J. Ocean Univ. China (Oceanic and Coastal Sea Research)
https://doi.org/10.1007/s11802-023-5336-8
ISSN 1672-5182, 2023 22 (3): 821-830
http://www.ouc.edu.cn/xbywb/
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Immune Response of the Ridgetail White Prawn *Exopalaemon carinicauda* After Exposure to the Dinoflagellate *Prorocentrum minimum*

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(Received January 20, 2022; revised March 10, 2022; accepted June 7, 2022)

Abstract The dinoflagellate *Prorocentrum minimum* is known to affect the normal physiological function of *Exopalaemon carinicauda* by inducing oxidative stress, apoptosis, and cellular injury. To study the effects of *P. minimum* on the immune defense system of shrimp, *E. carinicauda* were exposed to 5×10^3 cells mL^{-1} and 5×10^4 cells mL^{-1} of *P. minimum* for 336 h in treatment groups, while *E. carinicauda* cultured in filtered seawater was employed as control. The total hemocyte counts (THC), hemocyanin concentration (HEM), and the activity of alkaline phosphatase (AKP) in hemolymph serum, as well as expressions of six immunity-related genes in hemocytes, hepatopancreases and gills were determined. The exposure of *P. minimum* significantly reduced the THC, HEM concentration and AKP activity in hemolymph serum. Immunity-related genes expressed differently in hemocytes, hepatopancreases and gills. Compared with the control group, the expressions of *Crustin* and *proPO* in hemocytes were significantly up-regulated in the treatment groups, while the up-regulated expressions of *LGBP*, *Lysozyme* and *Serpin* were only found in the group exposed to 5×10^4 cells mL^{-1} of *P. minimum*. In the gills of *E. carinicauda* exposed to *P. minimum*, the down-regulation of *ALF*, *proPO* and *Serpin*, up-regulation of *LGBP* and *Lysozyme*, as well as unaffected *Crustin* were observed. In hepatopancreases, the up-regulated expressions of *LGBP*, *Crustin*, *Lysozyme*, *Serpin* and *proPO* (only in 5×10^3 cells mL^{-1} of *P. minimum* group) were found in the treatment groups. After exposure to *P. minimum* for 336 h, shrimps were injected with *Vibrio parahaemolyticus* and WSSV. The results showed that the mortality rates of shrimp in the treatment groups were significantly increased with a dose-dependent effect, which suggests that exposure to *P. minimum* may reduce the immunity of *E. carinicauda*. The research indicates that hemocytes and hepatopancreases play important roles in protecting the shrimp immune response to harmful algae, while the protection effect of hemolymph serum and gills may be suppressed. Since the exposure to *P. minimum* depressed the immunity of *E. carinicauda*, further studies are needed to confirm whether the presence of the algae will affect the susceptibility of shrimp to pathogens.

Key words *Prorocentrum minimum*; *Exopalaemon carinicauda*; immune response; immunity-related genes

1 Introduction

Microalgae are an important part of the ecological system in shrimp ponds and can provide a direct and indirect food to the shrimp at different developmental stages (Alonso-Rodrıguez and Páez-Osuna, 2003; Paezosuna, 2003; Lukwambe *et al.*, 2019). As an essential part of food chain, most species of microalgae are beneficial for shrimp culture, while some harmful algae can affect the physiological functions of the shrimp, induce disease, death, or delay the growth of shrimp, and can result in a serious economic loss in culture operations (Alonso-Rodrıguez and Páez-Osuna, 2003; Ge *et al.*, 2017; Pérez-Morales *et al.*, 2017; Yang *et al.*, 2018; Holland and Leonard, 2020; Yeganeh *et al.*, 2020). The harmful algae usually affect organisms by poisoning (Chen and Xie, 2005; Kotaki *et al.*, 2008; Galanti *et al.*, 2013; Yang *et al.*, 2018; Yeganeh *et al.*, 2020), causing anoxia (Zhu and Xu, 1993), or producing mucus (Alonso-Rodrıguez and Páez-Osuna, 2003).

The dinoflagellate *Prorocentrum minimum*, one of the most widespread harmful algae species (Heil *et al.*, 2005; Li *et al.*, 2015; Ajani *et al.*, 2018), often cause damages to marine aquaculture (Alonso-Rodrıguez and Páez-Osuna, 2003; Azanza *et al.*, 2005; Mu *et al.*, 2019). It has shown that high concentrations of *P. minimum* ($>10^3$ cells mL^{-1}) could cause mortality (Landsberg, 2002; Alonso-Rodrıguez and Páez-Osuna, 2003; Sierra-Beltrán *et al.*, 2005), but the toxicity mechanism is still not clear (Landsberg, 2002;

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Vlamis *et al.*, 2015). The dinoflagellate *P. minimum* often occur in shrimp ponds (Sierra-Beltrán *et al.*, 2005; Xu *et al.*, 2010), and high concentrations of this alga induce stress to shrimp, affect their survival, growth, and increase their susceptibility to viral diseases (Alonso-Rodrıguez and Páez-Osuna, 2003). Previous studies have shown that *P. minimum* (5×10^4 cells mL^{-1}) induced sublethal effects on the ridgetail white prawn *Exopalaemon carinicauda*, and caused oxidative stress, apoptosis, and cellular injury (Mu *et al.*, 2017, 2019). Since the normal physiological function of shrimp can be affected by *P. minimum*, the immune system is also likely to be involved.

