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Hybridization improved stress resistance in the Pacific oyster: Evidence from physiological and immune responses

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ABSTRACT

Recently, mass mortality of cultured Pacific oyster (*Crassostrea gigas*) has occurred frequently, which has aroused our attention to the stress tolerance of oyster. Hybridization is regarded as an effective way to produce genetic improvement in aquaculture, which can effectively introduce improved characteristics to the hybrids. In the present study, we evaluated the physiological and immune responses at different temperatures (16–36 °C) and salinities (15–35 psu) in two strains of *C. gigas*, a fast-growing strain "Haida No. 1" (HH) and an inbreeding line with orange shell color (OO), and their reciprocal hybrids HO (HH♀ × OO♂), OH (OO♀ × HH♂). Oxygen consumption rate (OCR), ammonia-N excretion rate (AER), superoxide dismutase activity (SOD), catalase activity (CAT) and contents of malondialdehyde (MDA) were determined on day 10 of the temperature and salinity exposure. Results showed higher stress resistance ability of the hybrids than their parents against environment challenge. For physiological parameters, the OCR of OH strain increased in the experimental temperature range, while the maximum values of HO, HH and OO strains were 2.013, 2.193 and 1.994 mg g^{-1} h^{-1} respectively at 31 °C; OCR and AER of OH strain was significantly higher than that of the other strains at lower salinity ($P < 0.05$). For immune parameters, the overall SOD and CAT activity of hybrids were lower than that of the other strains under temperature treatments. Besides, the overall MDA content of OH strain was lowest at experimental temperature and salinity, and the other three strains' MDA level ranked as: OO > HH > HO. Overall, the results suggest that environment changes could significantly affect the physiological and immune status of oyster, and hybrids may be more resistant to environment stresses than their parents. This study provides physiological and immune evidences for interpreting the stress resistant heterosis in this oyster hybrid system, which could help us in a better understanding and utilization of heterosis in oyster aquaculture.

1. Introduction

The Pacific oyster (*Crassostrea gigas*) is an important economic aquaculture species around the world, primarily due to its fast growth rate, high economic value and strong environmental adaptability. Currently, with the oyster industry being developed worldwide, a number of breeding programs of *C. gigas* have been successfully launched, leading to significant improvements of important traits, such as disease resistance (Dégremont et al., 2015), shell coloration (Han et al., 2019), growth and survival traits (de Melo et al., 2019). Although several attempts have been made to increase oyster production, massive mortality has been commonly reported for *C. gigas* for many years in some countries (Dégremont, 2011; Evans and Langdon, 2006; Rahman et al., 2019). This phenomenon has not been well solved until now, and brought heavy losses to oyster aquaculture industry in the world.

Hybridization breeding, which is defined as the mating of animals from different species, strains or inbred lines, produces genetic improvement, which may have strong selective value (Hedgecock et al., 1995). In aquaculture, hybridization was commonly used to increase growth rate, manipulate sex ratios, improve flesh quality, increase disease resistance, produce sterile animals and improve environmental adaptability (Bartley et al., 2001). Among mollusks, interspecific and intraspecific hybridization have been widely practiced and have aimed at producing excellent hybrids resulting from heterosis or the combination of desirable characteristics found in parental species (Kong et al., 2017). For instance, previous studies have demonstrated the heterosis from interspecific hybrids like survival rate and temperature tolerance of the hybrids between *Haliotis discus hannai* and *H. gigantea* were

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superior to the parent (Liang et al., 2014). An oyster strain produced by hybridizing the *C. hongkongensis* ♀ and *C. gigas* ♂ is characterized by high growth rate and enhanced salinity tolerance (Zhang et al., 2016a). Similarly, favorable heterosis for growth and survival rate has been obtained by the intraspecific hybridization among different culture strains of *C. gigas* (Kong et al., 2017; Han et al., 2020). Furthermore, the improvement of production traits of *C. gigas* has also been demonstrated in crossbreeding of inbred strains, and the yield increased with general diallel crosses among inbred parent strains (Hedgecock et al., 1995; Hedgecock and Davis, 2007).

Environmental factors (temperature and salinity) play vital roles in physiological metabolism and immune enzyme activities in aquatic animals (Pourmozaffar et al., 2019; Wang and Li, 2019). Aquatic animals are able to sense transient fluctuations and seasonal variations in temperature and salinity, and respond to these changes by actively adjusting their physiological and biochemical activities to adapt to the ambient environmental regime. It has been reported that large-scale mortality of bivalve was often closely related to the change of environmental factors such as temperature and salinity (Dégremont, 2011; Fuhrmann et al., 2016; Gajbhiye and Khandeparker, 2017). Some studies confirmed that the survival rate of some tolerant strains which were bred by hybridization breeding was greatly improved in extreme environment. For instance, the hybridization of the *C. hongkongensis* and *C. ariakensis* created the offspring which had greater growth and survival characteristics at high salinity than its parents, and it may be considered to be more suitable for regions with higher salinity in China (Huo et al., 2014). Considering that hybridization is meaningful for breeding stress resistant strains and improving the survival rate of hybrid progeny, it is necessary to examine the hybrid performance under different environments to provide some useful information for cultivating new strains and resisting to environmental stress.

