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Effect of Long-term Temperature Or Salinity Stress on the Activities of Immune-related Enzymes in Crassostrea Hongkongensis

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Abstract

High water temperature and high salinity are the main factors that cause the mass mortality of oyster Crassostrea hongkongensis. This study aimed to investigate the range of water temperature and salinity that produced stress effect on C. hongkongensis and assess the effects of long-term high water temperature and high salinity stress on the activities of superoxide dismutase (SOD), catalase (CAT), acid phosphatase (ACP), alkaline phosphatase (AKP) and lysozyme (LZM) in the serum of oyster. The results showed that 17-28℃ was the suitable temperature for oysters survival, and 30℃ had a stress effect on oyster, resulting in a significant increase in oyster mortality. The salinity at 18-24‰ was suitable for oysters survival, and it below 14‰ or above 26‰ had a stress effect on oysters, leading to a significant increase in oyster mortality. When oysters were stimulated with high water temperature (30 or 32℃), the activities of five serum enzymes were increased significantly on day 4. And then they were decreased significantly with the increasing of exposure time, for example, the activity of LZM was decreased significantly on day 14 and 21 ($p < 0.05$), and the activities of ACP and AKP were decreased significantly on day 21 ($p < 0.05$). High salinity (30‰) and low salinity (15‰) stress significantly enhanced ACP and AKP enzyme activities, but high salinity stress significantly decreased SOD and LZM activities ($p < 0.05$). For example, from day 4 to 17, SOD and LZM activities in 30‰ salinity group were significantly lower than those in other salinity groups. The present results indicated that long-term high salinity or high temperature obviously decreased the activities of immune-related enzymes in serum, which reduced the resistance of oyster to stress and resulted in the death of oyster.

Keywords

Crassostrea Hongkongensis; Salinity; Temperature; Stress; Immune-related Enzymes.

1. Introduction

Crassostrea hongkongensis, commonly known as "white meat", is the main cultured species in the coastal areas of Guangdong, Guangxi and Hainan Province, China. According to the China Fisheris Statistic Yearbook, 2021, the total output of oysters reached 5.42 million tons in 2020 with an increase of 3.8% over 2019 [1]. With the increasement of oyster farming density, mass mortality events of oysters frequently occurred, such as Alectryonella plicatula in Rushan Bay

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from August to september 1995 [2], C. gigas larvae during May to September 2008 in French [3] and C. hongkongensis during 2017 to 2019 in Qinzhou port, Guangxi province, China [4]. The high temperature and high salinity may be the important factors causing the mass death of C. hongkongensis.

Temperature and salinity are the important environmental factors that affect the growth and survival of shellfish. Previous studies mainly focus on the effects of temperature and salinity on the embryonic development and larval growth [5-7], feeding and metabolism [8-11], as well as immune-related gene expression and immune-related enzymes activity [12-15] of shellfish. Temperature directly affects the physiological metabolism [16] and immune function [17], and salinity is closely related to the regulation of shellfish osmotic pressure, and its changes can also cause a series of physiological responses of shellfish [18,19]. For example, high temperature and low salinity had a serious impact on shellfish immunity [12]; high temperature reduced the rate of feeding, the avtivity of metabolic enzymes and immune enzymes of Mytilus coruscus [14]; high salinity stress increased the activity of protein kinase and total antioxidant capacity of C. hongkongensis [20]. In addition, short-term (72 h) salinity stress significantly enhanced the immune-related enzymes activity such as SOD, CAT, ACP, AKP, LZM in the hemolymph and hepatopancreas of C. hongkongensis [21, 22]. On the whole, existing studies mainly focused on the effects of short-term temperature and salinity stress on oysters. However, the mass mortality of oyster was usually caused by long-term (more than 20 days) high temperature and high salinity stress. Therefore, it is necessary to investigate the effects of long-term temperature and salinity stress on the survival of oyster and study the immune-related enzymes activity of oyster.