The innate immune system is the first line of defense against infectious agents. Therefore it is important to investigate the harmful effects of *P. minimum* on the immune defense system of shrimp. Like other invertebrates, crustaceans lack adaptive immune systems and depend largely upon their innate immune mechanisms (Vazquez *et al.*, 2009; Huang and Ren, 2021; Kulkarni *et al.*, 2021). The innate immune system of crustaceans can be roughly divided into cellular immunity and humoral immunity. Hemocytes, as the main functional cells in the circulation system, directly involved in the cellular immunity and play a major role in humoral immunity (Gao *et al.*, 2017). Humoral immunity can recognize foreign material and activate cellular or humoral effector mechanisms to destroy invading pathogens (Vazquez *et al.*, 2009). In the present study, pattern recognition receptor *LGBP*, three antimicrobial peptides (AMPs) encoding genes including *ALF*, *Crustin*, and *Lysozyme*, prophenoloxidase *proPO* and *Serpin* were chosen as target immunity-related genes.

To study the harmful effects of *P. minimum* on the immune defense system of shrimp, *E. carinicauda* were exposed to different concentrations of *P. minimum* for 336 h, the total hemocyte counts (THC), hemocyanin (HEM) concentration, and activity of alkaline phosphatase (AKP) in hemolymph serum, as well as six important immunity-related genes in different tissues were investigated. To reflect the effect of *P. minimum* on the immunity of *E. carinicauda*, after the exposure to *P. minimum* for 336 h, shrimp were injected with *Vibrio parahaemolyticus* and WSSV, and the mortality rates were measured. *V. parahaemolyticus* and WSSV are common pathogens in shrimp and are often associated with mass mortality and economic losses (Flegel *et al.*, 2008; Lai *et al.*, 2015).

2 Materials and Methods

2.1 Animals

Healthy ridge tail white shrimp *E. carinicauda* (1.62 g± 0.16 g) were bought from a commercial farm in Ganyu, Jiangsu, China. The shrimps were raised in aerated seawater (salinity, 30; pH, 8.2; temperature 25℃±1.0℃) in 200 L plastic containers with continuous aeration, and were fed with commercial prawn pellets three times a day.

2.2 Microalga

The dinoflagellate *P. minimum* were isolated from a bloom at a farm in Laoshan, Qingdao, China (Mu *et al.*, 2017). They were cultured in sterilized seawater with f/2 medium under a 14 h:10 h (light:dark) cycle at 22–25℃. The seawater was filtered through a 0.45-μm membrane before use. Algae were harvested in the late exponential growth phase and fixed with Lugol's solution. Cell numbers were counted under microscope using a plankton counting chamber with a 100-μm orifice before the experiment.

2.3 Animals Treatments and Sample Collection

2.3.1 Bioassay 1 (exposure treatment)

Totally 630 shrimps were randomly distributed into nine plastic containers in three groups and each group was run in triplicate. Shrimps were exposed to 5×10^3 cells mL^{-1} and 5×10^4 cells mL^{-1} of *P. minimum* for 336 h in treatment groups and the culture media were changed every two days to ensure the algal concentrations. Shrimps cultured in filtered seawater were used as control.

After 6, 12, 24, 48, 96, 168 and 336 h, eight shrimps were randomly collected from each group. The hemolymph was harvested from the heart of shrimp using a 1-mL sterile syringe containing an equal volume of cold anti-coagulant buffer (0.34 mol L^{-1} sodium chloride, 10 mmol L^{-1} $EDTANa_2$, and 30 mmol L^{-1} trisodium citrate, adjusted to 780 mOsm kg^{-1} using 0.115 mol L^{-1} glucose at a pH 7.55 with osmolality) (Liu and Chen, 2004), then placed in a 1.5-mL sterile centrifugal tube to run at 3000 *g* for 10 min at 4℃. The supernatant was transferred to a new tube and stored at −80℃ for later determination of HEM concentration and AKP activity. Hemocytes were stored at −80℃ for RNA extraction. After being excised, gills and hepatopancreases were immediately put in liquid nitrogen and stored at −80℃ for RNA extraction.

2.3.2 Bioassay 2 (infection studies)

Totally 360 shrimps were randomly distributed into 18 plastic containers and divided into six groups for two injection experiments, while each group was run in triplicate. Shrimps were treated as Bioassay 1 for 336 h, and then transferred to normal seawater for infection experiments. In this study, *V. parahaemolyticus* was separated from the AHPND-infected *Litopenaeus vannamei*, and the WSSV crude extract was obtained from gills of WSSV-infected *L. vannamei*. The AHPND-infected *L. vannamei* and WSSV-infected *L. vannamei* were obtained from the Mariculture Disease Control and Pathogenic Molecular Biology Laboratory, Yellow Sea Fisheries Research Institute. The *V. parahaemolyticus* was incubated in tryptic soy broth (TSB) supplemented with 2% NaCl at 28℃ for 16 h. The concentration of bacteria was determined by plate count with TCBS agar. The WSSV crude extract was prepared following the method of Ge *et al.* (2015). In brief, 0.1 g gills were homogenized quickly on ice with 0.9 mL phosphate-buffered saline (PBS), and centrifuged (1200 r min^{-1}, 20 min) at 4℃, filtered through 0.45 μm membrane, and the supernatant was stored at −80℃.

For the *V. parahaemolyticus* experiment, each shrimp was injected with 20-μL of PBS-diluted *V. parahaemolyticus* so-

lution (10^5 CFU g^{-1}). For the WSSV experiment, each shrimp was injected with 20-μL WSSV solution, which was a 1000-fold dilution of WSSV crude extract by PBS. Shrimps in control groups were injected with 20-μL of PBS. The number of dead shrimp at each sampling time was counted, and the cumulative mortality was obtained according to the formula of Ge *et al*. (2015):

$$S(\%) = (D_1 + D_2 + \cdots D_t) / N_t \times 100 ,$$

where S is the cumulative mortality; D is the number of death; t is the sampling time; N is the total number of shrimp.

