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Effects of dissolved oxygen on survival and immune responses of scallop (*Chlamys farreri* Jones et Preston)

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Abstract

This experiment investigated the effects of dissolved oxygen (DO) on the survival and immune responses of scallop *Chlamys farreri*. The scallops (initial mean dry weight of soft tissue 1.52 ± 0.10 g) were cultivated in the seawater with different DO levels (8.5, 6.5, 4.5, and 2.5 mg l^{-1}, respectively) for 21 d. Each treatment had triplicate groups of 35 animals. During the experimental period, the scallops were fed with *Spirulina maxima*, and water temperature ranged from 15.2 °C to 17.5 °C, salinity from 29.5‰ to 32.5‰ and pH from 7.5 to 8.2. Survival, specific growth rate (SGR) and total haemocyte count (THC) were examined at the end of the study, and superoxide dismutase (SOD), acid phosphatase (ACP) and alkaline phosphatase (ALP), were examined at 12 h, 24 h, Day 7, Day 14 and Day 21 after being exposed to the graded DO levels. The lower DO levels (2.5 and 4.5 mg l^{-1}) resulted in lower survivals of scallops, and the survival (81.7%) at 2.5 mg l^{-1}DO was significantly lower than those (100.0%) at 8.5 and 6.5 mg l^{-1} DO. Similarly, the SGR and THC of scallop gradually reduced with decreasing DO levels, and reached significant levels at 2.5 mg l^{-1} DO ($P < 0.05$). At higher DO levels (8.5 and 6.5 mg l^{-1}), the SOD activity maintained rather stable during the entire sampling period. At lower DO levels (4.5 and 2.5 mg l^{-1}), however, the SOD activity significantly increased at 12 h, and then significantly decreased to the levels below the normal. At the two lower DO levels, ACP activities had no significant changes before Day 7, and then declined to the levels that were significantly lower than the normal. Significantly higher ALP activity was only observed at 12 h in the treatment of 2.5 mg l^{-1} DO, but in all other treatments and sampling times it fluctuated in a narrow range. In conclusion, less than 4.5 mg l^{-1} DO reduced the survival and depressed the immune responses of *C. farreri*.

Keywords: Scallop; *Chlamys farreri*; Dissolved oxygen; Survival; Immune responses

1. Introduction

The scallop *Chlamys farreri* is one of the commercially important bivalves, and has been widely cultured along the coastal areas in northern China [1]. In recent years, the production has been sharply dropping down due to high mortality [2]. The annual economic loss was estimated to be more than four billion Yuan in 1997 and 1998 in Shandong

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province [1]. Some scientists believed that the high mortality of *C. farreri* resulted from high density culture and other environmental stresses [2,3]. Wu et al. also reported that large-scale mortality of *C. farreri* was caused by severe hypoxia as a result of eutrophication [4].

It is well known that oxygen is indispensable for all aerobic organisms [5]. Unlike the open sea, where environmental conditions are typically stable, DO in coastal areas often markedly fluctuates due to photosynthesis and respiration. Hypoxic events may be seasonal or diel [5]. Seasonal hypoxia often develops as stratified water prevents the oxygenated surface water from mixing downward. Low DO then occurs in the lower waters where respiration and sediment deplete oxygen faster than it can be replenished. Diel cycles of hypoxia often appear in unstratified shallow habitats where nighttime respiration can temporarily deplete DO. Hypoxia may also appear in the upper water of eutrophic water bodies on calm and cloudy days, when more oxygen is consumed than is produced by photosynthesis and when atmospheric aeration is limited. In China, the scallops are usually reared in net-cages. The foulings on the surface of the net-cages can seriously reduce the free exchange of water inside and outside of the net-cages [2]. Therefore, the DO concentration in net-cages and the effects of DO on the physiological and immunological responses of scallop are of primary concern. If oxygen insufficiency lasts out, death will ultimately occur, although some aerobic animals also possess anaerobic metabolic pathways, which can delay lethality for short time periods (minutes to days) [6].