China is the top oyster-producing country worldwide with total oyster production reaching 5.23 million tons in 2019 (BOF, 2020). The Pacific oyster are widely cultured and become the dominant commercial oyster species in northern China. In order to improve the Pacific oyster industry in China, a series of breeding programs have been carried out, and several new varieties with fast growth (Li et al., 2011) and rare shell color (Han et al., 2019) have been successfully bred. However, like other countries, China has also reported numerous episodes of mortality of Pacific oyster in recent years, which has brought great losses to oyster industry in China (Chi et al., 2021).

In this study, intraspecific hybridization between two strains of *C. gigas*, a fast-growing strain "Haida No. 1" and an inbreeding line with orange shell color, was conducted. Two physiological parameters (oxygen consumption rate and ammonia-N excretion rate) and activities of three immune-related enzymes (superoxide dismutase, catalase, and malondialdehyde content) were measured at different temperatures and salinities. These experiments are expected to provide the guidance for future hatchery breeding of new variety with high survival rate and practical application of heterosis in oyster culture.

2. Materials and methods

2.1. Biological material

The oysters used in this study were a selected strain "Haida No. 1" and an orange shell variant of the Pacific oyster. The "Haida No. 1" strain was produced by artificial selection for fast growth annually since 2006 and the orange shell variant was an inbreeding line with left and right shell color. A complete diallel cross among "Haida No. 1" strain (HH) and orange shell variant (OO) produced four strains, including two parental strains HH (HH♀ × HH♂), OO (OO♀ × OO♂) and two hybrid strains HO (HH♀ × OO♂), OH (OO♀ × HH♂). All oysters grew in the same area located in Rushan Bay, Shandong province, China (36.4°N, 121.3°E) and were transferred to Yantai Haiyi hatchery, China for 1 month of conditioning at a pool with 24 m^3 seawater (temperature: 24 °C; salinity: 30 psu) prior to experiment. The mean growth traits of sampled oysters were measured (Table 1). Soft tissues of the oysters in each chamber were dissected, and dried at 80 °C for 48 h after the physiological measurement was completed, and then the dry meat weight (DW: g) was individually determined.

2.2. Experimental design

The oysters were randomly selected from four strains held in the rearing pool and divided into five temperature treatments and five salinity treatments. The levels for each temperature and salinity were determined according to the previous studies about optimal oyster rearing conditions (Zhang et al., 2018; Wang and Li, 2019) and the range of environmental conditions of aquaculture area in the Yellow Sea (Chu et al., 2005; Liu et al., 2008). Test temperature of 16, 21, 26, 31, and 36 °C, and test salinity of 15, 20, 25, 30, and 35 psu were used in this experiment, which were gradually adjusted from ambient at a rate of 1 °C/day and 2 psu/day respectively. Water temperature was maintained by water bath with immersed heaters or water chiller (HC-150A, 33ILEA, China) and a temperature regulator, while water salinity was adjusted by diluting natural seawater with filtered fresh water or adding sea salt and measured by a refractometer (ATAGO). When the test temperature and salinity was reached, it was maintained for 10 days. The salinity was kept stable at 30 psu in the temperature treatments, and the temperature was kept stable at 26 °C in the salinity treatments. During the experiment, oysters were fed with a mixture of *Isochrysis galbana* and *Platymonas subcordigoramis*.

2.3. Measurements of the physiological parameters

Closed-chamber respiration method was employed to determine the oxygen consumption rate (OCR) and ammonia excretion rate (AER). In order to reduce the organic matter produced by food metabolism and fecal excretion, acclimated oysters were kept unfed for 24 h. Four similar-sized oysters from different strains were transferred to respiration chambers respectively at the test temperature or salinity level, and seawater was fully aerated for 24 h to assure oxygen saturation before taking measurement. A blank chamber with no oyster served as the control, and then subtracted from the experimental units to correct for autogenic trends. The experiment lasted for 3 h. All chambers are sealed with liquid paraffin to ensure that they were airtight. Water samples were siphoned into a brown glass bottle to take the measurement, and dissolved oxygen (DO) and ammonia were measured before and after the experiment using a DO meter (YSI, 600XL, USA) and hypo-bromate oxidimetry method (Li, 1995). In order to minimize measurement errors, each treatment was analyzed in three replicate samples and measured three times of each sample. The condition index is the main indicator that oysters use the available inner shell cavity for somatic cell and gonadal tissue growth and reflect physiological and nutritional status (Rainer and Mann, 1992). Condition index (CI) was calculated for each group by the following equation (Lawrence and Scott, 1982):

$$\text{Condition index} = \frac{\text{dry meat weight (g)}}{\text{whole wet weight} - \text{shell wet weight (g)}} \times 100$$

OCR and AER were calculated using the following equations (Valverde et al., 2006):

$$\text{OCR} = (DO_0 - DO_t) \times V/(DW \cdot t)$$

$$\text{AER} = (N_t - N_0) \times V/(DW \cdot t)$$

The initial and final concentration of DO and ammonia are denoted by subscripts 0 and t, respectively, V is the volume of the respiration chamber (L), DW is the dry weight of *C. gigas* (g), and T is the time between the initial and final measurements (h).

The oxygen: nitrogen (O:N) atomic ratio (based on the oxygen uptake and ammonium nitrogen excretion) was used to estimate the proportion

Table 1
Biological characteristics of four strains of *C. gigas* used in the experiment.

