

## Seawater acidification affects the immune enzyme activities of the Manila clam *Ruditapes philippinarum*\*

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**Abstract** Ocean acidification leads to changes in physiological and immune responses of bivalves, but the effect on the immune enzyme activities of the Manila clam, *Ruditapes philippinarum*, when the pH is lower than the normal value has not been studied in detail. In this study, experiments were conducted to determine how pH (7.4, 7.7, 8.0) affects the immune enzyme activities in the gill and hemocytes of the Manila clam. Membrane stability and phagocytosis increased with decrease of pH from 8.0 to 7.7 and then decreased at pH 7.4. The total protein content in the hemocytes and gills decreased with decreasing pH. Lysozyme content in the hemocytes increased with decreasing pH, and the differences were significant among the different pH groups ( $P < 0.05$ ). Adenosine triphosphatase activity at pH 7.4 was significantly higher than in the other two groups, but no significant difference was observed between pH 7.7 and 8.0. Catalase activity decreased from pH 8.0 to 7.7 and then increased at pH 7.4, and the differences were significant among the experimental groups ( $P < 0.05$ ). These findings provide valuable information about the immune response of *R. philippinarum* to reduced water pH and insights for future research investigating exposure of bivalves to elevated CO<sub>2</sub> conditions.

**Keyword:** seawater acidification; immune enzyme; *Ruditapes philippinarum*

### 1 INTRODUCTION

Seawater acidification refers to the reduced pH in oceans that results from increased anthropogenic carbon dioxide (CO<sub>2</sub>) emissions (Caldeira and Wickett, 2003; Feely et al., 2004; Sabine et al., 2004). Many experimental results have confirmed that ocean acidification leads to changes in marine carbonate systems, which have direct effects on physiological and immune functions of marine bivalves such as respiration, metabolism, reproduction, biological mineralization, energy budget, and hemocyte parameters (Ding et al., 2012; Wang et al., 2015; Sui et al., 2016a, b; Hu et al., 2017). For example, sperm motility of the clam *Tegillarca granosa* was decreased and sperm half-life was shortened under low pH conditions (Zhao et al., 2012). In addition, the fertilization rate (sperm:egg ratio 10:1) of *T. granosa* after acidification was significantly lower than that of

the control group. Caldeira and Wickett (2003) reported devastating damage to marine species, especially the formation of shells, caused by ocean acidification. Talmage (2011) showed that the calcification ability of bivalve larvae was weakened at high concentrations of CO<sub>2</sub>. Wang et al. (2015) indicated that physiological energetics of juvenile *Mytilus coruscus* are able to acclimate to CO<sub>2</sub> acidification with little physiological effect. To date, however, the effect of seawater acidification on the immune functions of shellfish has not been studied in detail. A few studies confirmed that the immune system function of shellfish can be damaged when the

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seawater pH is lower than the normal value (Michaelidis et al., 2005; Fan et al., 2006). The increasing degree of acidification of sea water also likely affects the immune function of shellfish (Huang et al., 2016; Wu et al., 2016; Sui et al., 2017).

The Manila clam is an ecologically and commercially important bivalve found along the west coast of North America, Europe, and throughout Asia. It has become one of the most important aquaculture species because of its fast growth, low mobility, strong adaptability, and short growth cycle. In coastal areas of southern and northern China, *R. philippinarum* aquaculture has produced good economic and social benefits. Studies of the Manila clam have documented sensitivity to several stressors, including temperature, salinity, pathogens, and food availability (Paillard, 2004; Inoue et al., 2007; Yan et al., 2009), but few studies have investigated the impact of ocean acidification (Xu et al., 2016). The present study investigated the effects of seawater acidification on immune enzyme activities of the Manila clam. The results provide valuable information about the immune response of *R. philippinarum* to reduced water pH and insights for future research investigating exposure of bivalves to elevated CO<sub>2</sub> conditions.