Some studies showed that low DO affected the behavior of Baltic clam *Macoma balthica* [7], haemolymph osmotic pressure of abalone *Haliotis diversicolor supertexta* [8], oxygen consumption of greenlip abalone *Haliotis laevigata* [9], metabolism of oyster *Crassostrea virginica* [10] and periwinkle *Littorina littorea* [11], survival and growth of oyster *C. virginica* [12,13]. The bivalve mollusc *Scapharca inaequivalvis* depressed oxygen consumption rate at low oxygen tensions [14]. The growth of juvenile greenlip abalone *H. laevigata* declined because of low oxygen concentration [9]. Cumulative mortality of the abalone *H. diversicolor supertexta* at 2.05 and 3.57 mg l^{-1} DO was significantly higher than that at 7.70 mg l^{-1} DO [15]. DO also affects immune responses of molluscs [15] and crustaceans [16–18]. When *H. diversicolor supertexta* was exposed to low DO for 24 h, the total haemocyte count, phenoloxidase activity, respiratory burst, phagocytosis, and clearance efficiency significantly decreased [15]. Santovito also reported that low DO decreased the activity of SOD in digestive gland of the Mediterranean mussel *Mytilus galloprovincialis* [19].

No information from experimental studies was available on the effects of hypoxia on the survival, growth and immunity of *C. farreri*. However, scallops reared in net-cages often encounter the problems of low DO. Therefore, this present study is aimed to examine the survival, growth and immune responses of *C. farreri* under graded levels of DO. Haemocytes play pivotal roles in internal defence in molluscs that lack acquired immunity [20–23]. Many humoral factors also take part in immune defence, of which, SOD, ACP and ALP have been reported to correlate well with immune competence of molluscs [24–27]. As a free radical elimination enzyme, SOD is essential to minimise the oxidative damage to host cells in the immune defence [28,29]. The decreasing SOD activity shows a reduced immune ability in tiger shrimp *Penaeus monodon* exposed to lower salinity or higher salinity [30], or in freshwater giant prawn *Macrobrachium rosenbergii* injured with dopamine [31]. When challenged with β-glucan [29] or zymosan [28], white shrimp *Litopenaeus vannamei* and Chinese prawn *Fenneropenaeus chinensis* showed increasing SOD activity. ACP is a typical lysosomal enzyme, and is involve in killing and digesting microbial pathogens during immune responses [24]. When it encountered pathogens, the freshwater snail *Biomphalaria glabrata* showed an elevated ACP levels in serum [24]. ALP is a polyfunctional enzyme, and also takes part in immune defence [32]. In *C. farreri*, some studies indicate that SOD, ACP and ALP are closely relevant to immune competence [27,33–37]. So the THC, SOD activity, ACP activity and ALP activity were examined to monitor the immune responses of this scallop under graded DO levels.

2. Methods and materials

2.1. Rearing animals

The *C. farreri* (initial mean shell height 5.12 ± 0.09 cm, initial mean dry weight of soft tissue 1.52 ± 0.10 g) were obtained from a single spawn at the hatchery of Taipingjiao Seashore Laboratory of Ocean University of China and acclimated in flow-through tanks for 2 weeks before the experiment. During this period, DO in seawater in the tanks was recorded in the range of 8.0 to 9.0 mg l^{-1}. Scallops were fed with *S. maxima* with a concentration of 5.0×10^4 cells ml^{-1} seawater during the acclimation and experimental periods.

2.2. Experimental design

Four DO levels of 8.5, 6.5, 4.5, and 2.5 mg l^{-1} were designed. The experimental system was comprised of hermetic service tanks and test tanks. The service tanks were placed at a higher place and the volume were 1000 l each. The test tanks were placed at a lower place and the volume was 60 l each, in which 35 scallops were stocked. Each treatment had three replicates. Graded DO levels were obtained by adjusting the ratio of pure nitrogen and oxygen bubbling into the seawater of service tanks. DO levels were constantly monitored with a DO meter (YSI 556 MPS, USA), and adjusted whenever necessary. Seawater with certain DO level flowed from the service tanks to the test tanks through hermetic pipes at a rate of 20 l h^{-1}. The mean DO levels of the treatments were measured as 8.75 ± 0.04 mg l^{-1}, 6.48 ± 0.03 mg l^{-1}, 4.57 ± 0.04 mg l^{-1} and 2.66 ± 0.04 mg l^{-1}, respectively.