Strain	Shell height (mm)	Shell length (mm)	Shell width (mm)	Live weight (g)	Dry meat weight (g)	Shell wet weight (g)	Condition Index
OH	69.41 ± 5.45	39.56 ± 6.02	23.53 ± 4.49	28.30 ± 6.50	0.26 ± 0.09	17.59 ± 4.80	2.51 ± 1.09
HO	68.68 ± 7.55	39.23 ± 5.17	22.19 ± 4.70	29.66 ± 6.33	0.31 ± 0.17	18.40 ± 4.81	2.93 ± 1.71
HH	67.35 ± 8.20	40.00 ± 4.52	21.66 ± 4.71	27.85 ± 5.69	0.32 ± 0.12	16.96 ± 3.09	3.20 ± 1.44
OO	63.79 ± 8.20	35.45 ± 6.82	21.49 ± 5.41	22.44 ± 6.96	0.22 ± 0.07	15.35 ± 4.87	3.31 ± 1.47

of proteins, lipids and carbohydrates for metabolism as follows:

$$O : N = (OCR/16)/(AER/14)$$

Q_{10} (temperature coefficient), a parameter to describe the rate of change in a biological or chemical system as a consequence of increasing the temperature by 10 °C, was calculated for *C. gigas* according to the Van't Hoff equation (Bayne and Newell, 1983).

$$Q_{10} = (R_2/R_1)^{10/(t_2-t_1)}$$

where t_1 and t_2 represent the temperature of two group trials respectively, and R_1 and R_2 represent corresponding OCR under each temperature group.

2.4. Enzyme activity assays

After exposure to different temperatures and salinities for 10 days, the gill tissues of four oysters from each rearing pool were sampled for the analysis of enzyme activities. Gill tissues were frozen with liquid nitrogen immediately after sampling in the 2 mL Eppendorf tube and stored at −80 °C until analyzed. Prior to analysis, the frozen gill samples were thawed on ice. About 500 mg tissue was homogenized (10% w/v) on ice in 0.86% saline with a homogenizer (IKA, Germany). The homogenates were immediately centrifuged at 3500 rpm for 20 min at 4 °C, and the supernatants were subjected to enzyme activity assays. Total protein concentration was determined following Bradford's method (Bradford, 1976) using Coomassie brilliant blue.

2.4.1. Activities of antioxidant enzymes

Superoxide dismutase (SOD) activity was determined with a commercial kit (Nanjing Jiancheng, China), using WST-1 method. SOD can catalyze the disproportionation of superoxide anion (O_2^-) to generate hydrogen peroxide (H_2O_2) and elemental oxygen. The raw superoxide anion, catalyzed by xanthine oxidase, may react with water-soluble tetrazole salt (WST-1) to form blue formazan. Because SOD inhibited the above reactions, the OD value of formazan was inversely proportional to the activity of SOD. SOD activity is expressed as units per mg protein (U/mgprot).

Catalase (CAT) activity was detected according to Góth (1991) using a commercial kit (Nanjing Jiancheng, China). The reaction system including extract (100 μL), phosphate buffer (4.60 μmol, pH 7.4), and H_2O_2 (6.5×10^{-3} μmol) were incubated 1 min at 37 °C, then immediately terminated by ammonium molybdate, and the remaining hydrogen peroxide combined with ammonium molybdate to form a yellow compound. The absorbance was measured spectrophotometrically at 405 nm, where it had its maximum absorbance. One unit of CAT catalytic activity is defined as the amount of enzyme required to decompose of 1 μmol hydrogen peroxide per second and the enzyme activity was expressed as units per mg protein (U/mgprot).

2.4.2. Lipid oxidative damage parameters

The content of malondialdehyde (MDA) was carried out spectrophotometrically with the thiobarbituric acid method according to Esterbauer and Cheeseman (1990) with a commercial kit (Nanjing Jiancheng, China). The extract was added to the same volume of 1% thiobarbituric acid in a 95 °C water bath for 40 min. After cooling, thiobarbituric acid reactive substance was centrifuged at 3500–4000 rpm for 10 min and the supernate were measured at 532 nm against a blank control consisting of 5% trichloroacetic acid mixed with 1% thiobarbituric acid. Tetraethoxypropane was used as a standard. MDA content is expressed as nmol/mgprot.

2.5. Statistical analysis

Results were expressed as the mean ± standard deviation (SD) and analysis of all data was performed using SPSS 20.0. Two-way ANOVA was used to analyze the impacts of environmental factors (temperature and salinity), strains (HO, OH, HH, and OO) and their interaction on physiological parameters' levels and immune-related enzyme activity. Then, within a certain environmental factor (temperature and salinity), or within a certain strain (HO, OH, HH, and OO), one-way ANOVA followed by least significant difference (LSD) analysis was used to compare the physiological parameters' levels and immune-related enzyme activities among different strains or among different environments.

3. Results

3.1. Physiological parameters

No oyster was dead during the temperature and salinity exposure. Two-way ANOVA analysis showed significant differences in the physiological parameters' levels between different strains and temperature or salinity (Fig. 1; Table 2).