2.4 Determination of Various Immune Parameters

2.4.1 Total hemocyte counts (THC)

The THC values were observed directly under a light microscope using a hemocytometer, and 200 μL anticoagulant hemolymph was used to count the number of hemocytes. The THC was expressed as cells mL^{-1}.

2.4.2 Hemocyanin (HEM) concentration in the hemolymph

To assess the HEM concentration, 900 μL of double distilled water was added to 100 μL of serum sample, mixed well, and the absorbance measured at 335 nm by Multiskan spectrum (Thermo, USA). An extinction coefficient of 17.26 was used to calculate the HEM concentration.

2.4.3 Activity of alkaline phosphatase (AKP) in the hemolymph

The activity of AKP was measured using kits from Jiancheng Bioengineering Institute (Nanjing, China).

2.4.4 Expression of immunity-related genes

Samples were thawed on ice, total RNA was extracted from hemocytes, gills, and hepatopancreases using TRIzol reagent (Takara, Dalian, China). The RNase-free DNase (Takara, Dalian, China) was used to purify the remaining genomic DNA. The quality and concentration of nucleic acids were checked by spectrophotometry (A260/A280) and electrophoresis on 1.5% agarose gel. The cDNA template for the real-time quantitative PCR (qPCR) analysis was prepared using a PrimeScript™ Real-time PCR Kit (Takara, Dalian, China), and stored at −20℃.

Primers for real-time qPCR are shown in Table 1. The qPCR was performed with SYBR® Premix Ex Taq™ II (TaKaRa, Japan) on the ABI 7500 System (Applied Biosystems, USA). The total volume was 20 μL, including 10 μL of SYBR® Premix Ex Taq™ II, 2 μL of the diluted cDNA, 0.8 μL of each primer, 0.4 μL of ROX Reference Dye II and 6 μL of deionized water. The PCR program was performed as follows: one cycle of 95℃ for 30 s, 40 cycles of 95℃ for 5 s and 60℃ for 34 s. The expression levels of the target genes were analyzed by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1 Primers used for qPCR

Gene	Primer	Sequence (5'−3')
18S[a]	18S-F	TATACGCTAGTGGAGCTGGAA
	18S-R	GGGGAGGTAGTGACGAAAAAT
LGBP[b]	EcLGBP-F	GGTCCAACACTTCACCCACT
	EcLGBP-R	CGGCTTTCTCGCTGATACTG
ALF[b]	EcALF-F	GGTTCATCCTGCTGTCCTG
	EcALF-R	GAGCCCCATAATCCAACAAT
Crustin[b]	EcCrustin-F	CCCAACGAGATCGAAGTGAT
	EcCrustin-R	GCGTGAGTGGAGTGTAGCAA
Lysozyme[b]	Eclys-F	TGAACTTGCTACTGTGCTGGA
	Eclys-R	GTTGATGGCTTCCGTGTTG
proPO[b]	EcProPO-F	AAACGATTCGACTACCATCTCCA
	EcProPO-R	GTTCATTCGGTTTCCCCTCTC
Serpin[b]	EcSerpin-F	AGGCGCGAATCTACTCTCCA
	EcSerpin-R	CCACTCATCACCAAAGACATCAA

Notes: [a] Internalcontrol gene; [b] Target gene.

2.5 Statistical Analysis

All data are presented as means ± standard error (SE). A one-way analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to analyze the statistical difference between treatments, using SPSS 19.0 for windows (SPSS Inc, Chicago, IL, USA). Significant differences were set at $P<0.05$.

3 Results

3.1 Effects of *P. minimum* on THC

Effects of *P. minimum* on THC are shown in Fig.1. Compared with the control group, THC of *E. carinicauda* in 5×10^4 cells mL^{-1} of *P. minimum* group decreased significantly at 12−168 h ($P<0.05$, Fig.1), and reached the lowest value at 96 h (about a quarter of control), then increased and recovered to the control level at 336 h. THC values of *E. carinicauda* treated with 5×10^3 cells mL^{-1} of *P. minimum* were only decreased at 12−48 h.

3.2 Effects of *P. minimum* on HEM Concentration

The exposure of shrimp to *P. minimum* significantly affected the HEM concentrations in the hemolymph ($P<0.05$, Fig.2). The HEM concentrations in *P. minimum* groups decreased at 12 h, and had the lowest value at 96 h (0.55-fold and 0.39-fold of control in low and high concentrations of *P. minimum*, respectively), and then increased and recovered to the control level after 336 h.

3.3 Effects of *P. minimum* on Activity of AKP

As shown in Fig.3, the activities of AKP in the hemolymph were significantly reduced by the presence of *P. minimum* ($P<0.05$). In *P. minimum* treatment groups, the activities of AKP decreased significantly at 6 h, and returned to normal levels at 12−48 h; after 48 h, the activities of AKP dropped sharply and reached the lowest value at 96 h (0.25-fold and 0.10-fold of control in low and high concentrations of *P. minimum*, respectively).

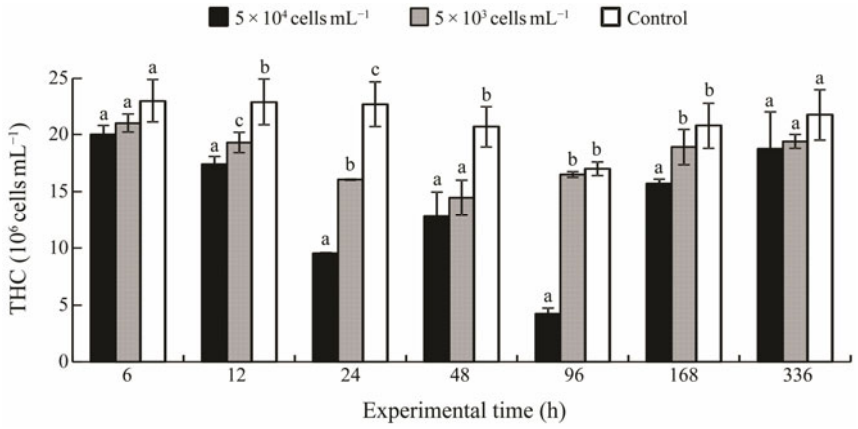

Fig.1 Effects of *P. minimum* on the total hemocyte counts of *E. carinicauda*. Data in all cases are expressed as means± standard error (SE) (n=8). Different letters above bars indicate significant differences (P<0.05) among treatments.