During the experimental period, temperature, salinity and pH were measured twice a day, and ammonium-N (indophenol blue spectropotometric method [38] and nitrite-N (diazotisation method [39]) were measured once every 2 days. Water temperature ranged from 15.2 °C to 17.5 °C, salinity from 29.5‰ to 32.5‰ and pH from 7.5 to 8.2. Ammonium-N and nitrite-N remained negligible levels.

2.3. Haemolymph collection

To collect haemolymph, four scallops from each tank at each sampling were randomly sampled at the designed intervals of 12 h, 24 h, Day 7, Day 14 and Day 21, and rinsed with filtered seawater (0.45 μm). Haemolymph was withdrawn from the pericardial chamber using a 1 ml syringe and 22-gauge needle [40]. Subsequently, the haemolymph was centrifuged at 2000 *g* for 10 min at 4 °C with a TGL-16 G centrifuge. The supernatant was separated and stored at −70 °C for examining the activities of SOD, ACP and ALP later. The scallops were not released back to the tanks after sampling of haemolymph. Four scallops from each tank at each sampling were used for measuring the activities of SOD, ACP and ALP.

2.4. Indices examination

Specific growth rate of the dried weight of soft tissue of the scallops was measured at the end of this experiment. The soft tissue dissected from 8 scallops was dried at 105 °C to constant weight. SGR was calculated by the following formula:

$$\text{SGR}\left(\%\text{d}^{-1}\right) = \{\text{Ln}\,(\text{final weight}) - \text{Ln}\,(\text{initial weight})\}/\text{days} \times 100$$

Total haemocyte count (THC) was measured at the end of this experiment. Haemolymph was collected as mentioned above. To avoid rapid aggregation and morphological changes, haemocytes for cell counting were added with 2.5% glutaraldehyde (in 0.1 M PBS, pH 7.2) and quickly counted with an optical microscope. Five scallops from each tank were used for measuring THC.

Superoxide dismutase (EC 1.15.1.1) activity was measured by observing the inhibition of ferricytochrome C reduction at 550 nm [41]. Aliquots of serum were added to a solution of 50 mM potassium phosphate buffer (pH 7.8), 50 μM ferricytochrome C, and 15 mM xanthine. Then xanthine oxidase (0.2 U ml^{-1}) was added to initiate the reaction whilst the decrease in absorbance was recorded for 5 min. SOD activity is reported as units per mg serum protein.

Alkaline phosphatase (EC 3.1.3.1) activity was determined with spectrophotometry using *p*-nitrophenyl phosphate (Sigma) as the substrate [42]. The reaction mixture containing 0.2 ml serum, 1.8 ml of 0.1 M carbonate (Na_2CO_3/$NaHCO_3$) buffer and 0.2 ml of 20 mM substrate was incubated at 37 °C for 30 min. Then 1 ml of 1 M sodium hydroxide was added to stop the reaction. The hydrolytic product, yellow *p*-nitrophenyl, was measured at 405 nm by an UV 751 GD spectrophotometer. One unit of enzyme specific activity was defined as the amount of enzyme being able to release 1 μM *p*-nitrophenol per minute per mg serum protein under the assay conditions.

Acid phosphatase (EC 3.1.3.2) activity in serum was determined with spectrophotometric method [43]. A tube containing 0.2 ml serum, 0.5 ml 0.1 M sodium citrate buffer (pH 4.8) and 0.2 ml 10 mM *p*-nitrophenol phosphate (Sigma) was incubated in a 37 °C water bath for 30 min. Then 0.5 ml of 1 M NaOH was added to stop the reaction. The hydrolytic product was measured at 405 nm using an UV 751 GD spectrophotometer. One unit of enzyme specific

activity was defined as the amount of enzyme being able to release 1 μM *p*-nitrophenol per minute per mg serum protein under the assay conditions.