## 2 MATERIAL AND METHOD

### 2.1 Material

Eight hundred and ten *R. philippinarum* specimens (33.6±1.8 mm shell length) were collected from the intertidal shores of Liangshui Bay (39°04'14.41"N, 122°01'47.70"E), Liaodong Peninsula, Northeast China, in May 2015. Clams were transported to an aquarium facility where they were allowed to acclimate to laboratory conditions for 2 weeks prior to experimentation. Clams were held in recirculating seawater that mimicked natural conditions. Temperature, salinity, pH, and light: dark cycles were maintained at constants of 9.0±0.31°C, 32.12±0.08, 8.1±0.05, and 12 h:12 h, respectively.

### 2.2 Experimental setup

To control the seawater pH, nine recirculating systems were established. Each system contained 100 L of seawater and had three chambers (filter, temperature controlled, and gas mixing). CO<sub>2</sub> gas was used in the gas chamber of these systems to control pH. Gas flow was adjusted using a mass flow controller. After 36 h, the systems reached a stable state. The experiment included three systems each for

pH 7.4, pH 7.7, and pH 8.0; the latter, which served as the control, was bubbled with ambient air only (Xu et al., 2016). Ninety clams were kept in a sand substrate in each of the nine tanks for 3 months under the three pH conditions. Clams were fed twice daily with an equal mixture of the microalgae *Chaetoceros muelleri*, *Nitzschia closterium*, *Chlorella vulgaris*, and *Isochrysis galbana* to fulfill their nutritional requirements. A constant food supply (20 000 cells/mL) was maintained over the duration of the experiment. Over the following 90 days, clams were subjected to increasing temperature regimes. In all temperature was simultaneously increased ca. 0.3°C per day from 9 to 25°C. clams were maintained at 25°C for 40 days.

### 2.3 Measurement

At the end of the experiment, three individuals from each of three replicate tanks of each experimental group were selected to collect hemocyte and gill tissue for immune activity analyses. Mean values were used in data analysis to avoid pseudoreplication.

Throughout the duration of the experiment, constant low pH conditions were maintained. Dissolved oxygen content (DO), Temperature, salinity, and pH were recorded every day. TA was measured using an alkalinity titrator. Partial pressure of CO<sub>2</sub> in seawater (*p*CO<sub>2</sub>), dissolved inorganic carbon (DIC), and CaCO<sub>3</sub> saturation state for aragonite ( $\Omega_{\text{ara}}$ ) and calcite ( $\Omega_{\text{cal}}$ ) were calculated on the basis of the pH, temperature, TA, and salinity of the seawater, following the method of Wang et al. (2015).

#### 2.3.1 Measurement of cell membrane stability of clams

To measure cell membrane stability, a 50-μL aliquot of hemocytes was collected in an enzyme-linked immunosorbent assay (ELISA) plate and incubated at 4°C for 45 min. The sample was washed twice with normal saline that did not adsorb hemocyte cells (100 μL). Next, 200 μL of 0.004% neutral red solution were added, and the mixture was incubated at 24°C for 3 h. The excess dye was washed off with normal saline, and the sample was added to the enzyme-labeled plate to mix with 200 μL of alcohol with pH adjusted corresponding to the experimental groups. The ELISA plate then was placed into the enzyme standard micropipette reader (FluorMax 2000, Shanpu Biological Technology Co. Ltd., Shanghai, China) to read the value at optical density at 550 (OD<sub>550</sub>).

### 2.3.2 Phagocytic activity of hemocyte cells

To measure phagocytic activity, 50  $\mu\text{L}$  samples of Manila clam hemocytes were placed into an ELISA plate and incubated at  $4^{\circ}\text{C}$  for 1 h. Non-adherent hemocyte cells were washed away with two 100  $\mu\text{L}$  washes of physiological saline. After adding 50  $\mu\text{L}$  ( $50 \times 10^7/\text{mL}$ ) of neutral red dyed yeast suspension (Xiuming Biotechnology Co. Ltd., Shanghai, China) at  $20^{\circ}\text{C}$  for 30 min, 100  $\mu\text{L}$  of 7-Benzoyloxy-4-trifluoromethyl coumarin were added to terminate the reaction. Excess yeast particles were washed away with normal saline, and the sample was mixed with 100  $\mu\text{L}$  of alcohol with pH adjusted corresponding to the experimental groups. The plate was placed in the microplate reader (Molecular Devices, Sunnyvale, California, USA) and readings were taken at  $\text{OD}_{550}$ . Phagocytic activity was expressed as the number of yeast particles per mg of protein.