2. Materials and Methods

2.1. Experimental Materials

A total of 1875 oysters Crassostrea hongkongensis at 2 years old with a shell height of 7-10 cm were purchased from Hantang Aquatic company, Jiangmen City, Guangdong Province, China. All oysters were placed in several 100-L tanks after removing the surface attachments and raised in the Aquatic Animal Breeding Health Room of Jinan University for 7 days. During the domestication period, the salinity of 50 L artificial seawater was adjusted to 20‰ and the water temperature was maintained at 17°C, all water was replaced with new water every 3 days, and the oxygen in water was supplied with air pumps. Moreover, the oysters was fed with seawater chlorella twice a day, and photoperiod was 12 h:12 h. The oysters were checked daily for survival and dead oysters were removed.

2.2. Experimental Design and Methods

2.2.1. Effects of Temperature Stress on the Survival of C. Hongkongensis

Six temperature groups including 17, 20, 25, 26, 28, and 30°C were conducted in the experiment. Each group contained 75 oysters in a 100-L tank with 50 L artificial seawater and had three replications. The seawater salinity was 20‰, and the daily management methods were same as that in 2.1. The dead oysters were counted and removed every day. The temperature was maintained by heating rods. The cumulative mortality of C. hongkongensis in each group was counted on day 7.

2.2.2. Effects of Salinity Stress on the Survival of C. Hongkongensis

Ten salinity groups including 6, 10, 14, 18, 20, 22, 24, 26, 28 and 30‰ were conducted in thirty 100-L tanks containing 50 L artificial seawater. Each salinity group had 3 replications with 75 oysters in each tank. The seawater temperature was mantained at 17°C and other management methods were same as that in 2.1.

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2.2.3. Effects of Temperature on the Activities of Immune-related Enzymes

Five temperature treatment groups including 17, 20, 25, 30, and 32°C were conducted in fifteen 100-L tanks containing 50 L artificial seawater. Each group had triplication and each replication contained 75 oysters. The seawater salinity was 20‰, and the daily management method was same as 2.1.

Five oysters were randomly selected from each tank on day 0, 4, 7, 14, 21, 28, and 35 days. The sampled oysters were cut a small opening at the left and right shell seams close to the oyster adductor muscle. Then about 2 mL of blood sample was collected by a 2-mL injection syringe from the adductor muscle. The blood was placed into a pre-cooling centrifuge and centrifuged at 1600 g for 5 min at 4°C. The supernatant was collected and stored at -80°C refrigerator prior to use. The activities of SOD, CAT, ACP, AKP and LZM were detected using enzyme activity kits (Nanjing Jiancheng Institute of Biology) according to the manufacturer's instructions.

2.2.4. Effects of Salinity on the Activities of Immune-related Enzymes

Four salinity groups including 15, 20, 25, and 30‰ were conducted in twelve 100-L tanks containing 50 L artificial seawater. Each group had triplication and each replication contained 75 oysters. The seawater temperature was maintained at 17°C, and the daily management method was same as 2.1.

Five oysters were randomly selected from each tank on day 0, 4, 7, 14, 21, 28, and 35 days. The blood samples and the enzyme activities were collected and determined according to 2.2.3.

2.3. Statistical Analysis

All analyses were performed in SPSS 19.0 (IBM) and all pictures were drawn with Graph Pad Prism 8.02 (GraphPad Software). The differences among groups were analyzed with one-way ANOVA. Multiple comparisons were analyzed with Student-Newman-Keul's (SNK) test when ANOVA showed significant differences. The results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of Temperature and Salinity Stress on the Survival of C. Hongkongensis

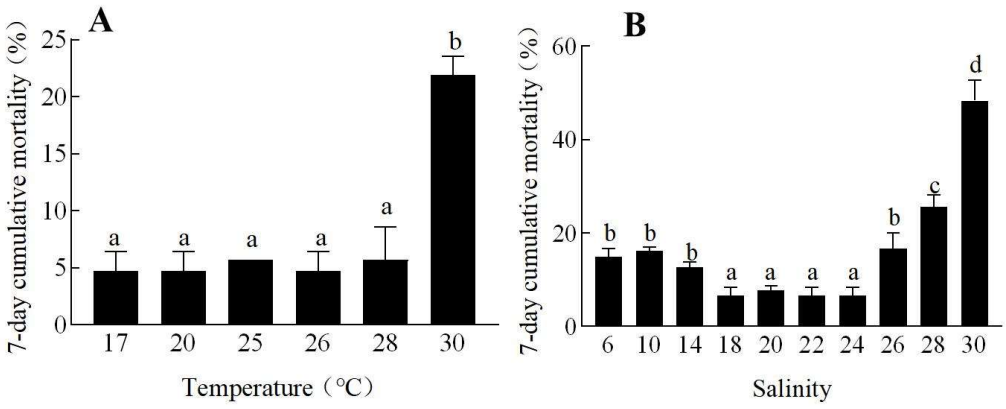

Figure 1. Effect of temperature or salinity on the mortality of Crassostrea hongkongensis