3.1.1. OCR

In the range of temperature (16–36 °C), OCR increases in OH strain in opposition to the effect in the other strains (HO, OO and HH) which reach a maximum at 31 °C (Fig. 1A). The maximum values of OCR for OH, HO, HH, and OO strains were 2.789, 2.013, 2.193 and 1.994 mg g^{-1} h^{-1}, respectively, in thermal treatments, and the minimum values of OCR were 0.659, 0.646, 0.573 and 0.960 mg g^{-1} h^{-1} respectively. At the temperature of 36 °C, the OCR of the OH strain was significantly higher than that of the other strains ($P < 0.05$), while no significant differences were observed between the four strains of OCR at the other temperatures. The highest OCR in the OH strain of *C. gigas* were detected at higher experimental salinities, with values of 2.400 mg g^{-1} h^{-1} at 25 psu (Fig. 1B). Similar trends were also observed in the HO, HH and OO strains. Notably, the hybrid population OH had significantly higher OCR than the parental populations (HH and OO) and the hybrid population HO at the experimental salinity ($P < 0.05$).

3.1.2. AER

AER of different strains of *C. gigas* was significantly affected by an increase in temperature and reached the highest level at 36 °C (Fig. 1C). Statistically, temperature of 26, 31 and 36 °C formed a homogeneous group in terms of excretion in the OH and OO strain, whereas 16 and 21 °C yielded the lower relative rate of ammonia excretion in the four strains. The hybrid population OH had significantly higher AER than other three populations at the optimal temperature of 26 and 31 °C ($P < 0.05$), with values of 0.147 and 0.149 mg g^{-1} h^{-1} respectively. With increasing salinity from 15 to 35 psu, AER of the OH strain firstly increased to highest level at 30 psu and then decreased to a lower level at 35 psu, while AER of the HO, HH, OO strain increased to highest level at

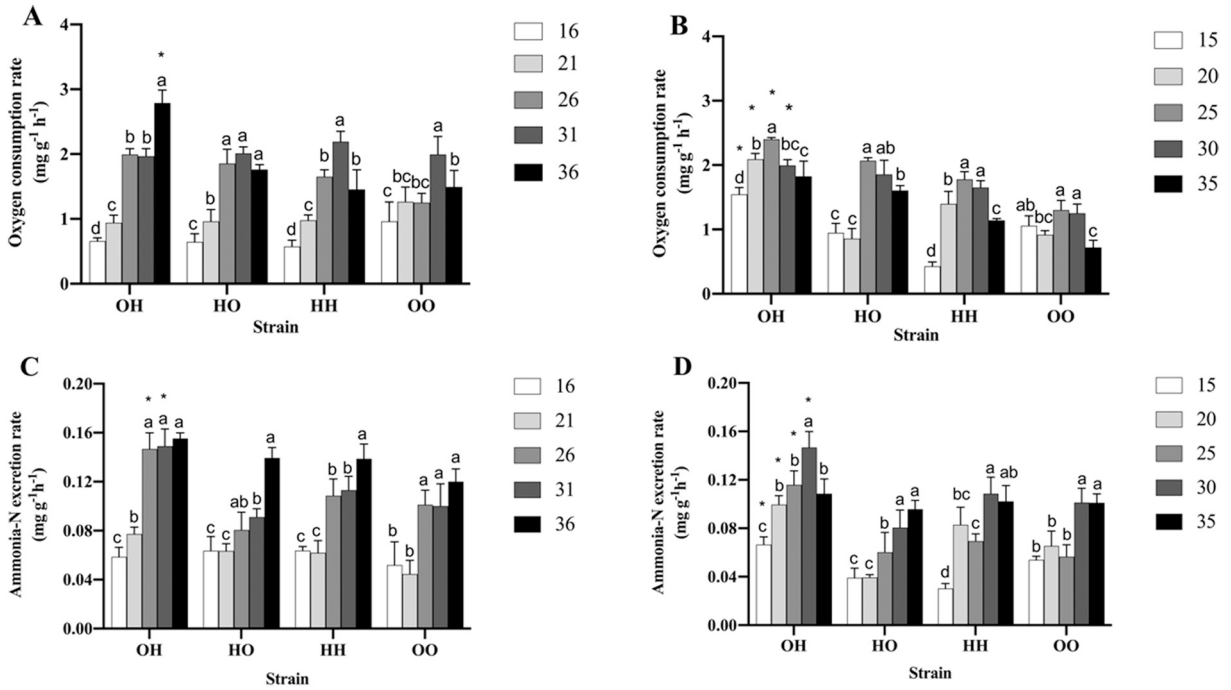

Fig. 1. Oxygen consumption rate and ammonia excretion rate of four strains under different temperature (A and C) and salinity (B and D). Different lowercase letters indicate significant differences ($P < 0.05$) in OCR and AER between different temperature or salinity within a same strain. Asterisk (*) is used to mark the point where the OCR or AER of a strain reached the highest level at a certain temperature or salinity.

30 and 35 psu respectively (Fig. 1D). At the salinity of 35 psu, no significant differences were observed between the four strains of AER, while the AER of the OH strain was significantly higher than that of the other strains at the other salinities ($P < 0.05$).

3.1.3. O:N

The O:N ratios at temperatures of 16, 21, 26, 31, and 36 °C ranged from 9.83 to 15.72 in OH strain, 8.89 to 20.10 in HO strain, 7.86 to 16.97 in HH strain and 10.81 to 24.84 in OO strain respectively (Table 3). The atomic O:N ratios of four strains were smaller at the temperature of 16 and 36 °C. The O:N ratios of the OH, HO, HH and OO strain at salinities of 15 to 35 psu ranged from 11.89 to 20.30, from 14.66 to 30.04, from 9.77 to 22.39 and from 6.26 to 20.13 respectively (Table 3).