Serum protein concentrations were measured with the Foline phenol reagent using bovine serum albumin as the standard [44].

2.5. Statistical analysis

Data (Mean ± S.E.M.) from each treatment were subjected to one-way ANOVA using SPSS 11.5 for windows. When overall differences were significant ($P < 0.05$), Tukey's multiple range test was used to compare the mean values between individual treatments.

3. Results

DO significantly affected the survival of *C. farreri* ($P < 0.01$, Fig. 1). At the end of the experiment, survival rates at 8.5, 6.5, 4.5 and 2.5 mg l^{-1} DO were (100.0 ± 0.0) %, (100.0 ± 0.0) %, (88.3 ± 6.0) % and (81.7 ± 6.7) %, respectively. The survival at 2.5 mg l^{-1} DO was significantly lower than those at 8.5 and 6.5 mg l^{-1} DO ($P < 0.01$).

The SGR was significantly affected by DO ($P < 0.05$, Fig. 2). The scallops reared at higher DO (8.5 and 6.5 mg l^{-1}) had a significantly higher SGR than that at lower DO (2.5 mg l^{-1}, $P < 0.05$).

Low DO significantly reduced the THC of scallops ($P < 0.05$). The THC (1.22×10^7 ml^{-1}) of *C. farreri* at the highest DO level (8.5 mg l^{-1}) was significantly higher than that (6.59×10^6 ml^{-1}) at the hypoxia level (2.5 mg l^{-1}). The THC of scallops exposed to 2.5 mg l^{-1} DO decreased by 46% compared with that at 8.5 mg l^{-1} DO (Fig. 3).

The effect of DO on the SOD activity in scallop serum is shown in Fig. 4. The SOD activities at 8.5 and 6.5 mg l^{-1} DO showed no significant differences during the experimental period. While the SOD activities at 4.5 and 2.5 mg l^{-1} DO at 12 h was significantly higher than that at 8.5 mg l^{-1} DO ($P < 0.01$), which suggested a rapid elevation after exposure to lower DO. After the initial elevation, the SOD activities at low DO decreased rapidly, especially at 2.5 mg l^{-1} DO ($P < 0.01$). The SOD activities at Day 7, Day 14 and Day 21 at 2.5 mg l^{-1} DO were significantly lower than those at 8.5 and 6.5 mg l^{-1} DO. The SOD activity at 4.5 mg l^{-1} DO only significantly decreased at Day 21. No differences were found among different DO levels at 24 h.

The effect of DO on the ACP activity in the serum of *C. farreri* is shown in Fig. 5. The ACP activities at 8.5 and 6.5 mg l^{-1} DO had no significant differences during the experimental period. The ACP activities at 4.5 and 2.5 mg l^{-1} DO did not change significantly before Day 7 ($P < 0.01$) while they began to decrease at Day 14. The ACP activities at 2.5 mg l^{-1} DO at Day 14 and Day 21 were significantly lower than those at 8.5 mg l^{-1} DO ($P < 0.01$). However, the ACP activity at 4.5 mg l^{-1} DO only significantly decreased at Day 21. The activities at 2.5 mg l^{-1} DO from 12 h to Day 7 have a gradual elevation while having no significant difference ($P > 0.01$).

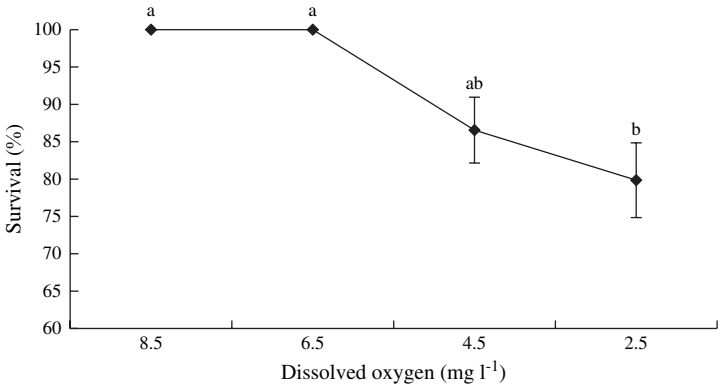

Fig. 1. Survival of the scallop, *Chlamys farreri,* reared under graded DO levels for 21 days. Data with different letters were significantly different ($P < 0.05$), (mean ± S.E.M., $n = 3$).