### 2.3.3 Total protein content in the hemocytes and gill tissue

The total protein content of the gill tissue and hemocytes was determined using the Coomassie brilliant blue method. Gill tissue was weighed (g) and mixed with physiological saline (mL) at a volume ratio of 1:9, and the sample was ground using a mortar and pestle. The sample was placed in a centrifuge tube and centrifuged for 10 min at 3 000 r/min. The supernatant was used as the experimental sample. Absorbance value was measured at room temperature ( $18^{\circ}\text{C}$ ) at the wavelength of 595 nm (1 cm light transmittance path). Total protein content was calculated as follows:

$$\text{Protein concentration (g/L)} = \frac{\text{OD}_{\text{determined}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \times C_s,$$

where  $C_s$  is the concentration of the standard, which was 0.563 g/L.

### 2.3.4 Determination of lysozyme (LSZ) content

The LSZ content of the gill tissue was determined using a LSZ determination kit following the instructions provided by the manufacturer (Jian Cheng Bio-engineering Institute, Nanjing, China). The contents of each tube were mixed well and kept at  $37^{\circ}\text{C}$  in a water bath for 15 min, then immediately transferred to an ice water bath ( $<0^{\circ}\text{C}$ ) for 3 min. The absorbance value of each tube was measured at room temperature ( $18^{\circ}\text{C}$ ) at the wavelength of 530 nm (1 cm light transmittance path). LSZ content ( $\mu\text{g/mL}$ )

was calculated as follows:

$$\frac{\text{OD}_{\text{determined}} - \text{OD}_{\text{control}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \times$$

standard sample concentration  $\times$

concentration of test sample before dilution.

### 2.3.5 Determination of adenosine triphosphatase (ATPase) activity

This analysis was conducted following the instructions provided in the ATPase determination kit (Jian Cheng Bio-engineering Institute). The sample was mixed well and kept at  $45^{\circ}\text{C}$  in a water bath for 20 min after all steps were completed, followed by immediate cooling to room temperature. The absorbance value of each tube was measured at the wavelength of 660 nm (1 cm light transmittance path). ATPase enzyme activity was calculated using the following formula:

$$\frac{\text{OD}_{\text{determined}} - \text{OD}_{\text{control}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \times$$

standard tube concentration  $\times$  sample dilution  $\times 6$ .  
protein content (mg/mL)

### 2.3.6 Catalase (CAT) activity

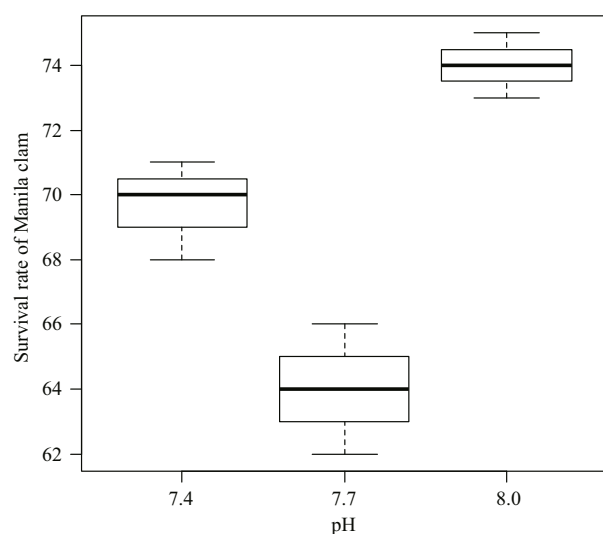
Samples were processed following the instructions provided in the CAT determination kit (Jian Cheng Bio-engineering Institute, Nanjing). The mixture in each tube was shaken and the absorbance value of each tube was measured at room temperature at the wavelength of 405 nm (0.5 cm light transmittance path). CAT activity was calculated using the following formula:

$$A_{\text{CAT}} (\text{U/mg prot}) = \frac{(\text{OD}_{\text{control}} - \text{OD}_{\text{test}}) \times 271 \times [1/(60 \times W)]}{C_{\text{test}} (\text{mg prot/mL})},$$

where  $A_{\text{CAT}}$  is the activity of CAT in the tissue,  $C_{\text{test}}$  is the protein concentration of the sample tested, and  $W$  is the amount of the sample (mg).