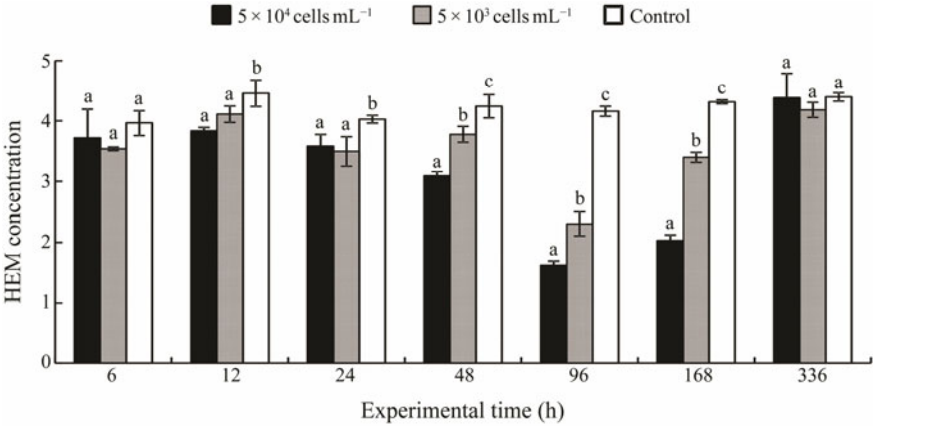

Fig.2 Effects of *P. minimum* on the hemocyanin concentrations in hemolymph of *E. carinicauda*. Data in all cases are expressed as means±standard error (SE) (n=8). Different letters above bars indicate significant differences (P<0.05) among treatments.

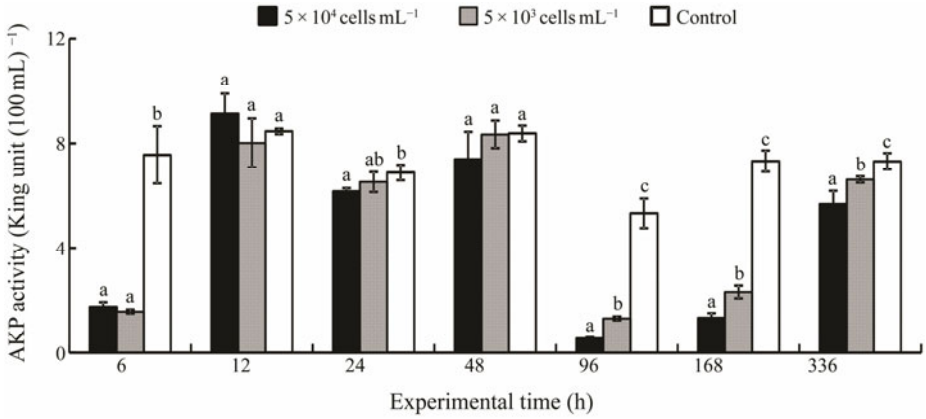

Fig.3 Effects of *P. minimum* on the activity of AKP in hemolymph of *E. carinicauda*. Data in all cases are expressed as means±standard error (SE) (n=8). Different letters above bars indicate significant differences (P<0.05) among treatments.

3.4 Effects of *P. minimum* on the Expression of Immunity-Related Genes

3.4.1 Effects of *P. minimum* on the expression of immunity-related genes in hemocytes

The effects of *P. minimum* on the expression of immunity-related genes in hemocytes are presented in Fig.4. Compared to the control, the *LGBP* expression in 5×10^3 cells mL^{-1} of *P. minimum* treatment only increased after 336 h (P<0.05), while the mRNA expression levels of *LGBP* were evoked immediately by 5×10^4 cells mL^{-1} of *P. minimum* and reached peak level at 336 h (5.46-fold of control, P< 0.05) (Fig.4A). The expression of *ALF* was not significantly affected by exposure to *P. minimum* (P>0.05) (Fig.4B). The mRNA level of *Crustin* and *proPO* were up-regulated after exposure to *P. minimum*, and expressed as a dose-dependent relationship (Figs.4C and 4E). The mRNA level of *Lysozyme* in 5×10^3 cells mL^{-1} of *P. minimum* treatment

group was significantly down-regulated at 6–12 and 96–168 h ($P<0.05$), but up-regulated at 48 h ($P<0.05$). While in 5×10^4 cells mL^{-1} of *P. minimum* group, the mRNA level of *Lysozyme* was only significantly down-regulated at 6 h ($P<0.05$), and up-regulated at 12–96 h and 336 h (Fig.4D). The *Serpin* mRNA expression in 5×10^3 cells mL^{-1} of *P. minimum* group decreased significantly at 6–96 h ($P<0.05$), while in the group exposed to 5×10^4 cells mL^{-1} of *P. minimum*, expression of *Serpin* increased significantly during 12–48 h and at 336 h ($P<0.05$) (Fig.4F).