On day 7, the cumulative mortalities of oysters in 17, 20, 25, 26, 28, and 30°C groups were 4.76%, 4.76%, 5.71%, 4.76%, 5.71%, and 21.90%, respectively (Fig 1-A). There was no significant difference in mortality of oysters among 17°C to 28℃ treatment groups ($p > 0.05$), while the mortality of oysters in the 30°C treatment group was significantly higher than that in

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the other groups ($p < 0.05$). These results indicated that 17-28°C was the suitable temperature for survival, and 30°C had a significant stress on the oysters.

As shown in Figure 1-B, there was no significant difference in cumulative mortality among 18‰ to 24‰ salinity groups ($p > 0.05$). However, the mortality of oysters was increased significantly ($p < 0.05$) when the salinity below 14‰ or above 26‰. When the salinity was 30‰, the mortality of oysters reached 48.3%. Thus, the suitable survival salinity of C. hongkongensis was 18-24‰, and the salinity below 14‰ or above 26‰ had a significant stress effect on the adult oysters.

3.2. Effects of Temperature on the Activities of Five Immune-related Enzymes in Serum of Oysters

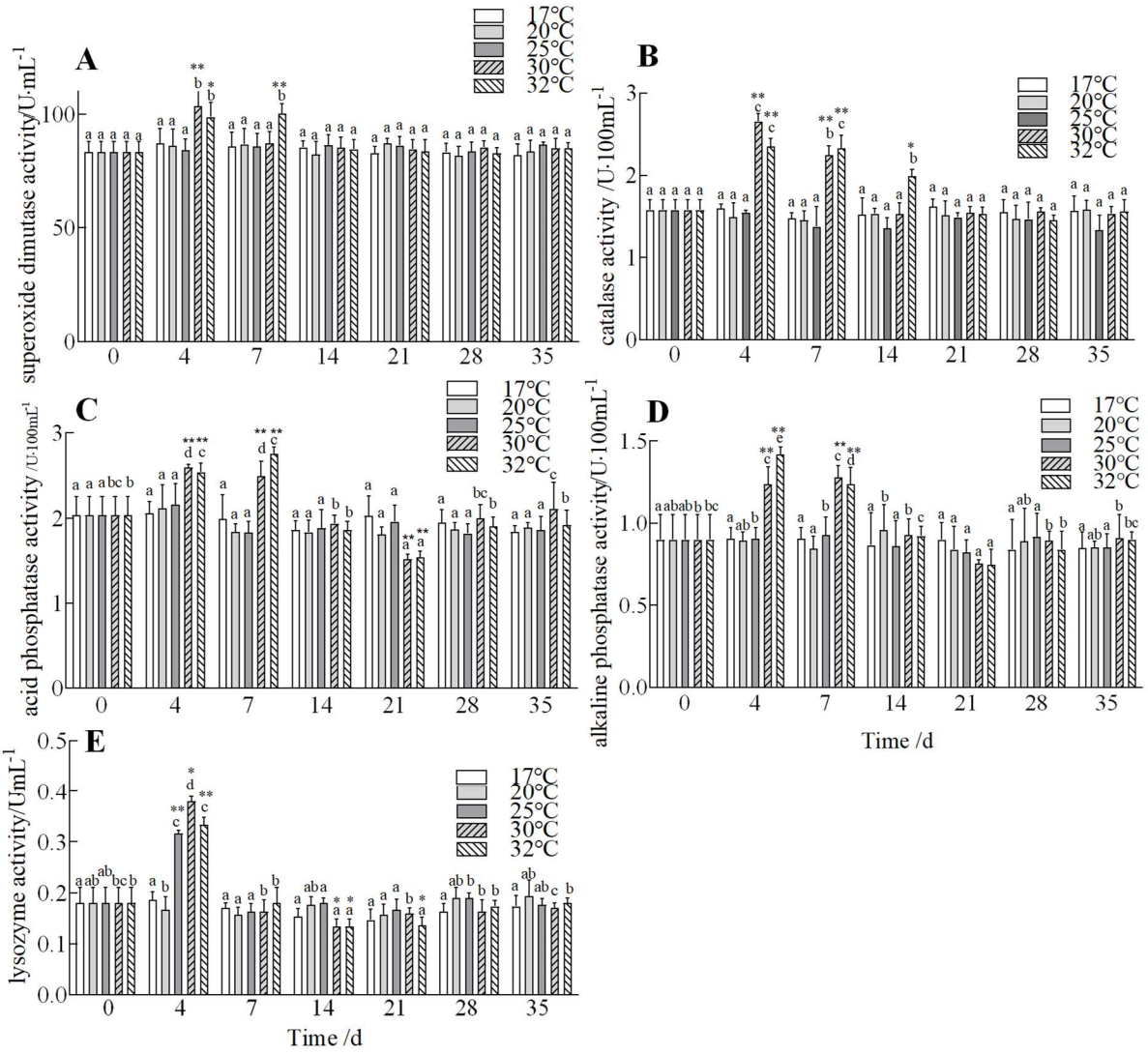

Figure 2. The changes of five immune-related enzymes activities in the serum of C. hongkongensis in response to temperature stress. In the same temperature treatment group, bars with different letters mean significant difference ($p < 0.05$).

Asterisks indicate a significant difference (*$p < 0.05$, **$p < 0.01$) between the experimental group and control group.

The change of SOD activity treated with different temperature was shown in Figure 2-A. The SOD acticity of oysters in 17, 20 and 25℃ groups showed no significant change during 35 days ($p > 0.05$). It in the 30 and 32℃ groups was increased significantly on day 4 ($p < 0.05$), then it

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was decreased on day 7 and 14, respectively, and showed no significant difference compared with other groups on day 21, 28, and 35 ($p > 0.05$). This indicated that high temperature stress increased SOD activity temporarily.