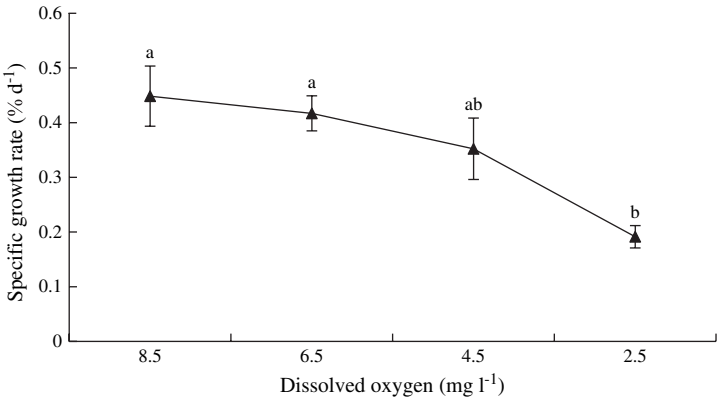

Fig. 2. Specific growth rate of the scallop, *Chlamys farreri*, reared under graded DO levels for 21 days. Data with different letters were significantly different ($P < 0.05$), (mean ± S.E.M., $n = 3$).

The ALP activities of all treatments at all sampling times had no significant difference except for that at 2.5 mg l^{-1} DO it was significantly higher at 12 h after being exposed to the low DO environment (Fig. 6).

4. Discussion

In the present study, long-term hypoxia (<4.5 mg l^{-1} DO) caused death of scallops. Similar results were observed in some other bivalves [14,15]. To determine the resistance of benthic organisms to oxygen deficiency, de Zwaan et al. [14] performed tests on a number of bivalves, *Chamelea gallina*, *Tapes philippinarum*, *Mytilus galloprovincialis* and *Schapharca inaequivalvis*. The 50% survival time of the individuals demonstrated that the most resistant animal was *S. inaequivalvis* with 19 d, followed in decreasing order by *M. galloprovincialis* with 16 d, *T. philippinarum* with 12 d and finally *C. gallina* with 6 d. The bivalves could delay lethality for short time periods at low DO while they are vulnerable to oxygen deficiencies for a long period. From our knowledge, no study reports the optimum DO level of *C. farreri*. According to this experiment, this scallop should not be chronically cultured in the seawater under 4.5 mg l^{-1} DO.

In this present study, hypoxia caused a remarkable growth decrease of *C. farreri*. Oxygen availability is one of the most limiting growth factors and chronic hypoxia may be an important environmental stressor influencing the growth of mollusc [9,10,13]. In the present study, the specific growth rate of the scallop started to decline at 4.5 mg l^{-1} DO and significantly decreased at 2.5 mg l^{-1} DO, hence, DO level below 4.5 mg l^{-1} could be considered as a hypoxia status for this animal.

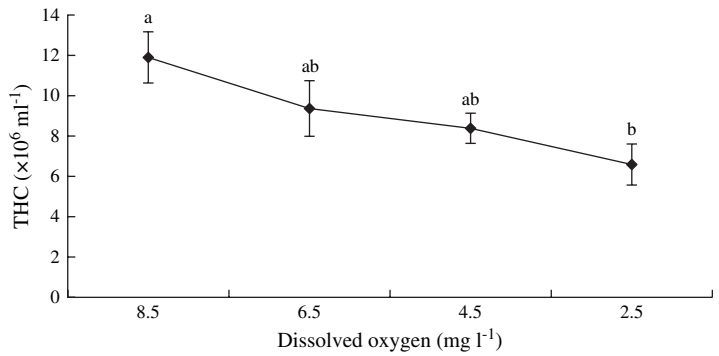

Fig. 3. Total haemocyte count of the scallop, *Chlamys farreri*, reared under graded DO levels for 21 days. Data with different letters were significantly different ($P < 0.05$), (mean ± S.E.M., $n = 3$).