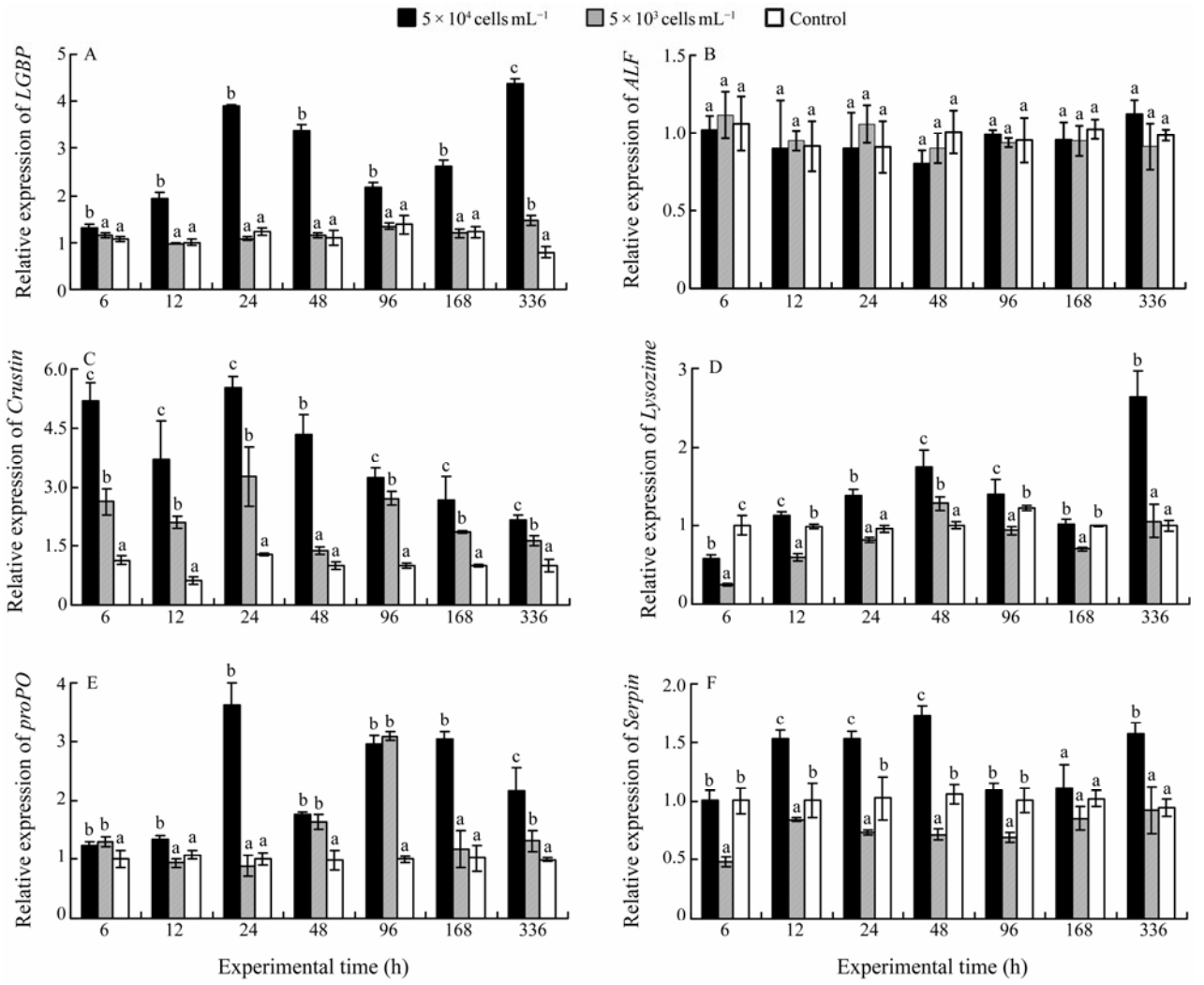

Fig.4 Effects of *P. minimum* on the expression of immunity-related genes in the hemocytes. Data in all cases are expressed as means ± standard error (SE) ($n=8$). Different letters above bars indicate significant differences ($P<0.05$) among treatments.

3.4.2 Effects of *P. minimum* on the expression of immunity-related genes in gills

The expressions of immunity-related genes in gills are shown in Fig.5. The mRNA level of *LGBP* was significantly increased during 6–12 h and 168–336 h in the group exposed to 5×10^3 cells mL^{-1} of *P. minimum* ($P<0.05$), and significantly increased during 6–12 h, and at 48 h in the group exposed to 5×10^4 cells mL^{-1} of *P. minimum* ($P<0.05$) (Fig.5A). The *ALF* mRNA expression was significantly reduced by the exposure of *P. minimum* ($P<0.05$), especially at 5×10^4 cells mL^{-1} of *P. minimum* (Fig.5B). The expression of *Crustin* was hardly affected by *P. minimum* exposure, while significant difference only occurred at 48 h and 6 h in low and high concentrations of *P. minimum* ($P<0.05$), respectively (Fig.5C). The mRNA levels of *Lysozyme* were evoked immediately by 5×10^3 cells mL^{-1} of *P. minimum* and reached peak level at 168 h (5.36-fold of control, $P<0.05$), while in the group exposed to 5×10^4 cells mL^{-1} of *P. minimum*, the levels were evoked only during 6–12 h (Fig. 5D). The expressions of *proPO* and *Serpin* had similar patterns, and were only significantly reduced by *P. minimum* exposure before 24 or 48 h (Figs.5E and 5F).