As shown in Figure 2-B, the CAT activity in the 17, 20, and 25°C groups also showed no significant change during 35 days ($p > 0.05$). While it in the 30 and 32°C groups were increased significantly on day 4 and 7 ($p < 0.01$), then it was decreased on day 14 and 21, respectively, and showed no significant difference compared with other groups on day 28 and 35 ($p > 0.05$). This indicated that high temperature stress increased CAT activity temporarily.

The change of ACP activity level was shown in Fig.2-C. The ACP activity in the 17, 20 and 25°C groups showed no statistical differences among all time point ($p > 0.05$). The ACP activity in the 30 and 32°C groups was increased significantly on day 4 and 7 ($p < 0.01$), then it was decreased and significantly lower than other groups on day 21 ($p < 0.05$). This indicated that high temperature stress could decreased ACP activity finally followed a transient increased period.

The AKP activity in the 17, 20 and 25℃ groups had no significant difference among all time points ($p > 0.05$) (Figure 2-D). The AKP activity in the 30 and 32°C groups were enhanced significantly on day 4 and 7, while it was decreased from day 14 to day 35 and significantly lower than the other groups. This result demonstrated that high temperature stress could decreased AKP activity followed a transient increased period.

There was no significant difference in LZM activity among all time points in the 17 and 20°C groups ($p > 0.05$). However, the LZM activity in the 25, 30 and 32°C treatment groups were significantly higher than other groups on day 4 ($p < 0.01$). The LZM activity in the 30 and 32°C treatment groups were decreased and significantly lower than the other groups on day 14 (Figure 2-E). This indicated that high temperature stress caused a significant decrease in LZM activity.

3.3. Effects of Salinity on the Activities of Five Immune-related Enzymes in Serum of C. Hongkongensis

The SOD activity in different salinity groups was shown in Figure 3-A. There was no significant difference on the SOD activity in 20‰ and 25‰ salinity groups among all time points ($p > 0.05$). The SOD activity in the 15‰ salinity group was increased significantly on day 4 and 7 ($p < 0.05$), while it in the 30‰ salinity group was decreased significantly on day 4, 7, and 14 ($p < 0.05$).