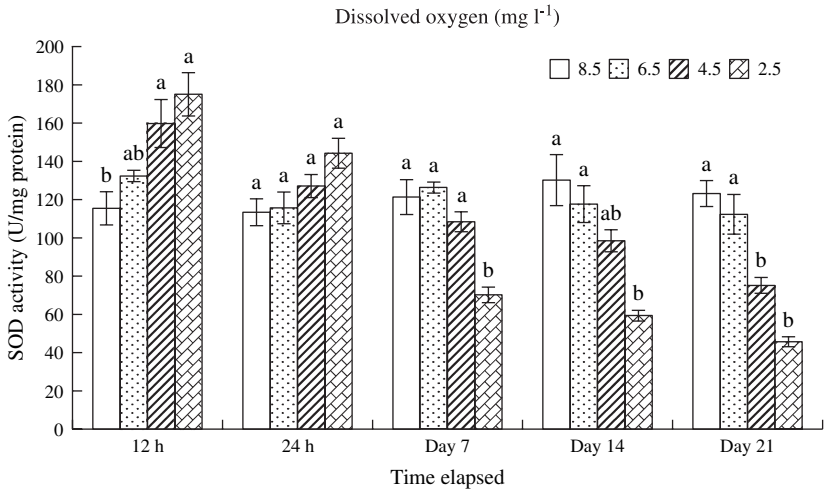

Fig. 4. Superoxide dismutase activity (mean ± S.E.M., $n = 3$) in the serum of *Chlamys farreri* exposed to different DO levels at different sampling times (12 h, 24 h, Day 7, Day 14 and Day 21). Data with different letters are significantly different ($P < 0.05$).

Haemocytes are important to the immune responses of bivalve, and are involved in the inflammatory response, wound recovery, the respiratory burst, encapsulation and phagocytosis [45]. Phagocytosis is considered an important way to control and eliminate invaders. It is well known that the life cycle, food supply, diseases, pollutants and other environmental stress affect the circulating haemocyte count both in quantity and quality [9,43]. In addition, the circulating haemocytes and non-circulating haemocytes exist in a state of dynamic flux [20]. The number of circulating haemocytes is affected by the migration from the tissues and proliferation of the cells [20,44]. DO has been reported to affect THC in abalone [15] and shrimp [16,46]. Following 24 h exposure to 2.05 mg l^{-1} DO, the abalone *H. diversicolor supertexta* showed a decrease in THC by 27% [15]. *Litopenaeus vannamei* exposed to hypoxia at 30 torr for 48 h decreased its THC by 40.4% [46]. In this study, the THC of the scallops exposed to 2.5 mg l^{-1} DO significantly decreased by 46%. Low THC resulted in immune depression [47]. Further research is needed to clarify whether the decrease in THC results from reduced proliferation of haemocytes or movement of cells from circulation into tissues [15,25,44,48].

The catalytic function of SOD was discovered by McCord and Fridovich [49]. This enzyme virtually exists in all O_2-respiring organisms, and its major function is to scavenge superoxide anion. SOD has been known as the important

