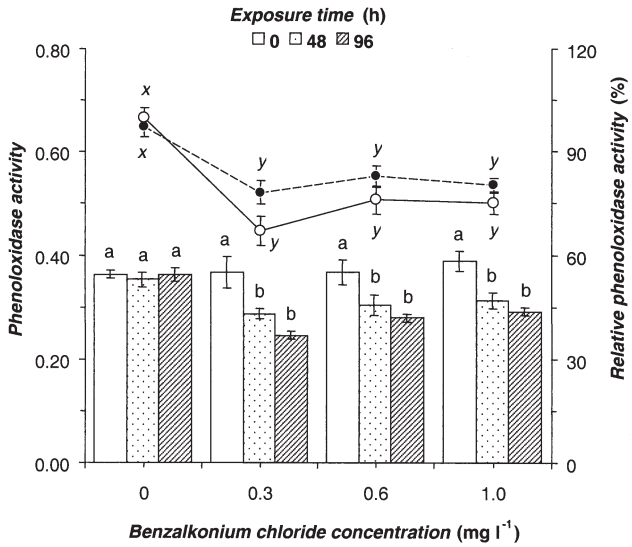

Fig. 1. *Macrobrachium rosenbergii.* Mean (±SE) phenoloxidase activity and relative phenoloxidase activity in hemocytes at 0, 48 and 96 h exposure to BKC. Each bar represents the mean value (± SE) from 6 determinations. Values for phenoloxidase activity at the same BKC concentration with different letters (a, b) were significantly different ($p < 0.05$) among the prawns at 0, 48 and 96 h. Data for relative phenoloxidase activity (●, ○) with different letters (x, y) are significantly different ($p < 0.05$) for different BKC concentrations at 48 (●) and 96 h (○)

although this might depend on other pond-water parameters. On the other hand, concentrations as low as 0.3 mg l^{-1} do affect its virulence to *Macrobrachium rosenbergii*, suggesting that additon of BKC to pond water might prevent *L. garvieae* infections. It is well known that media or environmental parameters can affect the growth of pathogens and their production of bacterial enzymes and toxins (Weinberg 1985, Arp 1988). For example, Ramesh et al. (1989) indicated that environmental parameters can influence the growth of luminous bacteria, and we have previously shown that temperature, pH, ammonia, nitrite and copper sulfate can influence the growth and virulence of *L. gariviae* (Cheng & Chen 1999, 2002, Cheng & Wang 2001, Cheng et al. 2002). In summary, both ammonia and BKC decrease the virulence of *L. garvieae* to *M. rosenbergii* while copper sulfate (0.4 mg l^{-1}) increases the virulence (Cheng & Wang 2001).

In addition, the present study has shown that BKC in rearing water at levels above 0.3 mg l^{-1} increases the susceptibility of *Macrobrachium rosenbergii* to *Lactococcus garvieae* infection. We have also shown in previous studies that susceptibility to *L garvieae* infection is also increased by high pH levels, high temperatures and the presence of ammonia, nitrite and copper sulfate in the rearing water (Cheng & Chen 1998b, 2002, Cheng & Wang 2001, Cheng et al. 2002).

Circulating hemocytes of *Macrobrachium rosenbergii* differ with size, season, body weight and molting cycle (Cheng & Chen 2001). For example, THC are highest and lowest in autumn and winter, respectively. In addition, the lowest THC are obtained at the D_3 molt

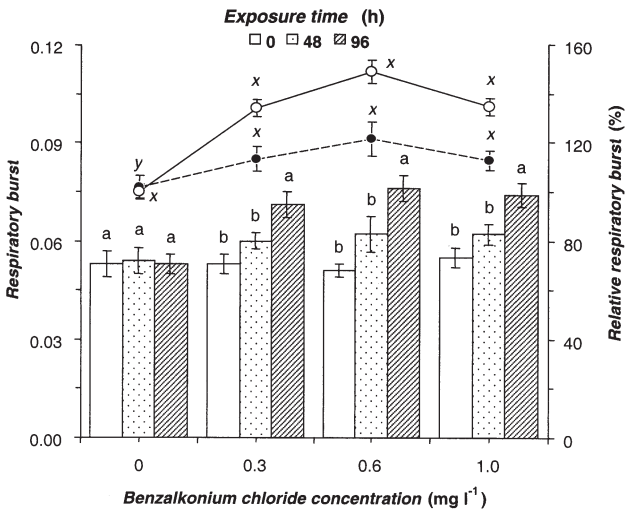

Fig. 2. *Macrobrachium rosenbergii.* Mean (±SE) respiratory burst and relative respiratory burst in hemocytes at 0, 48 and 96 h exposure to different concentrations of BKC. See Fig. 1 for statistical data