The CAT activity in the four salinity groups showed no significant change among all sampling time points ($p > 0.05$) (Figure 3-B), indicating that salinity stress had no significant effect on the CAT activity in the serum of C. hongkongensis.

As shown in Figure 3-C, the ACP activity in 20‰ salinity group had no significant changes among all sampling time points ($p > 0.05$). ACP activity in 15‰ and 25‰ salinity groups was increased significantly on day 4 and 7, and it in the 30‰ salinity group was increased significantly on day 4, 7, and 14 ($p < 0.01$). This indicated that high-salinity and low-salinity stress caused a transient increase in ACP activity.

The AKP activity in the 15‰, 20‰, and 25‰ salinity groups had no significant changes among all time points ($p > 0.05$) (Figure 3-D). However, the AKP activity of the 30‰ salinity group was increased significantly on day 4 and 7 ($p < 0.01$), indicating high salinity stress only caused a short-term increase in AKP activity.

The LZM activity in the 15‰, 20‰, and 25‰ salinity groups showed no significant changes among all sampling time points ($p > 0.05$, Figure 3-E). The LZM activity in the 30‰ salinity group was decreased significantly on day 4, 7, and 14 ($p < 0.05$). This indicated that high salt stress reduced the LZM activity of C. hongkongensis.

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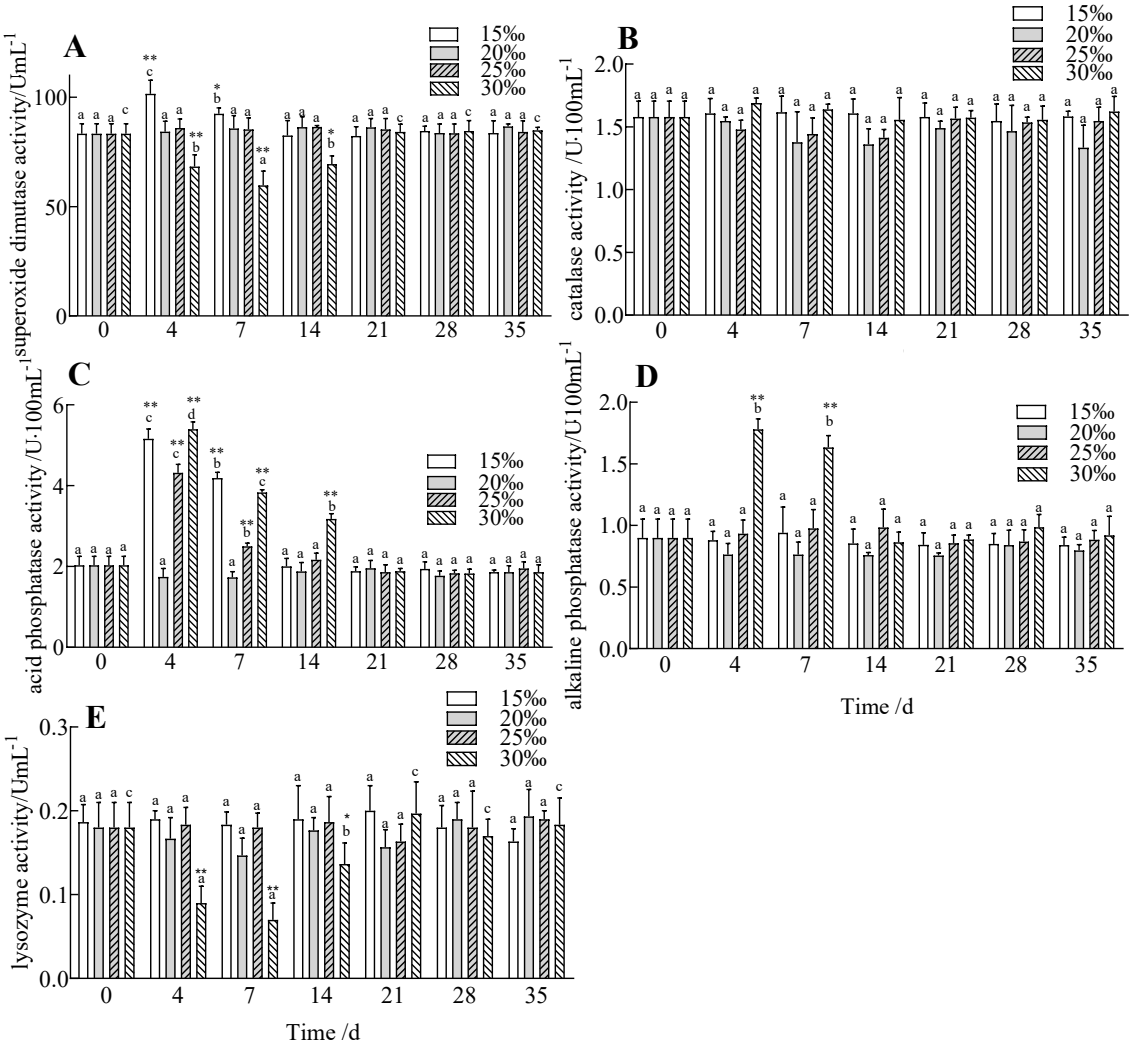