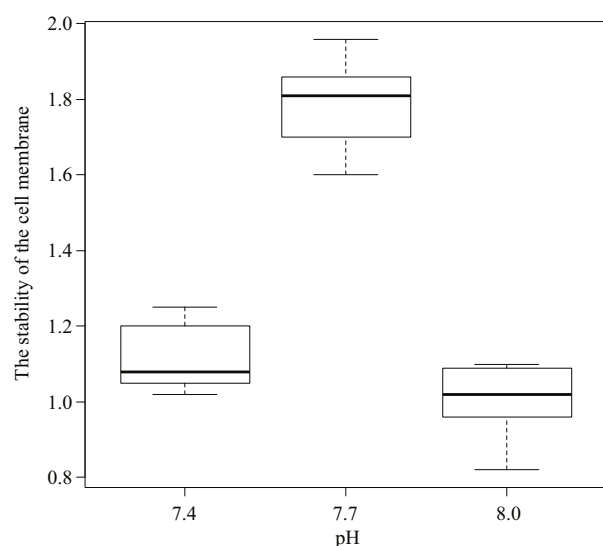
### 2.3.7 Statistical analysis

The objective of this study was to examine the immune enzyme activities of Manila clam reared at different pH values. All analyses were performed using the Statistical Package for  $R$  statistical software. The potential effects of pH on immune enzyme activities were analyzed using one-way analysis of variance. Significant effect was tested using Tukey's honest significant difference (HSD) test. Significance



**Fig.1 Survival rate of Manila clams under different pH conditions**

The error bars represent standard deviation, the same meaning as follows.



**Fig.2 Stability of the cell membrane of Manila clams under different pH conditions**

The error bars represent standard deviation.

level for all analyses was set at  $P < 0.05$ . Measurements are reported as the mean (of three replicates)  $\pm$  95% confidence limit as implemented in R.

### 3 RESULT

#### 3.1 Water chemistry

Carbonate chemistry parameters of seawater within each treatment are summarized in Table 1. The desired temperature and pH levels were successfully reached within each treatment. Salinity was maintained at

**Table 1 Seawater carbonate chemistry (mean  $\pm$  SD, sample size  $n=4$ ) during the experimental period**

	pH 8.0	pH 7.7	pH 7.4
Temperature ( $^{\circ}\text{C}$ )	25.62 $\pm$ 0.32	25.31 $\pm$ 0.22	25.11 $\pm$ 0.41
Salinity	32.84 $\pm$ 0.14	32.94 $\pm$ 0.10	32.86 $\pm$ 0.09
pH	8.01 $\pm$ 0.03	7.72 $\pm$ 0.07	7.43 $\pm$ 0.05
$A_T$ ( $\mu\text{mol/kg}$ )	2 320 $\pm$ 270	2 250 $\pm$ 90	2 780 $\pm$ 710
$p\text{CO}_2$ ( $\mu\text{atm}$ )	740 $\pm$ 127	1 021 $\pm$ 85	2 385 $\pm$ 969
DIC ( $\mu\text{mol/kg}$ )	2 267 $\pm$ 293	2 198 $\pm$ 80	2 730 $\pm$ 973
$\Omega_{\text{cal}}$	2.77 $\pm$ 1.12	1.89 $\pm$ 0.61	1.33 $\pm$ 0.81
$\Omega_{\text{ara}}$	1.78 $\pm$ 0.74	1.21 $\pm$ 0.41	0.85 $\pm$ 0.54
DO (mg/L)	5.31 $\pm$ 0.23	5.16 $\pm$ 0.35	5.43 $\pm$ 0.46

Temperature, salinity, total alkalinity ( $A_T$ ) and dissolved oxygen content (DO) were measured every day.  $\text{pH}_{\text{NBS}}$  was measured continuously during the experiment. Dissolved inorganic carbon (DIC), partial pressure of  $\text{CO}_2$  in seawater ( $p\text{CO}_2$ ) and saturation state for calcite ( $\Omega_{\text{cal}}$ ) and aragonite ( $\Omega_{\text{ara}}$ ) were calculated from the measured temperature, salinity, pH, and TA.