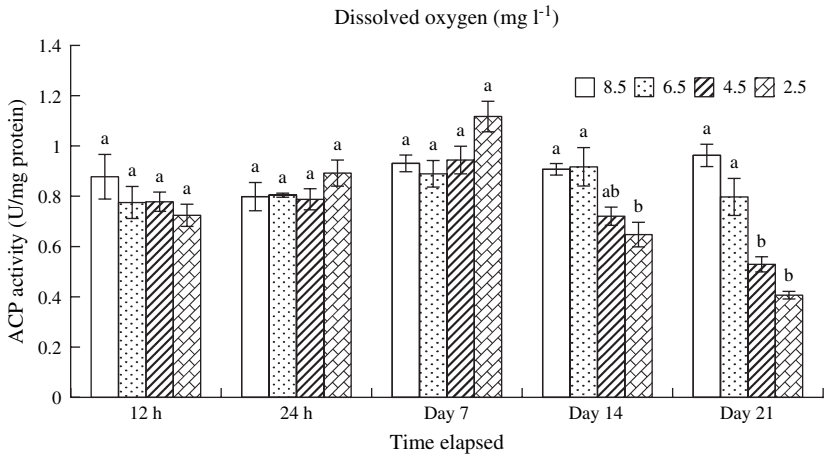

Fig. 5. Acid phosphatase activity (mean ± S.E.M., $n = 3$) in the serum of *Chlamys farreri* exposed to different DO levels at different sampling times (12 h, 24 h, Day 7, Day 14 and Day 21). Data with different letters are significantly different ($P < 0.05$).

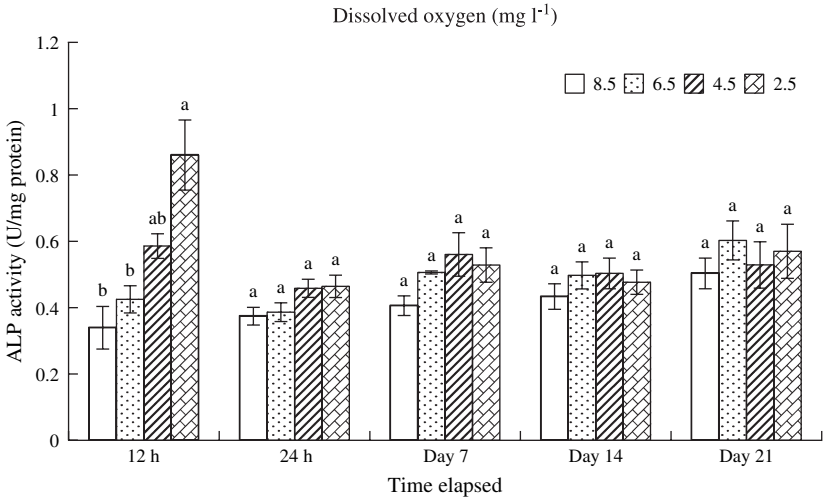

Fig. 6. Alkaline phosphatase activity (mean ± S.E.M., $n = 3$) in the serum of *Chlamys farreri* exposed to different DO levels at different sampling times (12 h, 24 h, Day 7, Day 14 and Day 21). Data with different letters are significantly different ($P < 0.05$).

immune factor in aquatic animal [25,26,28–31]. In *C. farreri*, the studies also indicate that SOD activity can reflect the immunological state [33,34,37].

As a free radical elimination enzyme, superoxide dismutase protects the cellular components from being damaged by reactive oxygen intermediates (ROIs) [50]. ROIs are produced by haemocytes during phagocytosis, and they are important to kill the invaders [51]. However, excessive ROIs are harmful to the host cells, and can cause cellular injury and death. Therefore, SOD is essential to minimise the oxidative damage to host cells during immune defence. The activity of SOD varies widely with many factors such as seasonality and reproductive cycle, age, tissue type and environmental stress [52]. The present study found a rapid elevation of SOD activities shortly after the exposure to low DO. Similar results were reported in fish [41]. Elevated activity of SOD is an indicator of oxidative stress. When oxygen concentrations decline to hypoxic levels, there is an increase in the production of ROIs [53]. And elevated SOD activity is needed to depress the damage from ROIs [19]. However, in this present study, SOD activity at Day 21 at low DO decreased to a significantly lower level. It indicates that a long period of hypoxia damaged the ability of response to ROIs, which caused the oxidative damage subsequently. The decreased THC also indicates the damage from the oxidative stress. The lower SOD activity at lower DO was not beneficial to depressing the oxidative damage to haemocytes and caused lower THC subsequently. The high levels of SOD at early stage at low DO really represent an adaptation to the oxygen stress [54], while decreased SOD activity at a later stage would render the organisms more susceptible to the oxidative stress related to their environment. Also, lower SOD activity suggests a poor physiological and immunological state of the scallops after a long period of hypoxia.