Figure 3. The changes of five immune-related enzymes activities in the serum of C. hongkongensis in response to salinity stress. In the same salinity treatment group, bars with different letters mean significant difference ($p < 0.05$). Asterisks indicate a significant difference (*$p < 0.05$, **$p < 0.01$) between the experimental group and control group.

4. Discussion

Temperature and salinity stress are the primary environmental factors that might cause mortality in farmed shellfish [4, 23]. When shellfish are in bad living temperature or salinity, they will have stress responses in various aspects, such as immune stress, energy metabolism response, and changes in feeding [10, 12, 24]. This study firstly clarified the range of temperature stress and salinity stress of C. hongkongensis, and then explored the effects of salinity or temperature stress on the activity of immune-related enzymes of oysters. SOD and CAT are important antioxidant enzymes in the humoral immunity of oysters, they can convert too many harmful reactive oxygen radicals generated in cells into oxygen molecules and water [25], which achieve the purpose of scavenging oxygen free radicals in the organism. Besides, lysosomal enzymes of shellfish mainly come from blood cells and hemolymph and play a dual role of defense and digestion [26]. Pathological changes of lysosomes can be used as indicators to detect the impact of environmental stress on marine organisms [27], among which ACP, AKP and LZM are three important lysosomal enzymes in shellfish. LZM plays an important role in the non-specific immune system and can destroy peptidoglycan components in bacterial cell wall, disintegrate cells, and induce the synthesis and secretion of other immune factors [28]. ACP is not only an important metabolic regulatory enzyme in shellfish, but also a landmark

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enzyme of lysosome, it can hydrolyze foreign bodies with phosphate esters on the surface in acidic environment and plays an important role in non-specific immune system [28]. AKP is a metal enzyme that regulates membrane transport and calcium and phosphorus metabolism in mollusks, and it aslo closely related to keratin secretion and shell formation [29].

Temperature is one of the main environmental factors affecting the growth and survival of bivalves. Uncomfortable or drastic changes in ambient temperature will have adverse effects on bivalves physiology and biochemistry [30], blood cell and enzyme activities, then reduce the body immunity, affect bivalves growth, development, and energy metabolism [31]. To explore the impact of long-term temperature stress on the survival rate and immune-related enzyme activities of C. hongkongensis, 17-32℃ was conducted as the experimental temperature range, which was mainly considered from the actual water temperature of oyster farming areas in Taishan and Yangjiang City, China. Presents results demonstrated that 17-28℃ was the suitable temperature for oyster survival, and 30℃ had a stress effect on C. hongkongensis. Previous reports indicated that temperature stress range for survial of shellfish might be different among different species, different development stages or different culture environments. For example, the temperature stress range of C. sikamea was below16℃ or above 32℃ [6], and it of young Haliotis discus hannai was below 14℃ or above 28℃ [32]. This study found that temperature stress range of C. hongkongensis was above 30℃. High temperature (30℃ and 32℃) increased the activities of SOD and CAT in the serum of C. hongkongensis on day 4 and 7, suggesting that C. hongkongensis could eliminate excessive reactive oxygen radicals by producing more SOD and CAT to avoid oxidative damage. This phenomenon was similar with other aquaculture animals such as Haliotis discus [33] and Litopenaeus vannamei [34]. However, Shi et al. [35] found that the SOD activity of hybrid abalone was decreased significantly under high temperature stress (30℃), which might be the result of disorder of antioxidant system. The present results found that the activities of ACP, AKP and LZM were increased shortly and then decreased significantly on the 21th day under high water temperature stress (30℃ and 32℃). The results showed that C. hongkongensis might suffer damage after producing a large number of active oxygen free radical to strengthen the function of self defense, but it might still cause a reversible damage to the host immunity. The similar phenomenon was also found in the four horn clam [36] and the Philippine clam [37]. In addition, temperature also affects the survival of shellfish from various aspects, such as feeding metabolism, digestive enzyme activities, energy budget and intestinal microbial diversity [8, 38-39].