32.88 $\pm$ 0.5, and dissolved oxygen remained at approximately 5 mg/L.

#### 3.2 Survival of Manila clams under different pH conditions

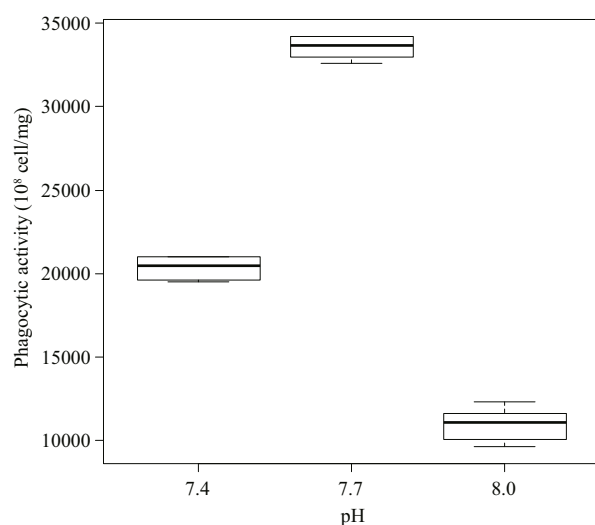
Survival rates of Manila clams at different temperatures on day 90 are shown in Fig.1. Results showed that the survival rate was not significantly different from pH 7.4 to 8.0.

#### 3.3 Cell membrane stability under different pH conditions

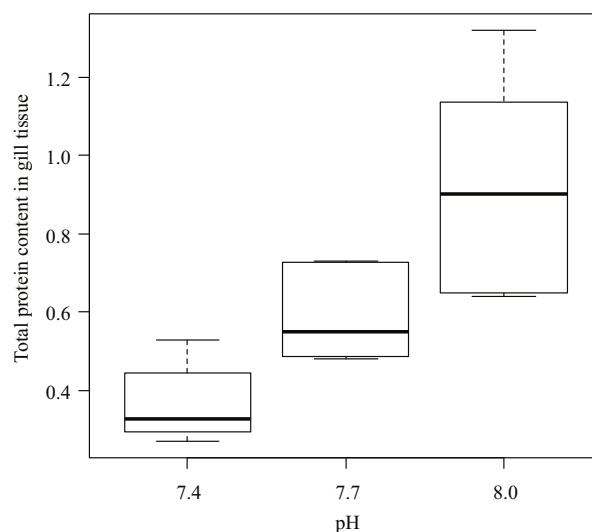
Figure 2 show the stability of the cell membrane of *R. philippinarum* under different pH conditions. As pH decreased from 8.0, the stability of the cell membrane increased to its peak value at pH 7.7 and then decreased at pH 7.4. Cell membrane stability did not differ significantly ( $P > 0.05$ ) between pH 8 and 7.4, but the difference was significant between pH 7.7 and the other two pH values ( $P < 0.05$ ).

#### 3.4 Phagocytic activity of hemocyte cells under different pH conditions

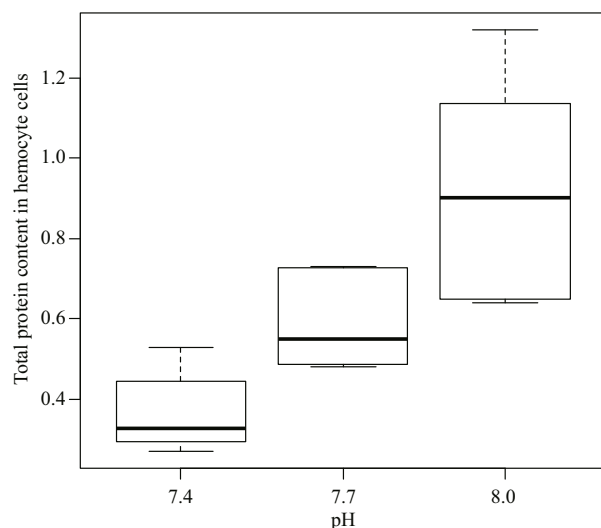
Differences in the phagocytic activity of Manila clam hemocyte cells under different pH conditions are shown in Fig.3. Phagocytic activity first increased as pH decreased from 8.0 to 7.7, where maximum activity was reached, and then it decreased at pH 7.4. Significant differences in the phagocytosis of the hemocyte cells were detected among the three pH groups ( $P < 0.05$ ).