3.1.4. Q_{10} coefficients

For the OH strain, the highest value was recorded at 4.50 between 21 °C and 26 °C, followed by 2.03 between 16 °C and 21 °C, 2.00 between 31 °C and 36 °C and 0.98 between 26 °C and 31 °C. In contrast, the highest Q_{10} value was recorded at 3.71 between 21 °C and 26 °C in HO strain, 2.90 between 16 °C and 21 °C in HH strain and 2.54 between 26 °C and 31 °C in OO strain (Table 4).

3.2. Enzyme activity assays

3.2.1. SOD and CAT

Two-way ANOVA showed significant effects of strains on SOD levels (Table 2). Among the four strains, the SOD levels in HH and OO strain significantly exceeded that of the OH and HO strain ($P < 0.05$), while HH and OO strain were not significantly different in SOD levels (Fig. 2A). In the salinity experiment (Fig. 2B), two-way ANOVA showed significant effects of salinity, strain and their interaction on SOD levels (Table 2). Notably, the overall SOD levels in OO strain significantly ($P < 0.05$) exceeded that of the other three strains.

Two-way ANOVA showed significant effects of temperature, strain and their interaction on CAT levels (Table 2). The CAT reached the highest level in HH and OO strain at 31 °C (Fig. 2C). In total, HO strain had the lowest CAT levels, while the other three strains showed no significant differences in CAT levels ($P > 0.05$). In the salinity experiment (Fig. 2D), two-way ANOVA showed significant effects of salinity and salinity × strain interaction on SOD levels (Table 2). At the salinity of 25 psu, CAT reached the highest in all evaluated strains.

3.2.2. MDA

Two-way ANOVA showed significant effects of temperature, strain and their interaction on MDA contents (Table 2). Among the four strains, OH strain got the lowest MDA level and the other three strains' MDA level ranked as: OO > HH > HO (Fig. 2E). In the salinity experiment, two-way ANOVA showed significant effects of salinity, strain and their interaction on MDA levels (Table 2). Similarly, OH strain got the lowest MDA level and the other three strains' MDA level ranked as: OO > HH > HO (Fig. 2F).

4. Discussion

A delicate balance exists between organisms and environmental factors, and breaking this balance often leads to occurrence of mortality. For instance, changes in temperature and salinity may influence the immune status of marine invertebrates, making them more susceptible to bacterial infection (Liang et al., 2014). Therefore, the adaptive capacity and recovery rate under environmental stresses are considered important indexes to evaluate aquaculture varieties. Hybridization breeding is considered to be an effective method of genetic improvement in fish and shellfish aquaculture, which brings desirable characteristics to the offspring such as stress resistance (Huo et al., 2014). In the present study, we compared and studied the physiological and immune responses of two strains of *C. gigas* and their hybrids to temperature and salinity stress. Our results reconfirmed that stressed temperature and salinity can influence oyster's physiological and immune responses. Moreover, we found that hybrid populations did response differently to thermal and salinity stress compared to their parents, both in the physiological level and immune level.

Table 2
Summary of two-way ANOVA results: effects of temperature and strain, and salinity and strain on physiological parameters and immune parameters of *C. gigas*.

Source of variation	df	OCR (mg g^{-1} h^{-1})			AER (mg g^{-1} h^{-1})			SOD (U/mgprot)			CAT (U/mgprot)			MDA (nmol/mgprot)		
		MS	F	P	MS	F	P	MS	F	P	MS	F	P	MS	F	P
Temperature	4	3.916	121.595	<0.001	0.014	109.806	<0.001	2241.62	0.429	0.787	3402.92	63.93	<0.001	96.333	18.068	<0.001
Strain	3	0.283	8.792	<0.001	0.003	26.115	<0.001	478,981	91.615	<0.001	174.085	3.27	0.031	1249.85	234.421	<0.001
Temperature × Strain	12	0.347	10.773	<0.001	0.001	3.983	<0.001	4099.93	0.784	0.663	418.006	7.853	<0.001	9.902	1.857	0.071
Salinity	4	1.464	86.452	<0.001	0.007	67.3	<0.001	33,230.7	14.532	<0.001	3415.19	35.863	<0.001	26.258	3.778	0.011
Strain	3	2.304	136.058	<0.001	0.005	47.82	<0.001	406,321	177.689	<0.001	167.19	1.756	0.171	1211.6	174.333	<0.001
Salinity × Strain	12	0.249	14.711	<0.001	0.001	4.735	<0.001	5652.52	2.472	0.016	334.561	3.513	0.001	27.462	3.951	<0.001

Note: df, degree of freedom; MS, mean square; F, means MS factor/MS error; P, means probability of significance.