3.4.3 Effects of *P. minimum* on the expression of immunity-related genes in hepatopancreases

The effects of *P. minimum* on the expression levels of six immunity-related genes in hepatopancreases are shown in Fig.6. The expression level of *LBGP* increased significantly at 6–12 h ($P<0.05$), and reached to the highest levels at 6 h (4.52-fold and 3.86-fold of control in low and high concentrations of *P. minimum*, respectively), then fluctuated (Fig.6A). The *ALF* mRNA expression level fluctuated and only significantly increased at 6 h and 168 h ($P<0.05$)

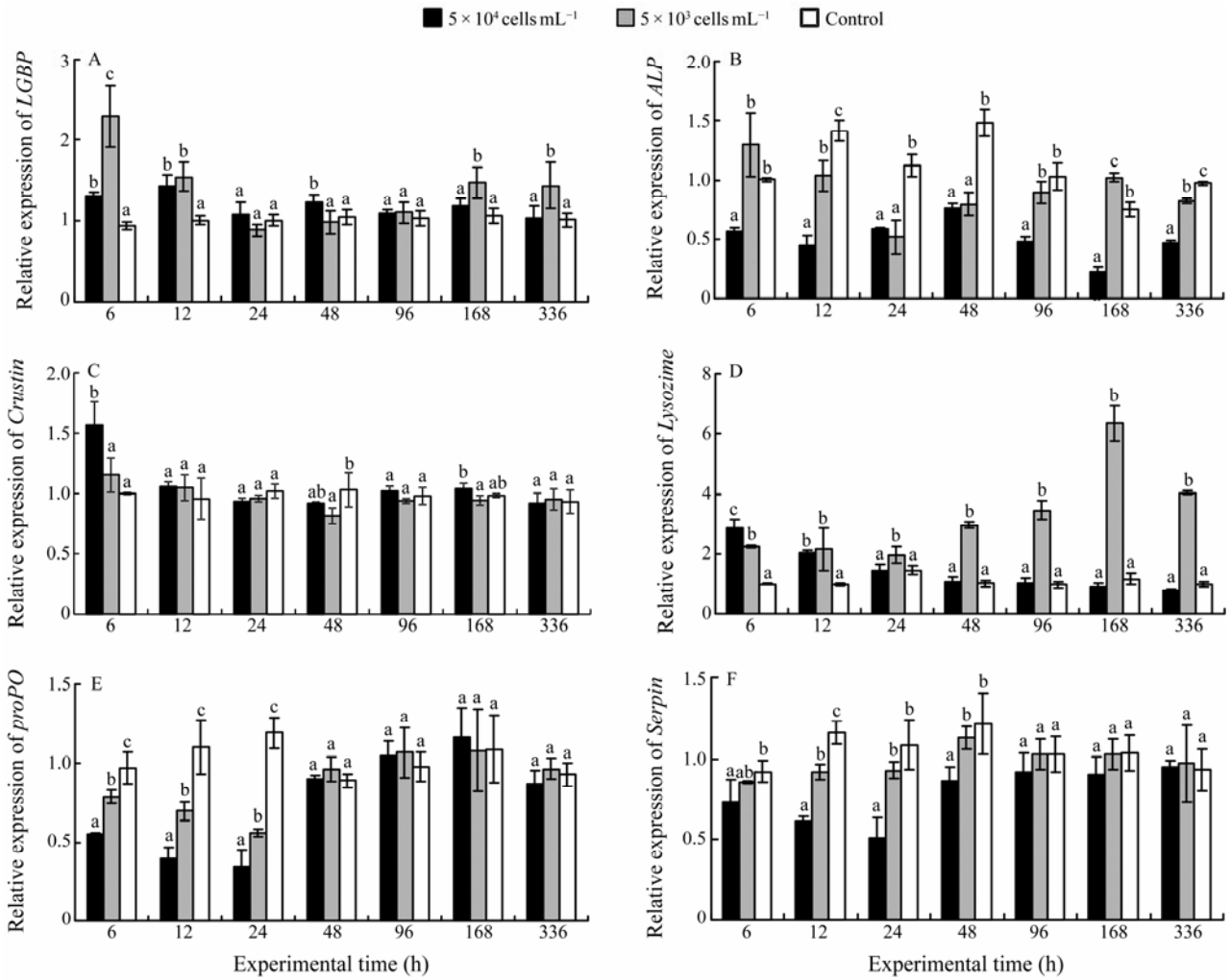

Fig.5 Effects of *P. minimum* on the expression of immunity-related genes in the gills. Data in all cases are expressed as means ± standard error (SE) ($n=8$). Different letters above bars indicate significant differences ($P<0.05$) among treatments.

(Fig.6B). The *Crustin* and *Lysozyme* mRNA expression levels were significantly up-regulated compared with the control group ($P<0.05$) (Figs.6C and 6D). The former reached peak level at 6 h (7.44-fold of control) and 24 h (3.11-fold of control) in the low and high concentrations group, respectively, while the latter reached peak level at 168 h (1.70-fold of control) and 96 h (2.40-fold of control), respectively. The *proPO* mRNA expression was only significantly up-regulated by 5×10^4 cells mL^{-1} of *P. minimum* after 24–168 h ($P<0.05$), and reached the highest value at 168 h (2.78-fold of control) (Fig.6E). The *Serpin* expression levels were significantly up-regulated at 48 h and 168 h in 5×10^3 cells mL^{-1} of *P. minimum* group ($P<0.05$), and during 48–336 h in 5×10^4 cells mL^{-1} of *P. minimum* group ($P<0.05$) (Fig.6F).

3.5 Cumulative Mortality of Shrimp After Injection of *V. parahaemolyticus* and WSSV

After exposure to *P. minimum* for 336 h, shrimps were injected with *V. parahaemolyticus* and WSSV, and the cumulative mortalities are shown in Fig.7.

In the *V. parahaemolyticus* injection experiment, the cumulative mortality in the low concentration group was not significantly increased before 48 h ($P>0.05$), while the cumulative mortality in the high concentration group was significantly elevated after 6 h ($P<0.05$). At 72 h after challenge, the cumulative mortalities of exposure groups were approximately 55% and 80%, which were significantly higher compared to the control group (22%, $P<0.05$) (Fig.7A).