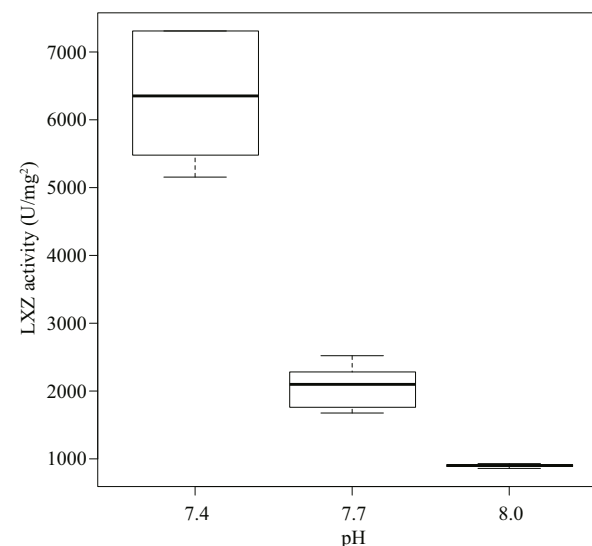
**Fig.3 Phagocytic activity of Manila clams under different pH conditions**



**Fig.5 Change of total protein content in gill tissue under different pH conditions**



**Fig.4 Change of total protein content in hemocyte cells under different pH conditions**



**Fig.6 Change of lysozyme activity in hemocytes under different pH conditions**

### 3.5 Effect of different pH conditions on total protein content in hemocyte cells

Figure 4 show the total protein content in the hemocyte cells of *R. philippinarum* under different pH conditions. The protein content in the hemocytes decreased with decreasing pH. There was no significant difference ( $P>0.05$ ) in total protein content between the pH 7.4 and 7.7 groups, but the content was significantly higher in the pH 8.0 treatment compared to the other two groups ( $P<0.05$ ).

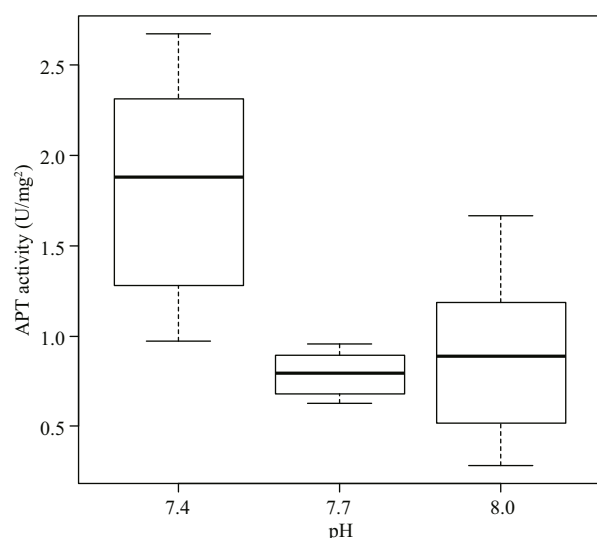
### 3.6 Effect of different pH conditions on total protein content in gill tissue

The total protein content in the gill tissue of

Manila clams under different pH conditions is shown in Fig.5. The content of total protein in gill tissue did not differ significantly ( $P>0.05$ ) when pH was 8 and 7.7.

### 3.7 LSZ activity under different pH conditions

The activity of LSZ in the hemocytes of *R. philippinarum* under different pH conditions is shown in Fig.6. The LSZ activity in the hemocytes increased with the decrease of pH, with the maximum value reached at pH 7.4. The value differed significantly among the three treatment groups ( $P<0.05$ ).