4.1. Physiological parameters

Oxygen consumption and ammonium excretion are good physiological variables of metabolic activity related to biotic (body size and food availability) and abiotic factors (temperature and salinity) (Sarà et al., 2008). OCR is a good indicator of the condition and fitness of organisms, as well as an accurate and sensitive index of the environmental and stress conditions (Guzmán-Agüero et al., 2013), while AER is a factor which limits production in aquatic species and reflects the energy lost as nitrogen (Yin et al., 2013). Many studies have demonstrated that OCR and AER were related to water temperature and salinity in marine mollusks (Nie et al., 2016; Wang and Li, 2019). In the present study, we found that OCR and AER of oysters tended to normally increase with temperature, up to a maximum or optimum limit beyond which they rapidly decreased. This is consistent with many other studies in mollusks, such as Manila clam *Ruditapes philippinarum* (Nie et al., 2016), Iwagaki oyster *C. nippona* (Wang and Li, 2019), and Yesso scallop *Patinopecten yessoensis* (Jiang et al., 2016). Of greater interests are the variations in OCR and AER among the 4 populations: (1) At the highest temperature (36 °C in this experiment), OH strain had higher OCR compared with this of HO, HH and OO strain; (2) The OCR of OH strain increased in the experimental range, while the HO, HH, OO reached the maximum at 31 °C; (3) At the temperature of 26 and 31 °C, OH strain had significantly higher AER compared with this of other three strains. Therefore, we may refer that the hybrid OH strain could have greater temperature tolerance limit than other strains. Similarly, the responses of OCR and AER were found to be significantly influenced by salinity. The length of the salinity acclimation period is higher than temperature because the bivalves need more time to modulate their osmotic pressure (Gosling, 2015). The change of osmotic pressure is probably one of the main factors of metabolism fluctuation of aquatic organisms caused by different salinities. Previous studies showed that the effect of salinity on AER has been often contradictory. Nie et al. (2016) suggested that when water is isotonic with normal body fluid, the energy used by aquatic organisms to regulate osmotic pressure is less, resulting in a lower metabolic level. However, Guzmán-Agüero et al. (2013) showed that under stress (hypertonic and hypotonic) conditions, the level of ammonia excretion would be affected by ingestion, resulting in lower ammonia excretion rate. Bougrier et al. (1995) argued that this contradictory can be explained by the difference in the process of animal acclimation. In our study, OCR and AER of four strains decreased to lowest level at lower salinity (15 and 20 psu), indicating that the effect of lower salinity on OCR and AER was more significant than that of higher salinity, which could be explained as the lower salinity was not conductive to oyster feeding. We also observed that the OCR and AER of hybrid OH strain was significantly higher than that of the other strains at lower salinity. This may indicate that OH strain has stronger adaptability to low salinity.

The O:N ratio of an organism is an important index of substrate (protein, lipid, and carbohydrate) and has proved useful in assessing the physiological response of bivalves to various stressful environments (Bayne et al., 1976). Theoretically, the O:N is 7 for catabolism of pure protein, whereas the ratio is 24 for catabolism of equal quantities of protein and lipids. Thus, greater O:N values correspond to higher levels of lipid and carbohydrate catabolism (Mayzaud and Conover, 1988). In the present study, the O:N for four populations reared at experimental temperatures ranged from 7.86 to 24.84 and at experimental salinities ranged from 6.26 to 30.04, which indicates equal utilization of proteins and lipids. Similar results were also reported for other bivalves (Hao et al., 2014).

The temperature coefficient (Q_{10}) is a parameter to describe the sensitivity of organisms to temperature increase and its value reflects the adjustment related to the enzymatic and physiological requirements for energy when temperature increases within the natural range (Manush et al., 2004). Saucedo et al. (2004) indicated that within an intermediate temperature range (23–28 °C), a combination of active respiration and

Table 3
The effects of different temperatures and salinities on the O:N ratio of four strains of *C. gigas*.

Strain	Temperature (°C)					Salinity (psu)				
	16	21	26	31	36	15	20	25	30	35
OH	9.83	10.65	11.89	11.56	15.72	20.30	18.38	18.13	11.89	14.70
HO	8.89	13.25	20.10	19.33	11.04	21.27	18.98	30.04	20.10	14.66
HH	7.86	13.80	13.31	16.97	9.18	12.38	14.75	22.39	13.31	9.77
OO	16.18	24.84	10.81	17.42	10.88	17.21	12.28	20.13	10.81	6.26

Table 4
Q_{10} coefficients in four strains of *C. gigas* calculated from different temperature ranges.

Temperature range	OH	HO	HH	OO
16–21	2.03	2.22	2.90	1.73
21–26	4.50	3.71	2.85	0.98
26–31	0.98	1.18	1.76	2.54
31–36	2.00	0.76	0.44	0.56

low ammonia excretion, together with Q_{10} values near 2, suggested the existence of compensatory mechanisms that allow the pearl oyster *Pinctada mazatlanica* to perform seasonal regulation during moderately warm temperature changes. In the present study, Q_{10} values obtained from OH strain were 2.03 and 2.00 between 16–21 °C and 31–36 °C, respectively, which indicated that the OH strain was well adapted to these temperatures because there were no strong modifications in metabolism. In addition, the hybrid populations (OH and HO) had a lower value of Q_{10} at 26–31 °C temperature intervals, indicating the changes in respiration of hybrid strains reached less sensitively than parental populations (HH and OO) at these temperatures and had a higher tolerance to this temperature intervals than parental populations.