There were few dead shrimp immediately after injection of WSSV, and massive mortalities occurred after approximately 24 h. The cumulative mortalities of shrimp were significantly affected by the exposure of *P. minimum*, and the final mortalities were about 55% and 70% in low and high concentrations of *P. minimum*, which were significantly higher compared with the control group (25%, $P<0.05$) (Fig.7B).

4 Discussion

It is known that the normal physiological function of *E. carinicauda* can be affected by the exposure of *P. minimum* (Mu *et al.*, 2017, 2019), which expands our knowledge of the impacts of *P. minimum* on shrimp and the immune response after exposure.

Hemocytes, as the bearers of cellular immunity and the providers of humoral immunity, play important roles in the

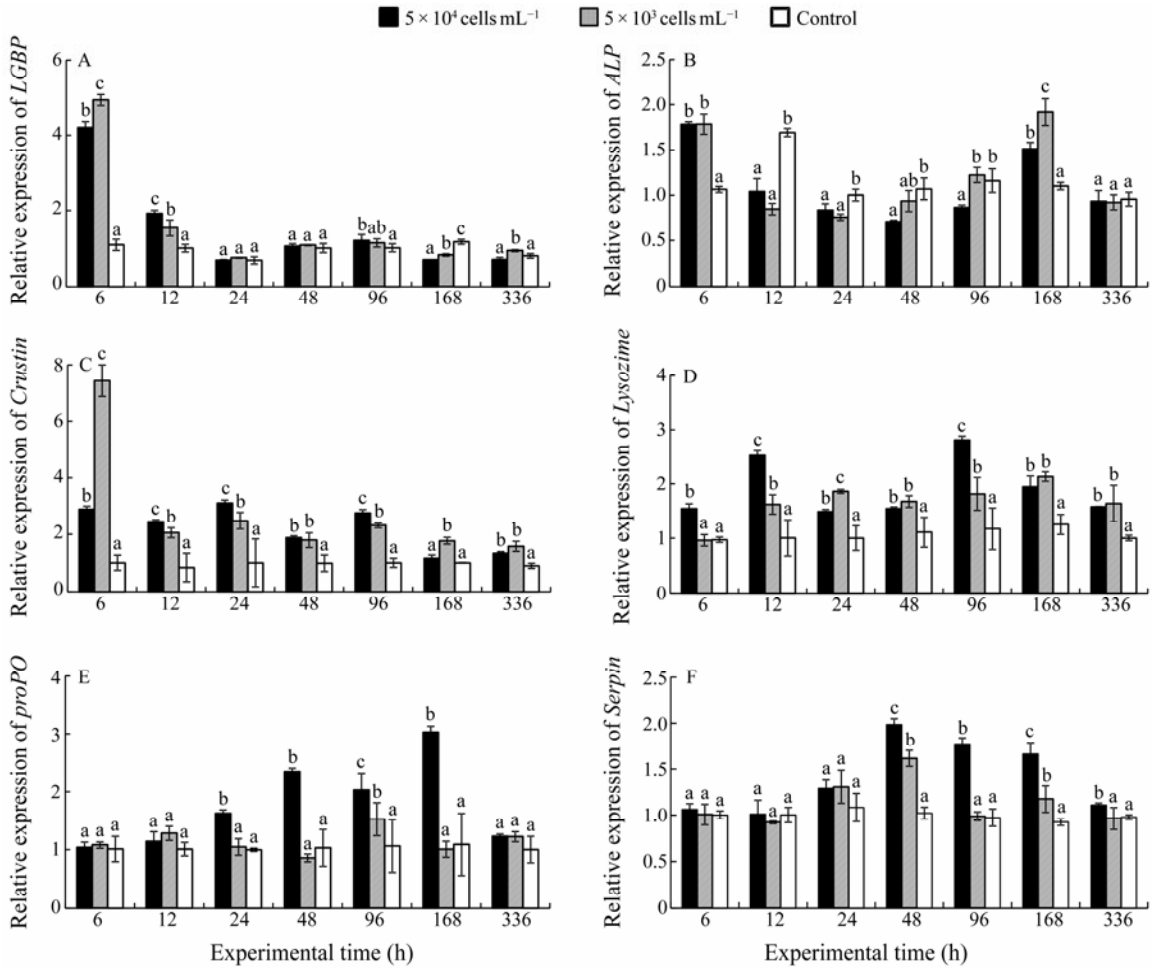

Fig.6 Effects of *P. minimum* on the expression of immunity-related genes in the hepatopancreases. Data in all cases are expressed as means±standard error (SE) (n=8). Different letters above bars indicate significant differences (P<0.05) among treatments.

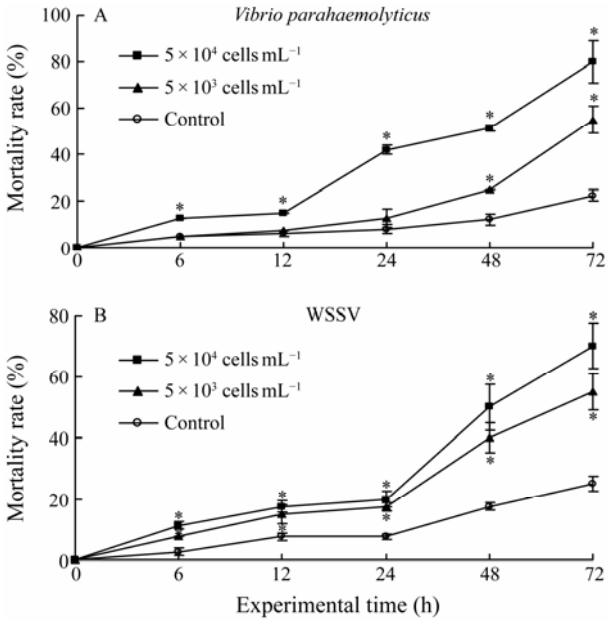

Fig.7 Cumulative mortality of shrimp after injection of *V. parahaemolyticus* and WSSV. Data in all cases are expressed as means±standard error (SE) (n=10). * means P<0.05 compared with control.