**Fig.7** Change of ATP activity in gill tissue under different pH conditions

### 3.8 ATPase activity under different pH conditions

Figure 7 show the ATPase activity in the gill tissue of Manila clams in different pH environments. The ATPase activity at pH 7.4 was significantly higher ( $P < 0.05$ ) than that in the other two groups, and no significant difference was observed between the pH 7.7 and 8.0 groups ( $P > 0.05$ ).

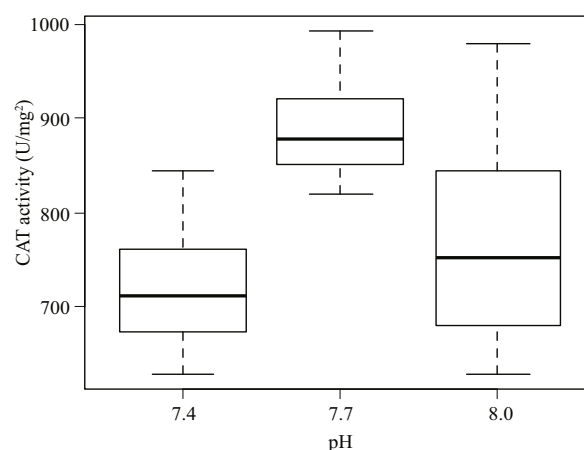
### 3.9 CAT activity under different pH conditions

The CAT activity in the gill tissue of *R. philippinarum* under different pH conditions is shown in Fig.8. CAT activity changed from pH 8.0 to 7.4. The highest value occurred at 7.7, and then it decreased at pH 7.4. CAT activity differed significantly ( $P > 0.05$ ) among the three experimental groups.

## 4 DISCUSSION

Sea water acidification has numerous effects on marine organisms, including plankton, fish, and bivalves (Matozzo et al., 2012; De Souza et al., 2014; Li et al., 2014; Meseck et al., 2016; Clements and Hunt, 2017; Taucher et al., 2017). Marine bivalves need to acclimate to their environment to avoid adverse effects on reproduction, the immune system, metabolism, growth, and survival (Sui et al., 2016a, b). In this respect, phagocytosis and the enzymes related to phagocytosis are important immune mechanisms. The results of the current study show that seawater acidification has an impact on the immune enzymes of *R. philippinarum*.

The results of the experiment revealed differences in cell membrane stability and phagocytosis of



**Fig.8** Change of CAT activity in gill tissues under different pH conditions

hemocytes in Manila clams reared under different pH conditions. Both factors first increased and then decreased with the decrease of pH, with maximum values occurring at pH 7.7, and both were higher in the pH 7.4 and 7.7 groups than in the pH 8.0 (control group) treatment. Seawater acidification affects cell membrane stability through intracellular ions and the acid-base status. Molluscs depend on an ion exchange mechanism to exchange  $H^+$  or  $HCO_3^-$  across cell membranes using protein carriers to minimize the effect of ocean acidification (Pörtner, 1996, 2004; Marchant et al., 2010; Parker et al., 2013). Feng (1988) showed that when foreign materials invade a living organism, their identification and adsorption are determined by the stability of the cell membrane and phagocytosis by hemocytes. Similar results were reported for the phagocytic activity of *Mytilus edulis* (Bibby et al., 2008; Sun et al., 2017). The results of the current study indicate that a moderate reduction in the pH of sea water will have a certain stimulating effect on the stability of the cell membrane and phagocytosis of *R. philippinarum*.

LSZ content is one of the most important physiological indexes that can be used to assess the non-specific immune function of animals. It is an important component in the extracellular killing of microorganisms by bivalve hemocytes, as it breaks down the bacterial cell wall by dissolving cell wall peptides (Cheng and Rodrick, 1975; Winston et al., 1996; Liu et al., 2003). The role of LSZ originally was to help digest food, but as the environment changed and natural selection occurred, LSZ gradually developed as a blood-borne immune function in shellfish. Bibby et al. (2008) reported that a reduction of LSZ activity involves in the disruption of cellular pathways (Bibby et al., 2008). Matozzo et al. (2012)

reported that decreased pH resulted in significantly lower LSZ activity in *Chamelea gallina* and *Mytilus galloprovincialis*. However, the results of the current study showed that LSZ content increased with decreased pH, with the maximum level at pH 7.4. This finding indicates that reduction of the pH of the sea water can increase the content of LSZ. Increased LSZ content helps the hemolymph to more actively defend against invaders.