stage and the highest at the C molt stage (Cheng & Chen 2001). In this study, we used C-stage *M. rosenbergii* throughout to ensure a similar defense capability. Although circulating hemocytes in crustaceans including *M. rosenbergii* can be affected by extrinsic factors such as temperature, pH, salinity, dissolved oxygen and ammonia (Truscott & White 1990, Le Moullac et al. 1998, Cheng & Chen 2000, 2001, Le Moullac & Haffner 2000), we found no significant difference in THC and DHC following 96 h exposure to BKC concentrations in the range of 0 to 1.0 mg l^{-1}. This was similar to results of our previous work, in which no significant differences in hemocyte counts were observed upon exposure of *M. rosenbergii* to ammonia, nitrite and copper (Cheng & Wang 2001, Cheng & Chen 2002, Cheng et al. 2002).

We found that exposure to BKC significantly reduced phenoloxidase activity in *Macrobrachium reosenbergii*. This was similar to the effect we previously found with ammonia and copper sulfate (Cheng & Wang 2001, Cheng & Chen 2002). It is known that environmental factors such as hypoxia (Le Moullac et al. 1998), temperature or pH (Cheng & Chen 2000, Le Moullac & Haffner 2000) and chemicals such as PCB 15 (Smith & Johnston 1992) can decrease or increase phenoloxidase activity. LeMoullac & Haffner (2000) suggested that the defense functions related to phenoloxidase activity and peroxinectin were reduced at the level of gene expression when *Penaeus stylirostris* was exposed to ammonia. A similar reaction may occur with BKC exposure in *M. rosenbergii.* We conclude that the observed increased susceptibility of *M. rosenbergii* to *Lactococcus garvieae* resulted from a reduction in resistance related to decreased phenoloxidase activity.

Production of superoxide anion increased in *Macrobrachium rosenbergii* following exposure to BKC, similar to the effect of exposure to ammonia, nitrite and copper sulfate (Cheng & Wang 2001, Cheng & Chen 2002, Cheng et al. 2002). In summary, exposure to ammonia-N at 0.55 mg l^{-1}, nitrite-N at 1.15 mg l^{-1}, copper sulfate at 0.3 and 0.4 mg l^{-1}, and BKC at 0.3 mg l^{-1} or greater in *M. rosenbergii* results in increased immunity or cytotoxicity (Muñoz et al. 2000).

Song & Hsieh (1994) reported that β-glucan had the strongest stimulatory effect on hemocytes of *Penaeus monodon* for generation of O_2^- (superoxide anion) and H_2O_2 (hydrogen peroxide). They also reported that O_2^- and H_2O_2 were more important in shrimp microbicidal activity than OCl^- (hypochlorites) and MPO (myeloperoxidase). Le Moullac & Haffner (2000) reported that injection of the fungicide propiconazole into the white shrimp *P. vannamei* (also known as *Litopenaeus vannemei*) increased the respiratory burst on Day 6, but caused a dose-dependent decrease on Day 13. Le Moullac et al. (1998) suggested that the superoxide anion decrease in hypoxic *P. stylirostris* was due to a decrease in THC, and that NADPH oxidase responsible for production of superoxide anion was not affected by hypoxia.

A small increase in the superoxide anion is considered beneficial in increasing resistance. However, too large an increase is considered to be cytotoxic (Cheng & Wang 2001). We consider the increase of the superoxide anion in *Macrobrachium rosenbergii* exposed to 0.3 mg l^{-1} BKC or greater to be cytotoxic. The increase in the superoxide anion resulted either from increased activity of NADPH oxidase (responsible for its production) or from a decrease in superoxide dismutase (SOD, responsible for scavenging superoxide anions). Further research, examining the activities of superoxide dimutase, catalase and peroxidase (Holmblad & Söderhäll 1999), is needed to understand the production of reactive oxygen intermediates under BKC stress.

In conclusion, our work suggests caution in the use of BKC for treatment of *Lactococcus garvieae* infections in growout ponds of *Macrobrachium rosenbergii*. The currently used levels of 0.5 to 2.0 mg l^{-1} (Lee et al. 1994) would be detrimental since concentrations as low as 0.3 mg l^{-1} can reduce the prawn's immune capacity and increase mortality. On the other hand, this detrimental effect is somewhat compensated by the decreased virulence of *L. garvieae* grown at the same concentration. Therefore, the recommended concentration of BKC for treatment of infection in prawn farms is less than 0.3 mg l^{-1}.

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