immune defense system of crustaceans (Söderhäll and Cerenius, 1992; Vazquez *et al*., 2009). As a result, the THC is an important indicator to evaluate the health status of crustaceans (Söderhäll and Cerenius, 1992). In the present study, the THC was significantly reduced by the exposure of *P. minimum*. Since the accumulation or infiltration of hemocytes is known to be in the hemocoelic space of gill of the shrimp exposed to *P. minimum* (Mu *et al.*, 2017), the reduced THC may be due to the migration of hemocytes to the gill tissue and may play a role in the immune response of the gill tissue. The involvement of hemocytes in the immune response of tissues has been confirmed in shellfish exposed to harmful algae (Estrada *et al.*, 2007a, 2007b, 2010; Galimany *et al.*, 2008; Yeganeh *et al.*, 2020).

Being a copper-containing respiratory protein, HEM can not only serve as an oxygen carrier to provide sufficient oxygen to the body (Nagai *et al.*, 2001), but also play a role in resisting the invasion of foreign pathogens and adapting to the environment (Destoumieux-Garzón *et al.*, 2001; Zhang *et al.*, 2004; García-Carreño *et al.*, 2008). The HEM concentrations in *P. minimum* treatment groups were significantly lower than those of the control group, and decreased with a dose-dependent relationship. The result indicates that the oxygen carrying capacity of haemolymph in shrimp exposed to *P. minimum* may be reduced.

The AKP is an important lysosomal enzyme and plays

a key role in invertebrate immune responses. Ge *et al*. (2017) found that AKP in the hemolymph of *E. carinicauda* increased following an AHPND-causing strain of *V. parahaemolyticus* infection, and suggested that the immune enzymes such as AKP play roles in resisting pathogen invasion, and may be used as a potential biomarker to measure the disease resistance of white shrimp. The activity of AKP was significantly reduced in the present study, suggesting that AKP might be inhibited by the harmful algal exposure.

Both Crustin and ALF are important antibacterial peptides in shrimp humoral immunity and have antibacterial and antiviral effects in crustaceans (Aweya *et al.*, 2021; Kulkarni *et al.*, 2021). In this study, the expressions of *Crustin* in hemocytes and hepatopancreases were up-regulated by the exposure of *P. minimum*, though the difference of *ALF* was not significant. While in gills the differences of *Crustin* were not significant, the expressions of *ALF* were down-regulated by the exposure of *P. minimum*. The results show that Crustin in hemocytes and hepatopancreases participates in the immune response of *E. carinicauda* exposed to *P. minimum*, and has a certain protective effect. The down-regulated expressions of *ALF* in the gills may inhibit the immune capacity of shrimp, which needs further verification in subsequent studies.

Lysozymes are usually used as an immune index in crustaceans to detect the immune function of organisms (Liu *et al.*, 2021; Yao *et al.*, 2021). In the experiment, the expression levels of *Lysozyme* showed an up-regulated trend, except for the hemocytes in the low concentration group. The results indicate that the *Lysozyme* gene of *E. carinicauda* is involved in the immune response after exposure to *P. minimum*.

The prophenoloxidase (proPO) system is a complex enzyme cascade system in the crustacean immune response. After being stimulated by pathogen infection or physical injury, proPO is converted into active phenol oxidase (PO) under the action of serine protease, and subsequently catalyzed to melanin. Melanin and its highly active intermediate products can inhibit the activity of extracellular proteases and chitinase of pathogen (Meng *et al.*, 1999). In this study, after being exposed to *P. minimum*, the *proPO* and *Serpin* genes were significantly up-regulated in the hemocytes and hepatopancreases, and significantly down-regulated in the gills, indicating that the proPO system was activated in hemocytes and hepatopancreases to defend against harmful algae, while the gills may not be able to resist well due to the tissue damage. LGBP is a pattern recognition receptor involved in the activation of the proPO system in shrimp and crabs (Cerenius and Söderhäll, 2004). The up-regulated expression of *LGBP* was found in each tissue of shrimp exposed to *P. minimum*. Changes in the expression of these genes suggest that the proPO system may be activated to participate in the defense response of shrimp.

After exposed to *P. minimum* for 336h, significantly more shrimps injected with *V. parahaemolyticus* and WSSV died. The results directly reflect the adverse effect of *P. minimum* on the immune system of *E. carinicauda*. Since the exposure of *P. minimum* depressed the immunity of *E. carinicauda*, further studies are needed to confirm whether the presence of the algae will limit the susceptibility of shrimp to pathogens.

5 Conclusions

In this study, *P. minimum* had an adverse effect on THC, hemocyanin, and the activity of AKP in the hemolymph of *E. carinicauda*. There was up-regulation of the immunity-related genes in hemocytes and hepatopancreases, and down-regulation of the expressions of *proPO*, *Serpin* and *ALF* in gills. The hemocytes and hepatopancreases play important roles in protecting the immune response of shrimp to harmful algae, while the immune protection effect in gills may be suppressed by the tissue damage.

Acknowledgements

This project was financially supported by the earmarked fund for National Key R & D Program of China (No. 2019YFD0900403), the Modern Agro-Industry Technology Research System (No. CARS-48), the Program of Shandong Leading Talent (No. LNJY2015002), the Central Public-Interest Scientific Institution Basal Research Fund of CAFS (No. 2020TD46), and the Scientific Research Start-Up Funding of Shandong Agricultural University (No. 72188).

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(Edited by Qiu Yantao)