4.2. Enzyme activity assays

Abele et al. (2002) suggested that exposure to environmental stress can induce the generation of reactive oxygen species (ROS), such as superoxide anions (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radicals

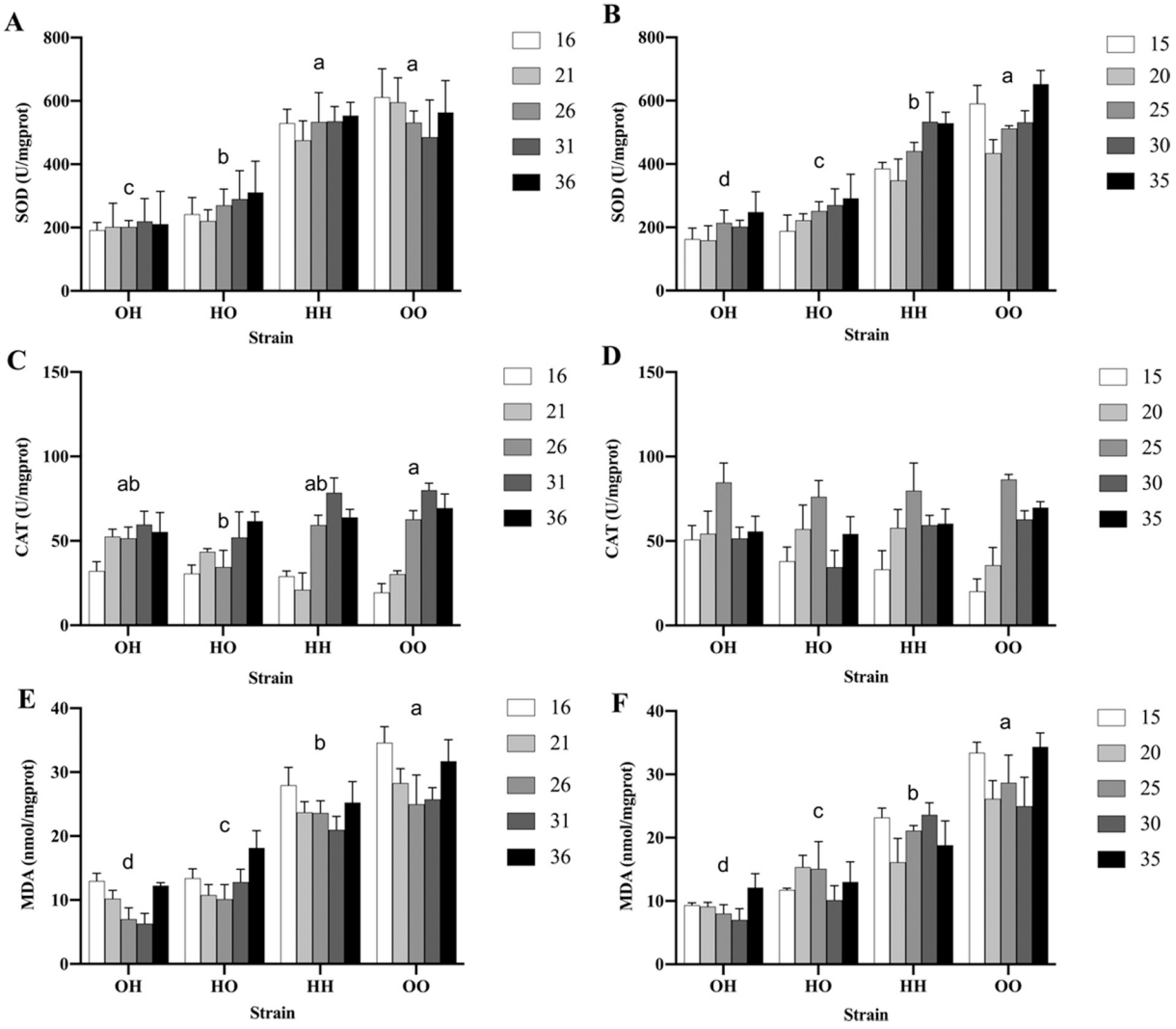

Fig. 2. Effects of different temperatures (A, C and E) and salinities (B, D and F) on immune-related enzyme activity of the four strains. Different lowercase letters indicate significant differences ($P < 0.05$) in overall enzyme activity among different strains.

(OH^-), singlet oxygen (O_2) and activate anti-oxidative defense systems. CAT and SOD appear to play an essential protective role in the ROS scavenging process. SOD catalyze the disproportionation of O^{2-} to H_2O_2 and O_2 and, in turn, CAT dismutase H_2O_2 into H_2O and O_2. Moreover, the levels of MDA, a product of lipid peroxidation, are often used as markers of oxidative stress (Zanette et al., 2011).