Besides THC, lysosomal response was considered as the most reliable effect observed in mussels in stress [55]. Lysosomal responses are widely accepted as cellular biomarkers of general stress. Acid phosphatase (ACP) is a typical lysosomal enzyme, and involves in killing and digesting microbial pathogens during immune responses [56]. ACP exists in the haemocytes and serum of bivalve [35,57,58]. In *C. farreri*, ACP activity in serum of *C. farreri* was 1.7 times as high as that in haemocytes [35]. Zhang et al. [34] has reported that ACP and ALP in serum and haemocytes of *C. farreri* were more important than other enzymes in immune defence. Previous studies show that the ACP activity in serum can reflect the immune state of this scallop [27,33,34]. Lower ACP activity was detected in this scallop in disease or stress state than in healthy state [33,34]. In this experiment, the trend of ACP activity at low DO was similar to that of SOD activity. The difference was that the SOD activity was significantly elevated at 12 h while ACP activity only has a slight elevation. Some studies show that environmental factors and other xenobiotics can cause structural and physiological changes such as lysosomal fragility and subsequent release of ACP [24,25,59,60]. In this study, the slightly elevated ACP activity in serum at low DO before Day 7 probably resulted from a reduction of lysosomal membrane stability [59,60]. However, the activities of this enzyme at low DO at Day 14 and 21 significantly decreased. A similar result was reported in clam, *Chamelea gallina* [25]. The observed decrease in ACP activity

under hypoxic stress is probably the result of several events, e.g. metabolic arrest due to anaerobiosis and alteration of the lysosomal integrity caused by hypoxic stress [11,59,60]. And the remarkable reduction in the number of circulating haemocytes also results in the decreased ACP activity [25]. The decreased ACP activity at low DO is deleterious for the animal to survive in a bad environment. Low ACP activity showed the reduction of immune ability of the scallop exposed in low DO for a long period.

Alkaline phosphatase (ALP) is a polyfunctional enzyme that hydrolyses a broad class of phosphomoester substrates and acts as a transphosphorylase at alkaline pH [61]. ALP also acts as an early marker of cell differentiation in the osteogenic lineage in bivalve mollusc *Pinctada maxima* [62]. ALP activity has been reported sensitive to heavy metals pollutants [63] and temperature [27]. There is no available information about changes of ALP activity under hypoxic stress. In this study, the ALP activities in serum of *C. farreri* exposed to low DO increased at 12 h, and decreased to normal level after 24 h, and then no further significant change was detected. Xing et al. [32] has reported that high ALP activity in this scallop indicated a strong immuno-competent ability. In this study, high activity at an early stage suggested a strong immune response when the scallops were exposed into low DO. However, the activity recovered to normal level after a long period of hypoxic stress. The result of this study showed that ALP activity was not sensitive to chronic hypoxia. Therefore, the ALP activity is not a suitable indicator of the immunity of *C. farreri* under chronic hypoxia except for the initial phase.

In this study, three enzymes were measured at different times during chronic hypoxia. All three enzymes increased at the beginning under low DO, which showed that low DO caused scallops in stress and induced the increasing activities of these enzymes. After initial elevation, the ALP activities decreased to normal level. However, SOD and ACP activities reached significantly lower level after a long period of hypoxia stress. The descended SOD and ACP activities efficaciously reflected the bad physiological state and poor immune ability of scallops under low DO stress, which were well consistent with survival.

In this present study, survival, SGR, as well as some indices reflecting the physiological and immune ability, namely, THC, SOD, ACP, ALP, were determined. It could be concluded that the stress of low DO decreased the survival of scallop *C. farreri*. The low level of THC, SOD and ACP at the end of the experiment revealed that low DO depressed the immune ability of *C. farreri*. The THC, SOD and ACP were sensitive indices of reflecting immune ability under the stress of DO.

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