Salinity would change osmotic pressure and cause physiological disorders and affect the health of oysters, among which variation of immune-related enzyme activity could be used to evaluate the health status of oysters [40]. In this study, The salinity at 18-24‰ was suitable for the survival of oysters, and it below 14‰ or above 26‰ had a stress effect on oysters, leading to a significant increase in oyster mortality. Yao [41] found that the optimal living salinity of larvae of C. hongkongensis was 10-25‰. The results indicated that the tolerance ability of larvae of C. hongkongensis to low salinity was stronger than that of adult oysters. It may be related to the fact that larvae of C. hongkongensis were born in a low salinity environment. The mortality rate of C. hongkongensis under high salinity stress was significantly higher than that under low salinity stress, which was consistent with the research results of She et al. [20]. The present results showed the SOD activity in the serum of C. hongkongensis was increased significantly on day 4 and 7 under low salinity stress (15‰), while it was decreased significantly from day 4 to day 14 under high salinity stress (30‰). This demonstrated that the antioxidant system and immunity of oysters were damaged under high salinity stress. However, Shi [22] found that the immune capacity of oyster was enhanced in short-term (48 h) under low salinity stress. Although having the similar change trend, the present research mainly focused on investigating the impacts of long-term (35d) salinity stress on immune related enzyme activity. The present

results also indicated that salinity stress had no significant effect on CAT activity of C. hongkongensis, which was inconsistent with the change activity of SOD. It was probably because the different immune-related enzyme had differenct induction sequence when oysters suffered salinity stress[22]. In addition, ACP, AKP and LZM showed different change characteristics under salinity stress. The activities of ACP and AKP were increased significantly first and then recovered under high salt stress, but the recovery time of ACP activity was longer than that of AKP. When water salinity is too high or too low, C. hongkongensis adjust osmotic pressure by consuming more ATP to adapt to external salinity, and inorganic phosphorus required for ATP synthesis can be generated by phosphate ester hydrolysis catalyzed by ACP and AKP [43]. Li [43] found that AKP activity of Litopenaeus vannamei could maintain high activity under higher salinity conditions compared with ACP, indicating that AKP might be less affected by high salinity than ACP, which was similar with the present results that the AKP activity recovery period was shorter than that of ACP. In this study, the LZM activity of C. hongkongensis had no significant change among all time points under low salinity stress (15‰), while it was decreased significantly from day 4 to day 14 with high salinity stress (30‰), suggesting that C. hongkongensis was more tolerant to low salinity than high salinity. High salinity levels could reduce the resistence ability of C. hongkongensis to pathogen infection, making them vulnerable to disease outbreaks. High temperature or high salinity stress could cause a temporary stress increase in the activity of immune-related enzymes such as LZM and SOD, but long-term stress might cause a significant decrease on these enzymes. Furthermore, the damage effect of high salinity on oyster was greater than that of high temperature stress.

This study showed that 17-28℃ was the suitable temperature for survival, and 30℃ or higher had a stress effect on C. hongkongensis, resulting in a significant increase in oyster mortality. The salinity at 18-24‰ was suitable for the survival of oysters, and it below 14‰ or above 26‰ had a stress effect on oysters. Moreover, C. hongkongensis had the characteristics of low salinity tolerance. Therefore, long-term monitoring of salinity and water temperature, especially salinity, is recommended to carry out in oyster farming areas. When the temperature and salinity are not suitable for the oysters survival, change the farming areas in time to reduce the death of oysters.

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