The main role of ATPase is to catalyze the decomposition of ATP into ADP and phosphate ions to release energy. However, in aquatic organisms, Na<sup>+</sup>K<sup>+</sup>-ATPase plays an important role in the regulation of osmotic homeostasis (Yadwad et al., 1990) by affecting active electrical transport across the gills (Parvez et al., 2006). Mg<sup>2+</sup>-ATPase plays an important role in ionic transport and oxidative phosphorylation and is responsible for the transepithelial regulation of Mg<sup>2+</sup> ions (Parvez et al., 2006). Ca<sup>2+</sup>-ATPase also plays a significant role in removing excessive calcium ions from the cytoplasm to maintain low Ca<sup>2+</sup> levels (Saxena et al., 2000). Thus, in some species changing pH had no effect on activity of Na<sup>+</sup>K<sup>+</sup>-ATPase (Seidelin et al., 2001), whereas in others increased Na<sup>+</sup>K<sup>+</sup>-ATPase activity was observed (Ishimatsu et al., 2005; Deigweiher et al., 2008). Increased ATPase activity with decreasing pH was observed in marine fishes such as cod (Melzner et al., 2009), whereas no significant change was detected in oysters (Lannig et al., 2010) and decreased activities were observed in the mussel *M. galloprovincialis* (Michaelidis et al., 2005). In the present study, ATPase activity of the Manila clam first increased as pH decreased, with the highest value at pH 7.7, and then it decreased to the lowest value at 7.4. As the pH decreased, more energy was used for acid-base regulation, resulting in elevated ATPase enzyme activities. However, an excessive decrease in pH along with long-term exposure induces metabolic depression (Barnhart, 1989; Rees and Hand, 1990) and decreased ATPase activities.

CAT exists in a wide variety of organisms and plays an important role in the process of removal of bacteria, pathogens, and toxic chemicals. The function of CAT was reported in shellfish such as *M. edulis* (Lin et al., 2000), *Sanguinolaria* sp. and *Sanguinolaria diphos* (Sun et al., 2008), *Ostrea gigas* and *T. granosa* (Xiao et al., 2003). These species use CAT to convert hydrogen peroxide into water (Estrada et al., 2007), which is considered to be an adaptive reaction of the organism to overcome stress derived from xenobiotics

(Geracitano et al., 2004). In response to toxic chemicals, CAT activity increases to reinforce the catabolic rate and/or to directly inhibit the enzymes (Viarengo et al., 2007; Qiu et al., 2013). In the current study, *R. philippinarum* exposed to different pH levels exhibited different CAT activities. As the pH decreased from 8.0 for the control group, CAT activity increased at pH 7.7 and then decreased at pH 7.4. Similar results were observed for the CAT activity of the scallop *Chlamys farreri* (Fan et al., 2006). Generally, a change in CAT activity helps the organism to neutralize the effect of harmful toxic chemicals and defend against disease. In the current study, CAT was effectively stimulated and defended against disease or toxic chemicals at pH 7.7, but at pH 7.4 the immune activity may have been inhibited, make the Manila clams more susceptible to invaders and toxic chemicals (Viarengo et al., 2007; Qiu et al., 2013).

## 5 CONCLUSION

In conclusion, reduction in pH resulted in changes in several immune enzymes that play important roles in the Manila clam's ability to tolerate elevated *p*CO<sub>2</sub> conditions. These changes in immune enzyme levels potentially affected the immune capacity of *R. philippinarum*. Results of this study provide valuable information about the immune response of *R. philippinarum* to reduced water pH and insights for future research investigating exposure of bivalves to elevated CO<sub>2</sub> conditions. Future studies should consider seawater acidification effects on the immune genes of the Manila clam *R. philippinarum*.

## 6 DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author upon request.

## 7 ACKNOWLEDGMENT

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