In marine environments, temperature has a strong effect on the function of immune defense system in mollusks. Previous studies have shown that enzymes play an important role in coping with temperature changes and protecting organisms from damage (Hao et al., 2014; Rahman et al., 2019). In this study, we found that SOD activity did not show significant difference under different temperature treatments. Similarly, there was no significant difference in SOD activity between *Mytilus galloprovincialis* and *Katelysia rhytiphora* at different temperatures, which was considered to be the result that SOD activity could neutralize high concentration of ROS in a short time (Rahman et al., 2019). The high CAT activity observed in the higher temperatures suggests that the oxidative stress is prone to peroxide radicals. This result is consistent with the elevated CAT activities in mussels *M. coruscusi* and *M. galloprovincialis* after exposed to stressors (Hu et al., 2015; Khessiba et al., 2005). The overall SOD and CAT activity of hybrids were lower than that of the other strains at temperature range. This may indicate that hybrids have a strong antioxidant defense mechanism, which is beneficial to protect cells from the adverse effects of oxidative stress. The change of salinity can induce oxidative stress in marine bivalves, which can eliminate ROS by increasing antioxidant defense (SOD and CAT activities) mechanism, thus responding to oxidative damage. In the current study, the activity of SOD in OH, HO and HH strain did not show significant difference at different salinities, but decreased first at 20 psu and then increased in OO strain. Notably, the overall SOD levels in OO strain significantly exceeded that of the other three strains, which indicated that OO strain was more vulnerable to oxidative damage. However, effects of salinity change on CAT activity seems to have different trends in mollusks. In the 28-day experiment, the antioxidant enzymes (including SOD and CAT) at lowest salinity increased significantly when *R. philippinarum* exposed to salinity levels (14 and 35 psu) (Velez et al., 2016). These results demonstrated that these enzymes are involved in defense system to prevent and block ROS, as well as repair mechanisms for oxidized components (Velez et al., 2016). Carregosa et al. (2014) showed that the three venerid clam species (*Venerupis decussata*, *V. corrugate* and *V. philippinarum*) significantly decreased the activity of CAT and SOD at the lowest tested salinities (0 and 7 g/L) due to valve closure, and significantly increased the activity of antioxidant enzymes (CAT and SOD) at a salinity of 14 g/L to cope with the increase of oxidative stress. Our results are in agreement with such findings since the CAT activity of four strains decreased at the lowest salinity (15 and 20 psu) and increased significantly at 25 psu. In contrast, the bivalve *C. rhizophorae* increased biotransformation (GSTs) activity under low salinities (9–15 psu) and control pH, while CAT activity was maintained under different salinities (9, 15, 25 and 35 psu) (da Silva et al., 2005). Similarly, the CAT and glutathione S-transferase activities in *C. gigas* gills did not change when exposed to salinity fluctuations of between 9 and 35 psu (Zanette et al., 2011).

Physiologically stressful conditions such as temperature and salinity changes can increase cellular damage in marine invertebrates due to an increased production of ROS, leading to the oxidation of the lipid membranes (Abele et al., 2002). It was reported that MDA content of the scallop *Mizuhopecten yessoensis* significantly increased and reached maximum value at 26 °C after 30 days of temperature exposure (Hao et al., 2014). Similarly, higher MDA levels were observed in the oysters kept at 25 psu salinity for 10 days when these oysters were compared to the groups kept at 9 and 35 psu salinity levels for the same amount of time, suggesting that there was an increase in the antioxidant defenses in the oysters kept at 9 and 35 psu salinity levels (Zanette et al., 2011). The results of the present study revealed that oysters tend to significantly increase lipid peroxidation because of the higher ROS production when the temperatures and salinities were outside the optimal values for the studied species (optimal values ranged from 21 to 31 °C of temperature; 25 to 30 psu of salinity). At the same time, the overall MDA content of OO strain was highest and the other three strains' MDA level ranked as: HH > HO > OH, suggesting that the hybrid species are not susceptible to oxidative damage under environmental stress.

4.3. Heterosis

Heterosis for growth and survival in the Pacific oyster have been documented by crossing inbred lines made by selfing hermaphrodites or by brother-sister mating within pedigreed families (Hedgecock et al., 1995; Hedgecock and Davis, 2007). Here, we discussed the potential of applying crossbreeding between selected strains to improve the stress resistance in *C. gigas*. Hybridization among selected strains offers the possibility for exploiting both the additive genetic variation accumulated within a strain and the non-additive genetic variation between strains. In the present work, the hybrid strains had greater temperature tolerance limit, stronger adaptability to low salinity and stronger antioxidant defense mechanism to those of parental strains. Such difference observed in reciprocal crosses could be partly linked to the genetic variation within populations and the level of the domestication of parental species (Zhang et al., 2016b). Meanwhile, analysis of physiological and immune parameters revealed that growth performance of OH and HO crosses was different, which indicated the existence of reciprocal effects. This may be due to the differences of crossing direction. Maternal effects could be a major contributor to the reciprocal effects (Bosworth et al., 1994). In addition, paternal effects, sex linked or cytoplasmic inheritance are other possible sources (Bentsen et al., 1998). This study showed undoubtedly the high tolerance of the hybris strains under extreme environments and it is possible to become dominant species under unsuitable environmental conditions.

5. Conclusions

We analyzed and compared the physiological and immune responses of two strains of *C. gigas*, and their hybrids to temperature and salinity challenge, hoping to get insights into the physiological and immune basis of the environment resistance heterosis observed in this hybrid system. Our results showed a positive role of hybridization in improving oyster's stress resistance ability. The hybrid strains had greater temperature tolerance limit and stronger adaptability to low salinity than their parents. Besides, overall enzyme activities suggested that hybrids had strong antioxidant defense mechanism, which may imply that hybrids were also more capable of dealing with environment stress. From the results, it is suggested that the two hybrid populations may perform better in actual mariculture.

Credit author statement

Lingxin Meng: Investigation, Methodology, Data curation, and Writing - original draft. **Qi Li**: Supervision and Writing - review & editing. **Chengxun Xu**: Writing review & editing. **Hong Yu**: Resources. **Shikai Liu**: Software. **Lingfeng Kong**: Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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